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Discovery of quantitative trait loci that mediate the effects of prenatal stress on cocaine and sensorimotor behaviors: Implications for gene by environment interactions that contribute to psychiatric disorders

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Psychological and Brain Sciences

by

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June 2017

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Manuscripts in review

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ABSTRACT

Discovery of quantitative trait loci that mediate the effects of prenatal stress on cocaine and sensorimotor behaviors: Implications for gene by environment interactions that contribute to psychiatric disorders

by

Jared R. Bagley

Gene by environment interactions may be important etiological factors that confer risk of numerous psychiatric disorders. Psychiatric disorders are found to be heritable, indicating genetic variants contribute to risk and resilience. In addition to genetics, early life stress confers significant risk. Prenatal stress (PNS) is associated with numerous disorders and alterations to affect and cognition that suggest profound and enduring consequences. Preclinical studies causally indicate the deleterious effects of PNS on models of psychiatric disorders, including effects on prepulse inhibition (PPI) and cocaine reward and locomotion. The intersection of genetics and PNS has been explored and PNS was found to interact with genetic background. PNS differentially alters PPI and cocaine reward and locomotion in the C57/6J (B6) and DBA/2J (D2) inbred mouse strains. These strains may serve as progenitors for populations

that can be utilized in forward genetic studies for discovery of quantitative trait loci (QTLs) that will facilitate discovery of PNS interacting variants. The following will present studies that utilized the BXD recombinant inbred mouse panel, derived from the B6 and D2 strains, to discover QTLs that interact with PNS to alter sensorimotor and cocaine-induced behaviors. A QTL by PNS interaction was discovered for PPI and acute cocaine locomotion. The BXD panel is a genetic reference population that allows for extensive accumulation and sharing of data across studies. Following discovery of these QTLs, publicly available BXD mRNA expression data was utilized to prioritize positional candidate genes. These efforts prioritized several positional candidate genes. In addition to offspring phenotypes, the maternal stress corticosterone response and effects of stress on dam-pup contact were assessed, as heritable maternal stress responses may contribute to strain differences in offspring phenotype, with implications for the interpretation of QTLs. Strain differences in the maternal corticosterone response and the maternal behavior response to stress associated with strain differences in PNS effects on male offspring cocaine phenotypes, suggesting a potential role for genetic variants that moderate the maternal stress response. The results obtained are a preliminary step in identifying genes that interact with PNS to confer risk of psychiatric disease.

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Chapter 1

Introduction

Psychiatric disorders exact devastating consequences on afflicted individuals and profound costs on society as a whole. Those who suffer with a psychiatric condition may be burdened with impaired daily functioning and a reduced lifespan (Murray et al., 2013; Walker, McGee, & Druss, 2015). The financial costs of these impairments were estimated at $2.5 trillion in 2010, with a projected cost of $6 trillion in the year 2030 (Bloom et al., 2012). Psychiatric disorders are common, with 1 in 5 diagnosed in a given year (Center for Behavioral Health Statistics and Quality, 2016). Because of the prevalence and often severe impact of psychiatric conditions, discovery of better treatments should be a top priority. However, better treatment may only be discovered with a sophisticated and comprehensive understanding of psychiatric disorder etiology. Despite progress in psychiatric disorder research, collectively, psychiatric diseases remain poorly understood both in terms of cause and optimal management.

Discovery of etiological factors will likely lead to improved prevention and treatment. Knowledge of etiological factors will allow for productive lines of neurobiological research that better elucidate the neuropathology of psychiatric disorders. An improved physiological understanding will provide opportunities for development of pharmacotherapeutics as well as non-drug therapies. Many psychiatric disorders are likely the result of a complex myriad of risk factors;

interactions between environmental and genetic factors occurring at proximal and distal times to the expression of psychiatric symptoms. Although a significant challenge, identifying and untangling these factors will improve the treatment of those who suffer from mental illness. The following will briefly review the genetics of psychiatric conditions, including current approaches in human and preclinical populations and potential genetic interactions with environmental factors. Particular attention will be given to gene by early life stress interactions and effects on cocaine related behaviors and pre-pulse inhibition, for relevance to the experiments presented in this dissertation.

Despite the poor understanding of psychiatric disorder etiology, substantial evidence points to genetic variants as an important contributing factor. Twin studies indicate that there is an effect of genetic variation on risk for developing most psychiatric disorders, with heritability ranging from moderate (0.33 for major depression) to high (0.80 for schizophrenia) (Kendler, 2001). Although heritability indicates that genetic variants are important etiological factors of psychiatric disease, these studies do not identify relevant genes, thus, additional approaches are required to elucidate the precise molecular entities driving disease development.

The discovery of psychiatric risk- or resilience-alleles is a promising avenue to enhance understanding of the etiology of psychiatric disease and can lead to new directions in neurobiological research. Genotyping is a non-invasive

procedure that has rapidly declined in cost due to technological advancements. These properties allow for the direct study of genetics in human psychiatric populations. Initial efforts involved linkage analysis and candidate-gene approaches. Linkage analysis is only suitable for large effect alleles and the candidate-gene approach requires biased selection of targets, which may greatly limit discovery of all relevant genes. In contrast, genome wide association studies (GWAS) are unbiased, genome-wide scans for genetic polymorphisms that associate with the phenotype under investigation. GWAS holds great promise for identifying risk and resilience alleles involved in psychiatric disorders. However, this approach is challenging. Psychiatric disorders involve complex traits under the influence of many alleles (Goldman, Oroszi, & Ducci, 2005). Furthermore, individual alleles often have very low effect size. Detection of a weak allele signal requires tremendous statistical power. Psychiatric GWAS may require many thousands of subjects for adequate power; initial failures of GWAS are largely thought to be due to low power. Despite these challenges, recent progress demonstrates the utility and applications of this approach. For example, 81 replicable alleles for schizophrenia have been identified by GWAS. (Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium, 2011). The variants identified in schizophrenia GWAS can be used to assign individuals a polygenic risk score. In addition to validating the GWAS findings, risk scores can be used to investigate the effects of these variants on a variety of phenotypes. A study of adolescents found that the polygenic risk score

was predictive of anxiety and negative schizophrenia symptoms (H. C. Jones et al., 2016). This approach highlights the feasibility of utilizing GWAS results, even prior to a deep biological understanding of the alleles, to predict disease risk. Perhaps individuals with high genetic risk can be targeted for early intervention and treatment prior to development of clinical schizophrenia. GWAS of other psychiatric disorders have demonstrable success, including drug abuse. GWAS conducted for smoking behavior detected alleles of the nicotinic acetylcholine receptor subunit alpha 3 (CHRNA3) and 5 (CHRNA5) that associates with smoking behavior (Tobacco and Genetics Consortium, 2010). The neurobiological effects of these alleles are currently under investigation (Ware, van den Bree, & Munafò, 2012). Furthermore, discovery of these alleles lead to interesting clinical findings, including an interaction of the CRHNA5 allele with nicotine cessation treatment. Those with the high risk genotype show greatest response to nicotine replace therapy, those with the intermediate risk genotype show a blunted response while those with the low risk genotype show no response to the therapy and the pattern is reversed in placebo treatment, with the low risk genotype demonstrating greatest abstinence (Bergen et al., 2013). Gene-treatment interactions may be of great value in the treatment of individual patients, in which therapies can be tailored to maximize efficacy and minimize adverse effects. These examples demonstrate the success of human GWAS to date, and indicate the promise of continued efforts.

Despite the potential for human subjects GWAS, there are limitations that hamper success. In relation to the aforementioned requirement for very large sample sizes, a significant consideration is the feasibility of sub-domain and quantitative phenotyping (M. Hall & Smoller, 2010). A psychiatric disorder may represent a collection of partially or fully independent traits, with substantial heterogeneity within a diagnosed population. However, human GWAS often utilize a case control design, in which a binary classification compares diagnosed subjects to control populations. This approach may reduce power by collapsing important variation (Van der Sluis, Posthuma, Nivard, Verhage, & Dolan, 2013). Greater success may come by characterizing quantitative traits associated with a disorder (Flint, Timpson, & Munafò, 2014). However, due to the large sample sizes required for GWAS, "deep" phenotyping of subjects is typically not feasible. In contrast, preclinical studies involving laboratory animals have good feasibility for characterizing quantitative traits. Additionally, traits are characterized under highly controlled conditions. Limiting environmental variability and measurement error reduces "noise" that may obscure allele effects. Furthermore, measurement of some phenotypes may be limited in human subjects due to ethical considerations (Schughart, Libert, & Kas, 2013). Similarly, genetic manipulations allow for causal inferences and exploration of biological effects that are not possible in human subjects due to ethical limitations (Schughart et al., 2013). These advantages indicate that preclinical genetic and genomic studies have excellent potential for generating unique contributions to

psychiatric genetics knowledge by enhancing our ability to elucidate detailed causation of behavioral variability. Greatest progress will be made if clinical and preclinical approaches are conducted in a complementary manner.

Cocaine Genetics in Pre-Clinical Models

Cocaine abuse is considered a neuropsychiatric condition in which the neurobiological response to cocaine leads to dysregulated and deleterious use (Volkow, Fowler, & Wang, 2003). Although many use drugs of abuse, such as cocaine, only a small minority go on to develop drug use disorders and it remains unclear why specific individuals are resistant or vulnerable to addiction (Johnston, O'Malley, Bachman, & Schulenberg, 2005; Merikangas & McClair, 2012). Cocaine abuse demonstrates a high heritability of 0.7 (Goldman et al., 2005). Identification of the genetic variants that mediate differences in human cocaine responsiveness is critical for a comprehensive understanding of substance abuse genetics. However, this pursuit looks to be a significant challenge. Non-Mendelian patterns of substance abuse inheritance indicate a polygenic influence (Goldman et al., 2005). Additionally, GWAS studies suggest the involvement of hundreds or thousands of alleles in substance abuse across drug class (F. S. Hall, Drgonova, Jain, & Uhl, 2013). To date, forward genetic research of substance abuse in human subjects has largely focused on alcohol and nicotine, with only one completed cocaine GWAS (Gelernter et al., 2014). As

such, our understanding of the genetics of cocaine addiction based on studies of human subjects is vastly incomplete, however, preclinical animal research has complimented cocaine genetics research with both reverse and forward genetic studies.

A large portion of research targeted at elucidating the genetics of cocaine responsiveness has focused on reverse genetics techniques. The advent of sophisticated techniques for genetic manipulation allow for the targeted manipulation of gene expression and function. Similar to human candidate-gene studies, selection of genetics targets is often founded on prior knowledge of drug pharmacodynamics and relevant neurobiology. Consequently, much research has focused on monoamine systems. The mechanism of action for cocaine involves reuptake inhibition of dopamine, serotonin and norepinephrine by interference with monoamine transporters (Benowitz, 1993). Increases in dopamine neurotransmission are thought to largely mediate the rewarding properties of cocaine (Wise, 1996). Dopamine transporter (DAT) knockout mice display largely eliminated cocaine locomotor responses and attenuated cocaine CPP and self-administration however these mice were still capable of experiencing cocaine reward and reinforcement (Sora et al., 2001). A double knockout of DAT and serotonin transporter (SERT) eliminated cocaine CPP, suggesting the involvement of SERT in cocaine reward and highlighting the likely genetic complexity of cocaine reward, which may often include gene-gene

interactions. This notion is further supported by the differential effect of DAT knockout on different strains of mice (Morice, Denis, Giros, & Nosten-Bertrand, 2004). While this is not a comprehensive review of reverse genetic approaches to cocaine abuse research, these studies highlight the benefits and limitations of this approach. Reverse genetic techniques provide a powerful method of experimentation that allows for causal genetic inference. However, the need for gene selection bias may greatly limit the identification of all cocaine-relevant genes. Tandem use of forward genetic techniques has the potential to greatly expand the number of candidate genes that can be further investigated by reverse genetics techniques.

Inbred strains provide a method of establishing the heritability of traits and can serve as a foundation for forward genetic studies. Repeated inbreeding leads to complete homozygosity, such that all members of a particular strain are genetically identical to all other members (Beck et al., 2000). Phenotypic differences between inbred strains can be attributed to the genetic variation that exists between the strains. The influence of genetic variation on cocaine-induced behaviors has been assessed by comparison of inbred strains. With respect to cocaine, much of the work has focused on the C57BL/6 (B6) and DBA/2 (D2) inbred strains. D2 mice demonstrate greater locomotor response upon initial cocaine dosing and greater locomotion sensitization to repeated cocaine administration (Cunningham, Dickinson, Grahame, Okorn, & McMullin, 1999; I. E.

M. de Jong, Steenbergen, & de Kloet, 2008; Inge E. M. de Jong, Oitzl, & de Kloet, 2007; Tolliver & Carney, 1994, 1995). However, there is discordance of locomotor responses with cocaine reward and reinforcement. B6 mice demonstrate greater cocaine reward as measured by conditioned place preference (CPP) (Cunningham et al., 1999; C. Orsini, Bonito-Oliva, Conversi, & Cabib, 2005; Cristina Orsini, Bonito-Oliva, Conversi, & Cabib, 2008; Seale & Carney, 1991). B6 mice also demonstrate higher rates of cocaine self-administration (Kuzmin & Johansson, 2000; Veen, Piazza, & Deroche-Gamonet, 2007). These data suggest that these strains differ in their response to cocaine with the B6 strain exhibiting relatively high responsiveness on measures of addiction-like behavior. These strain differences confirm that cocaine responsiveness is mediated by genetic variation and also indicate that the B6 and D2 strains may serve as valuable tools for identifying alleles that have relevance for cocaine addiction.

Although comparisons of a small number of inbred strains can indicate the heritability of a trait, the vast number of polymorphisms between strains will often preclude genome wide searches for associated alleles. However, populations can be derived from inbred strains by crossbreeding and then utilized for genome wide scans. These populations have been utilized to search for alleles that affect cocaine phenotypes. Much of this research has examined measures of cocaine induced locomotion (Boyle & Gill, 2001, 2009; Gill & Boyle,

2003; Miner & Marley, 1995b; Phillips, Huson, & McKinnon, 1998; Tolliver, Belknap, Woods, & Carney, 1994; Vendruscolo et al., 2009). More recent studies have focused on reward and reinforcement, with characterization of both cocaine-induced CPP and cocaine self-administration (Dickson et al., 2015; Philip et al., 2010a). The details of this research and the implications for the present experiments will be discussed further in Chapter 3.

Pre Pulse Inhibition Genetics in Preclinical Studies

Prepulse inhibition (PPI) is the attenuation of a startle response when preceded by a lower intensity stimulus (Swerdlow, Braff, & Geyer, 2000). PPI is observed across sensory systems, however acoustic PPI is frequently utilized for investigation. Both the startle response and PPI are highly conserved phenomena (Swerdlow, Braff, & Geyer, 1999). PPI procedures are also amendable to studies of human subjects, as such PPI is thought to be a behavioral phenotype with good potential for translational research. Furthermore, PPI deficits are observed in a number of psychiatric and neurological disorders including schizophrenia, bipolar disorder, autism, Huntington's disease and Tourette's syndrome (Kohl, Heekeren, Klosterkötter, & Kuhn, 2013). Deficits observed in psychiatric disorders and the translational potential indicate PPI to be a behavioral phenotype with rich potential for

discovery of genes in animal models that confer risk or resilience to psychiatric disease.

As with cocaine abuse related phenotypes, PPI has been investigated by reverse genetic techniques. Hypothesis driven selection of genes are targeted for manipulation (Powell, Weber, & Geyer, 2012). Abnormal dopamine and glutamate function are theorized to contribute to symptoms of schizophrenia. DAT KO mice display deficits in PPI suggesting a hyper-dopaminergic state can interfere with PPI (Ralph, Paulus, Fumagalli, Caron, & Geyer, 2001). Reduced expression of the NMDA receptor NR1 subunit causes PPI deficits; evidence in line with pharmacological research that suggests NMDA antagonists produce schizophrenic like symptoms and agonists may alleviate symptoms (Duncan et al., 2004). In addition to glutamate and dopamine systems, overexpression of corticotrophin releasing factor (CRF) in a transgenic mouse line causes deficits in PPI, implicating stress related genes as potential contributors to PPI deficits. This may be of particular relevance, given the involvement of stress in many psychiatric disorders (Agnew-Blais & Danese, 2016; Holtzman et al., 2013; Kendler, Karkowski, & Prescott, 1999; Koob et al., 2014). In addition to supporting dopamine/glutamate theories of schizophrenia and stress-psychiatric disorder connections, these lines of evidence generally suggest that PPI is a phenotype susceptible to alterations in gene expression and function.

Reverse genetic techniques may be of great utility in causally validating candidate genes that are discovered by genome wide scans or candidate-gene approaches in human subjects. This is particularly true of PPI, due to the feasibility of characterizing human subjects and comparing to animal models. Candidate-gene approaches have identified variants of the neuroregulin 1 (NRG1) and the disrupted in schizophrenia 1 (DISC1) genes as associating schizophrenia and with PPI deficits in human subjects (Mackie, Millar, & Porteous, 2007; Roussos, Giakoumaki, Adamaki, & Bitsios, 2011). NRG1 mutants in mouse have demonstrated PPI deficits, as well as the knock out of the NRG1 receptor ERBB2 (Barros et al., 2009; Chen et al., 2008; Wen et al., 2010). Both knockout of DISC1 and the DISC1L100P mutations demonstrate PPI deficits (Hikida et al., 2007; Lipina et al., 2010). These results reveal the utility in tandem human subjects and animal model approaches for genetic discovery. In addition to validating candidate genes, animal models may have utility for use in forward genetic screens of PPI.

Common inbred mouse strains demonstrate large between-strain differences in acoustic startle and PPI. These differences reveal heritability of PPI in mouse populations and indicate their utility for forward genetic approaches. Populations derived from crossbreeding some of these strains have been employed for QTL mapping of PPI (Fernández-Teruel et al., 2002; Joober, Zarate, Rouleau, Skamene, & Boksa, 2002; Leussis et al., 2009; Loos et al., 2012;

Palmer et al., 2003; Petryshen et al., 2005; Philip et al., 2010b; Samocha, Lim, Cheng, Sokoloff, & Palmer, 2010; Sittig, Carbonetto, Engel, Krauss, & Palmer, 2016; Vendruscolo, Terenina-Rigaldie, et al., 2006; Watanabe et al., 2007; Webb, McClay, Vargas-Irwin, York, & van den Oord, 2009). The details of this research and implications for the present experiments will be discussed further in Chapter 2.

Animal models have proven invaluable in genetics research. The literature discussed here demonstrates the utility of these models for investigation of cocaine related phenotypes and PPI. Reverse genetics has implicated and supported candidate genes. Furthermore, forward genetics is employed in both human and animal populations for the discovery of novel candidate genes. However, despite these efforts, many of the relevant genes are thought to remain undiscovered (Crow, 2011). Therefore, it is pertinent to consider experimental approaches that further increase the probability of gene discovery. These efforts may include consideration of environmental factors. Likely important for the etiology of all psychiatric disorders, environmental factors may interact with genetic variants to contribute to psychiatric disease. It is this interaction that may be exploited to discover new genes.

Early Life Stress as an Environmental Factor

Genetic variation does not account for all psychiatric disorder vulnerability, indicating environmental factors must also be identified and assessed (Goldman et al., 2005). Exposure to early life stressors, in both the prenatal and postnatal period, appear to contribute to psychiatric disease. Studies of human populations indicate that early life postnatal stressors associate with development of depression, schizophrenia, substance abuse and eating disorders (Carr, Martins, Stingel, Lemgruber, & Juruena, 2013) (Dube et al., 2003; Enoch, 2011). Early life stress is often measured in the postnatal period (i.e. early childhood), however exposure to prenatal stress may also have a role in psychiatric disorder etiology. Fetal development may pose a particularly vulnerable phase, in which tissues, including the central nervous system, are under profound and rapid development and thus sensitive to developmental insults. Exposure of the mother to stressors during pregnancy impacts fetal development, with enduring changes that appear to insidiously manifest as elevated vulnerability to a variety of psychiatric disorders. Prenatal stress has been associated with schizophrenia, autism, mood disorders and cognitive impairments (DiPietro, Novak, Costigan, Atella, & Reusing, 2006; Huizink, Robles de Medina, Mulder, Visser, & Buitelaar, 2003; Khashan et al., 2008; Kinney, Miller, Crowley, Huang, & Gerber, 2008; Laplante et al., 2004; Laplante, Brunet, Schmitz, Ciampi, & King, 2008; O'Connor, Heron, Golding,

Beveridge, & Glover, 2002; O'Connor, Heron, Golding, Glover, & ALSPAC Study Team, 2003). The associations of prenatal and postnatal stress exposure with a variety of psychiatric disorders indicate early life stress as environmental factor of potential significance for understanding the etiology of psychiatric disease. Accordingly, early life stress is the focus of preclinical research to confirm causality and elucidate the biological mechanisms.

Early life stress can be modeled in pre-clinical animal studies. Stress exposure in both the prenatal and postnatal period are investigated, however the following will focus on the prenatal period and its relevance to the present experiments. Stress is typically applied in the third trimester of mouse or rat gestation, which is estimated to be the neurodevelopmental equivalent of the first/second trimester of human pregnancy. However, such comparisons may be problematic when considering differences in scale and complexity of development between species. For example, rodent neurogenesis is thought to be largely complete in the prenatal period, however substantial human neurogenesis may continue to 2.5 years of age (Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013). Consequently, sensitive periods of development may differ between species, depending on the phenotype under study. Despite these caveats, early life stress in preclinical studies has enduring effects that often resemble the associated effects of early life stress in human subjects.

A variety of stressors have been utilized to elicit a stress response in pregnant animals, with repeated restraint of the dam as a common stressor employed in this model (Maccari & Morley-Fletcher, 2007). Restraint is confirmed as a stressor by the reliable activation of the hypothalamic pituitary adrenal (HPA) axis and subsequent increase in circulating glucocorticoids. PNS affects multiple behaviors in rodents that represent several domains of emotion and cognition including: anxiety, depression, social interaction, spatial memory and response to drugs of abuse (Weinstock, 2017).

PNS effects on affective behaviors are widely reported, with increases in anxiety and depressive-like behaviors. PNS decreased open arm time and entries in the elevated plus maze and decreased center time exploration in the open field test (G. A. Bennett, Palliser, Shaw, Walker, & Hirst, 2015; Bogoch, Biala, Linial, & Weinstock, 2007; Boulle et al., 2016; Fride & Weinstock, 1988; Gur et al., 2016; Laloux et al., 2012; Miyagawa, Tsuji, Fujimori, Saito, & Takeda, 2011; Patin, Lordi, Vincent, & Caston, 2005; Said, Lakehayli, Battas, Hakkou, & Tazi, 2015; Vallée et al., 1997; Zhang et al., 2016; Zuena et al., 2008). Males may be more sensitive to PNS effects on anxiety, although effects are reported for females (Said, Lakehayli, Battas, et al., 2015; Zuena et al., 2008). Increases in anxiety can be reversed by citalopram treatment, indicating predictive validity as a model for anxiety (Zohar, Dosoretz-Abittan, Shoham, & Weinstock, 2015). PNS also increases depressive-like behavior, as measured by increased

immobility in the forced swim test (Basta-Kaim et al., 2014; Rayen, van den Hove, Prickaerts, Steinbusch, & Pawluski, 2011; Sickmann, Arentzen, Dyrby, Plath, & Kristensen, 2015; Sierksma et al., 2013; Ślusarczyk et al., 2015; Sowa et al., 2015; Weinstock, 2007; Zhang et al., 2016). Both sexes appear to be affected, however females may be more sensitive (Sickmann et al., 2015; Sierksma et al., 2013; Weinstock, 2007). PNS-induced decreases in sucrose preference are also reported, indicating PNS may cause anhedonia (Ślusarczyk et al., 2015).

PNS causes deficits in learning and memory. Most widely reported are deficits in acquisition of platform location in the Morris water maze (Hosseini-Sharifabad & Hadinedoushan, 2007; H. Li et al., 2008; Lui et al., 2011; Modir, Elahdadi Salmani, Goudarzi, Lashkarboluki, & Abrari, 2014; Nazeri et al., 2015; Ratajczak et al., 2015; H. Sun et al., 2017; Suzuki et al., 2016; Yang, Han, Cao, Li, & Xu, 2006; Zhao et al., 2013). Deficits of memory retention as assessed on the final probe trial are reported in some but not most studies, indicating that acquisition may be most affected (Benoit, Rakic, & Frick, 2015; Modir et al., 2014; H. Sun et al., 2017). Other behavioral tests also indicate learning or memory deficits including increased error rate in radial arm maze, lowered retention of novel versus familiar arms in the y-maze (6 hours after exposure) and deficits in passive avoidance learning. Interestingly, three studies report PNS-induced improvement in learning or memory in cue-mediated Morris water maze, Barnes maze probe trial performance and female-specific improvement of spatial Morris

water maze (Benoit et al., 2015; Negrón-Oyarzo, Neira, Espinosa, Fuentealba, & Aboitiz, 2015; Zuena et al., 2008). However, a large majority of reports indicate PNS causes deficits in learning or memory. Sensitivity to these effects may differ between sexes. Females may be more sensitive to PNS effects on Morris water maze, however male effects are widely reported indicating PNS effects on learning and memory are not sex-specific. (H. Li et al., 2008; H. Sun et al., 2017; Zuena et al., 2008).

PNS and Cocaine

PNS may increase vulnerability to substance use disorders, including cocaine abuse. PNS has been shown to alter cocaine behaviors in adult offspring. Adult male PNS rats demonstrate an enhanced locomotor response to cocaine administration and both male and female PNS rats demonstrate enhanced locomotor sensitization to repeated cocaine dosing (Kippin, Szumlinski, Kapasova, Rezner, & See, 2008; Thomas, Hu, Lee, Bhatnagar, & Becker, 2009) PNS male rats are also reported to have higher cocaine intake during self-administration and to be resistant to self-administration extinction training (Kippin et al., 2008; Thomas et al., 2009). In addition to cocaine, PNS alters responsiveness to other drugs of abuse. PNS augments CPP of morphine, nicotine and benzodiazepines and increases self-administration of amphetamine and ethanol, indicating PNS may increase vulnerability to substance abuse

across drug class (Campbell, Szumlinski, & Kippin, 2009; Deminière et al., 1992; Lakehayli, Said, Battas, Hakkou, & Tazi, 2015; Said, Lakehayli, El Khachibi, et al., 2015; Yang, Li, et al., 2006). It is worth noting that no human studies have yet associated PNS with drug abuse liability; all measures of early life stress that associate with drug abuse vulnerability occurred in the post-natal period. However, these preclinical data suggest that PNS may confer risk for drug abuse and warrant further study of both behavioral effects and physiological mechanisms.

PNS and PPI

PNS has effects on PPI that extend into the adult period. The majority of studies report a deficit of PPI in PNS exposed offspring (Fumagalli, Bedogni, Perez, Racagni, & Riva, 2004; Koenig et al., 2005; Matrisciano et al., 2013; Matrisciano, Tueting, Maccari, Nicoletti, & Guidotti, 2012; Zubedat et al., 2015). However one study reports an increase (Lehmann, Stöhr, & Feldon, 2000) and another did not find an effect (Burton, Lovic, & Fleming, 2006). PPI deficits are associated with psychiatric disease, including some disorders associated with PNS exposure in humans (schizophrenia and autism) (Kohl et al., 2013; O'Donnell, O'Connor, & Glover, 2009). Interestingly, impairment of PPI development in human infants has also been associated with maternal social stress in the prenatal period (Huggenberger, Suter, Blumenthal, & Schachinger,

2013). Congruence of PNS effects between human and animal subjects suggests that preclinical studies have good potential to yield translational results.

Effects of PNS on cocaine responsiveness and PPI, in addition to effects on other behaviors and congruence with early life stress effects observed in clinical studies, suggest that PNS is an important etiological factor that should be further studied to understand the behavioral and physiologically pathology of psychiatric disorders. Furthermore, PNS may represent an environmental factor that interacts with genotype to determine developmental outcomes. This interaction may be utilized to discover genes that modify risk for psychiatric disease by conferring sensitivity or resilience to the effects of PNS.

Genotype X PNS Interactions

The phenotype of an organism is the product of genetic and environmental factors. However, genetic and environmental factors can interact, such that genetic variation mediates the effects of environment on phenotype. Consideration of gene x environment (GXE) interactions may benefit research in psychiatric disorder genetics. Psychiatric GWAS data indicate that alleles tend to have very small effect sizes (Goldman et al., 2005; F. S. Hall et al., 2013; Q.-R. Liu et al., 2006). This has pragmatic consequences, in that detection of some alleles may be difficult due to a weak signal. However, by stratifying samples into

environmental factors, the effect size of relevant alleles may increase and become detectable (Murcray, Lewinger, & Gauderman, 2009). Furthermore, detection of genetic loci that interact with an environmental factor can be followed with biological studies of gene effects that incorporate prior knowledge of environmental factor effects. This may guide hypothesis formation and benefit the often difficult task of determining the biological relevance of genes, once detected by genome wide scans.

Gene by environment interactions have been identified in the investigation of psychiatric disorders. Environmental factors include likely early life stressors (childhood maltreatment and trauma) and prenatal exposure to smoking and alcohol use. In addition to independent, main effects of these early life exposures, genotype at a number of genes interacts with these factors to modify risk. Alleles of the serotonin transporter linked polymorphic region (5-HTTPLPR) are found to interact with early life maltreatment and early life stress to increase risk of alcohol abuse and depression (Vergne & Nemeroff, 2006). 5-HTTPLPR alleles are the most extensively investigated genotypes in GXE studies and are found to interact with adult exposure to stress, in addition to early life exposure (Caspi, Hariri, Holmes, Uher, & Moffitt, 2010). Other genes are found to interact with early life stress and maltreatment including: neurotransmitter-metabolizing enzyme monoamine oxidase A (MAOA) (increase risk of conduct disorder), dopamine active transporter (DAT1) (increased risk of ADHD) and

corticotrophin-releasing hormone 1 (CRHR1) (increased risk of mood and anxiety disorders) (Nugent, Tyrka, Carpenter, & Price, 2011; Wermter et al., 2010). Collectively, this research demonstrates the relevance of gene by early life environment interactions for conferring risk of psychiatric disorders. However, these studies represent biased selection of genes for investigation. As with research of allele main effects, the search for the genes involved in GXE interactions may benefit greatly from unbiased, genome wide scans. Emerging methodology suggests this may be a promising approach in human subjects, however there are significant challenges including valid methods of exposure assessment (Winham & Biernacka, 2013). Preclinical animal studies allow for highly controlled exposure and may be of benefit when used in tandem with human genetics studies.

Preclinical Gene X PNS Interactions

Pre-clinical animal models are particularly useful in GXE interaction research. Controlled exposure to environmental factors reduces the confounds and measurement error that may often occur in human subjects research, allowing for a high probability of allele detection and experimental validation (Carhuatanta, Shea, Herman, & Jankord, 2014; Izídio et al., 2011; Reifsnyder, Churchill, & Leiter, 2000; Tarricone, Hingtgen, Belknap, Mitchell, & Jr, 1995; Vieira et al., 2000).

PNS has been investigated for interaction with genotype by reverse genetic techniques. A candidate-gene approach provides evidence for serotonin transporter (5-Htt) interaction with PNS, with effects that are congruent with human subjects studies. 5-Htt knockout mice, exposed to PNS, display increased depressive- like behavior, reduced social interaction and differential hippocampal gene expression that may be mediated, in part, by differential DNA methylation (Jakob et al., 2014; K. L. Jones, Smith, Edwards, Givens, & Beversdorf, 2010; Schraut et al., 2014; D. Van den Hove et al., 2011). SNAP-25 is a SNARE-associated protein implicated in neurotransmitter release (Tafoya, Shuttleworth, Yanagawa, Obata, & Wilson, 2008). The SNAP-25 gene has been implicated in schizophrenia and ADHD by GWAS and candidate-gene approaches (Lewis et al., 2003; Y.-S. Liu et al., 2016). Furthermore, altered SNAP-25 expression levels are found in schizophrenic patients (Thompson, Sower, & Perrone-Bizzozero, 1998). A SNAP-25 by PNS interaction was assessed by utilizing a mouse mutant for SNAP-25. This variant leads to impaired neurotransmitter release. PNS increased PPI deficits and impaired social interaction in SNAP-25 mutants, effects not observed in wild-type controls (Oliver & Davies, 2009). These results suggest that genes identified in human studies do interact with PNS to alter relevant phenotypes and generally support the relevance of gene by PNS interactions for psychiatric disorder etiology.

In addition to targeted genetic manipulation, inbred strains can also be utilized to identify GXE interactions. Comparison of two or more strains allows for assessment of strain by environment interactions that indicate one or more GXE interactions. Comparison of the Fischer 344 and Lewis inbred rat strains indicates PNS interacts with strain to impair avoidance conditioning, increase locomotor activity, reduce force swim immobility and lower pain thresholds (Stöhr et al., 1998). Comparison of the B6 and D2 inbred mouse strains provides evidence for strain-dependent increase in inter-male aggression (Kinsley & Svare, 1987).

This approach has been utilized by our laboratory to assess potential strain by PNS interactions that affect cocaine-related behaviors and acoustic startle/PPI. The B6 and D2 strains were subjected to PNS by a restraint stress protocol in the 3rd week of gestation. Adult offspring were then assessed for acute and sensitized cocaine locomotion and for cocaine-induced CPP, for 3 doses of cocaine (3, 10 and 30 mg/kg) (Kippin, Campbell, Ploense, Knight, & Bagley, 2015). PNS increased the magnitude of cocaine-induced CPP in both male and female B6 mice across all doses. However it did not affect CPP in the D2 strain. Furthermore, PNS increased cocaine locomotion in B6 males, but had no effect on D2 mice. PNS also differentially affected locomotion after saline injection. B6 PNS subjects demonstrate greater locomotion while D2 PNS subjects demonstrate reduced locomotion. In addition to cocaine behaviors, PNS

also interacts with strain to alter acoustic startle response (ASR) and PPI. PNS increases ASR in male D2 mice but decreases ASR in male B6 mice and impairs PPI in D2 mice but not B6 mice (Kippin et. al., unpublished data).

Taken together, the evidence collected in our laboratory indicates that PNS interacts with genetic background to determine the influence of PNS on cocaine behaviors and PPI. This suggests the existence of alleles that mediate vulnerability to the developmental effects of PNS. However, there does not appear to be a general genetic vulnerability to PNS, as the effects differ depending on the genotype. PNS affects cocaine CPP and locomotion in B6 but not D2 mice. The effects of PNS on saline-locomotion are opposite between B6 and D2 strains. Finally, PNS impairs PPI in D2 but not B6 mice. These differential effects across traits suggest that genetic vulnerability to PNS is mediated by alleles with unique, trait-specific roles. Therefore, multiple alleles should be involved in PNS vulnerability, with some or all of these alleles demonstrating trait specificity.

Strain by PNS interactions indicate that the B6 and D2 inbred strains are suitable as progenitors for populations that can be utilized in forward genetic screens. The BXD recombinant inbred strains are derived from the B6 and D2 strains and can be utilized for genome wide scans for genotype-phenotype associations. Characterization of PNS effects on a sample of BXD strains will

facilitate the discovery of the alleles that interact with PNS to alter cocaine responsiveness and PPI.

Utilizing the BXD Panel to Identify the Genes in Gene by PNS Interactions

A powerful preclinical method for associating genetic loci with phenotypic variation is the use of specialized animal populations for quantitative trait locus (QTL) mapping (Gora-Maslak et al., 1991; Plomin, McClearn, Gora-Maslak, & Neiderhiser, 1991). This method is a preliminary step in identifying alleles that mediate variation in phenotype. Characterization of a trait in a genetically heterogeneous population often yields a continuous distribution of trait values. A trait with a continuous distribution is known as a quantitative trait, and is thought to be under polygenic influence. Phenotypic variation between subjects can then be associated with the known genetic variation between subjects. This approach can simultaneously reveal multiple genetic loci, known as quantitate trait loci (QTLs) (Flint, 2003; Johnson, DeFries, & Markel, 1992; Plomin et al., 1991). QTLs are defined intervals on the genome that associate with trait variation and therefore likely contain the alleles that influence the trait in question. QTL mapping is an unbiased, genome-wide search for associated alleles that can be employed for any trait that demonstrates heritability in a subject population. Heritability can be assumed when the trait in

question differs between the progenitor strains. However, differences in the parental strain phenotype are not necessary (Plomin et al., 1991).

A variety of animal populations can be utilized for QTL mapping. They can be broadly categorized as either populations of genetically unique individuals or panels of inbred strains. Individually unique populations are often derived by crossbreeding two or more inbred strains to produce a genetically homogenous F1 population that are heterozygous for progenitor alleles. Crossbreeding the F1 animals produces a genetically heterogeneous F2 generation due to recombination. These animals can then be phenotyped and genotyped and the association between phenotype and genotype is assessed to produce a QTL map. Advantages of this method include a relatively rapid breeding scheme to produce the F2 generation. However, each animal is genetically unique, and therefore genome-types are not reproducible. This limits the phenotype group for each genome-type to a sample of 1 and may allow for environmental noise to skew genome-type values. Irreproducibility also precludes assessment of genetic correlations between genome-types. Additionally, only one generation of recombination produces low recombination fractions, which causes large haplotype blocks and limits QTL resolution. This, however, can be ameliorated by advanced intercross lines (AIL) in which successive outbreeding occurs for more than one generation and decreases haplotype length and consequently increases QTL resolution.

Panels of inbred strains can be utilized for QTL mapping, and often include recombinant inbred (RI) strains. RI strains are derived by cross-breeding two or more progenitor inbred strains (F0), followed by breeding the completely heterozygous F1 population. Due to genetic recombination, the F2 generation is a genetically heterogeneous population of animals that each possess unique combinations of progenitor strain alleles. Successive generational inbreeding of these mice can then fix these unique combinations, leading to a panel of RI strains. Each RI strain is genotyped for polymorphisms between the progenitor strains across the entire genome. Strains from the RI panel can then be phenotyped for a given trait. RI strains are infinitely reproducible and allow for phenotyping multiple individuals per strain, which may increase the accuracy of the strain phenotype values. Each strain only requires genotyping once, allowing for reduced cost and effort in subsequent studies. Furthermore, strain means can be subject to genetic correlations between phenotypes, both within a study of multiple phenotypes and between studies. Genetic correlations indicate shared genetic polymorphisms between traits. Genetically correlated traits can be subject to factor analysis which may allow for quantification of a latent constructs that are less subject to environmental variance and improve QTL mapping power. Despite the advantages, RI strains are time consuming and costly to produce. Often, poor breeding performance limits the number of strains that can be produced and utilized. Low strain numbers limit the power to detect QTLs. Furthermore, large

haplotype blocks can limit QTL resolution, although this can be ameliorated with AIL breeding schemes, at the expense of further time and cost.

Differential effects of PNS in B6 and D2 strains indicate that polymorphisms between these strains must interact with PNS. These data suggest the B6 and D2 strains can serve as progenitors for populations that can be utilized for mapping gene by PNS interactions. The BXD RI panel is derived from the B6 and D2 strains and currently consists of 120 commercially available and genotyped strains (Peirce, Lu, Gu, Silver, & Williams, 2004; Taylor et al., 1999). The BXD panel was initially produced by B.A. Taylor. The panel was expanded by Pierce et. al. in 2004 to 89 strains and again in 2016 to 120 strains. The second and third BXD panel expansions were produced by advanced intercross breeding to create BXD strains with increased recombination and therefore improved QTL mapping resolution (Peirce et al., 2004). The online resource www.genenetwork.org contains BXD genotypes and software for rapid BXD QTL mapping. This resource also contains BXD phenotypes uploaded by researchers that can be rapidly compared and assessed for genetic correlations and common QTLs. These phenotypes include strain mRNA expression levels for many tissues that can be utilized for systems genetics analysis and prioritization of positional candidate genes in behavioral or physiological QTLs (Bubier & Chesler, 2012).

The BXD RI strain panel will be employed to identify genetic factors that interact with early environmental stress to modulate adult behavior. Multiple strains from the BXD RI panel, under both PNS and control conditions, will be phenotyped for sensorimotor behavior and cocaine responsiveness, followed by discovery of QTLs that interact with PNS and main effect QTLs. PNS interacting QTLs are loci that harbor genetic polymorphisms that confer sensitivity or resilience to PNS. Furthermore, PNS interacting QTLs will be compared between phenotypes in order to identify any overlapping QTLs that suggest a common variant mediates the affects of PNS across phenotypes. Similarly, the effects of PNS will be subject to genetic correlation analysis in order to determine if the affects of PNS across phenotypes may be mediated by common alleles. Significant QTLs will be investigated by bioinformatics analysis and prior knowledge of gene function in order to prioritize candidate genes. Lastly, the maternal glucocorticoid response to restraint stress as well as the effects of restraint stress on dam-pup interaction in the postnatal period will be assessed. The maternal stress response (both immediate endocrine and latent behavioral) may be a heritable trait. Heritable differences in the maternal stress response may produce differential changes in adult behavior of prenatally-stressed offspring across BXD strains. Associations between strain variability of the maternal stress response and PNS effects on offspring phenotype will be determined in order to assess strain variance of the maternal stress response as a mediator of PNS effects.

The use of the BXD panel to identify QTL by PNS interactions is a critical step in identifying genetic polymorphisms that interact with PNS to influence cocaine responsiveness and sensorimotor gating. Discovery of these polymorphisms may allow for an enhanced understanding of psychiatric genetics and serve to advance neurobiological research of psychiatric disorders.

Chapter 2

Discovery of QTL by PNS interactions for sensorimotor behaviors

Prepulse inhibition (PPI) is a measurement of sensory gating that is thought to be an endophenotype related to multiple psychiatric and neurological disorders. PPI deficits are observed in schizophrenia, bipolar disorder, autism, Huntington's disease and Tourette's syndrome (Kohl et al., 2013). PPI is highly conserved across mammalian species and widely studied in laboratory animal models with the intent of elucidating the neurobiology of these PPI-associated psychiatric and neurological disorders.

PPI-associated disorders are heritable (Geschwind, 2011; Kendler, 2001; Nopoulos, 2016; N. R. Zilhão et al., 2017; Nuno R. Zilhão et al., 2015). However, with the exception of Huntington's disease, the genetic etiology of PPI-associated disorders is poorly understood. Identification of the genetic variants that mediate risk and resilience for these disorders will greatly improve understanding and treatment of psychiatric disease. PPI is a heritable trait in mouse and rat populations, indicating genetic variants influence the PPI phenotype in these populations. Identification of these variants may implicate genes that have relevance for PPI-associated disorders. Heritability in laboratory animal populations suggests that they are suitable for genome-wide QTL mapping studies. This approach has been taken using multiple strategies including inbred strain panels, recombinant inbred strains, chromosome substitution strains, F2 crosses and backcrosses (Brigman, Mathur, Lu, Williams, & Holmes, 2009; Fernández-Teruel et al., 2002; Joober et al., 2002; Leussis et al.,

2009; Dahai Liu et al., 2003; Loos et al., 2012; McCaughran, Bell, & Hitzemann, 1999; Palmer et al., 2003; Petryshen et al., 2005; Philip et al., 2010b; Samocha et al., 2010; Sittig et al., 2016; Vendruscolo, Terenina-Rigaldie, et al., 2006; Webb et al., 2009). These efforts have identified multiple loci on the mouse and rat genome that associate with PPI variance. In some cases, QTL discovery has been followed by prioritization of positional candidate genes by prior information on gene function and bioinformatics, including mRNA expression data. In one case, a candidate gene (fabp7) is strongly supported by further experimental evidence (Watanabe et al., 2007), however, most candidate genes nominated by PPI QTL studies await experimental validation.

QTLs for PPI, discovered to date, have not accounted for all genetic variance, suggesting that PPI associated QTLs remain to be discovered (Brigman et al., 2009; Loos et al., 2012). This is may be largely due to inadequate power. However, in addition to increased power, the discovery of PPI associated genes may benefit by incorporating environmental factors that allow for identification of GXE interactions. Prenatal stress (PNS) is associated with some psychiatric diseases, including schizophrenia and autism (Khashan et al., 2008; Kinney et al., 2008). In addition to genetic variants, PNS and early life postnatal stress exposure are thought to be substantial etiological factors for psychiatric disease (Carr et al., 2013). Furthermore, the consequences of PNS exposure may be moderated by genetic variants, in GXE interactions that ultimately cause the

development of a psychiatric disorder (Wermter et al., 2010). Therefore, utilization of mapping strategies that target the identification of PNS interacting variants may lead to new discoveries of psychiatric-implicated genes. This strategy may be particularly successful in preclinical models due to highly controlled environmental exposure and reliable characterization of endophenotypes, such as PPI. Discovery of genetic variants that interact with PNS to modify the PPI phenotype may have special relevance for psychiatric disorders and improve understanding of early life GXE interactions.

We have identified gene by PNS interactions by characterizing the effects of PNS on B6 and D2 mouse strains. Strain by PNS interactions were observed for the acoustic startle response (ASR) and PPI. These interactions indicate that genetic variance between these strains mediates variance in these sensorimotor phenotypes. In order to identify these genetic variants, we utilized BXD recombinant inbred strains. BXD strains are derived by crossbreeding B6 and D2 mice to produce inbred strains with unique combinations of B6 and D2 alleles. These strains can be used for QTL mapping. The effects of PNS on ASR and PPI were characterized in multiple BXD strains. We predicted heritable effects of PNS on ASR and PPI. Between strain variance in the effects of PNS on ASR and PPI was utilized to map for QTLs that interact with PNS to alter ASR and PPI. These efforts will serve as a preliminary step in identifying genes that interact

with PNS to modify sensorimotor behaviors and have relevance for PPI-associated psychiatric disorders.

Methods

Subjects

BXD strains (n=21) (The Jackson Laboratory, Bar Harbor, MI) were housed in a temperature- and humidity-controlled vivarium on a 12-h light–dark cycle. All mice were maintained on ad libitum mouse chow and water access. All procedures were approved by the University of California at Santa Barbara Institutional Animal Care and Use Committee and conducted in accordance with the National Institute of Health (NIH) Guide for Care and Use of Laboratory Animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

Breeding

BXD strains were purchased from the Jackson Laboratory to establish a breeding colony in UCSB facilities. The offspring of this colony were used for timed breeding. Adult males and females, at 8 to 24 weeks of age, were paired for four days. Pregnancy was confirmed by weight gain and the dams were

assigned to PNS or control conditions. Females that failed to conceive were re-subjected to the breeding procedures in future cohorts. Male breeders were used for multiple cohorts. Impregnated females were only used to generate a single litter of offspring which were used in behavioral experiments.

In order to limit litter effects, a minimum of 4 litters was represented in each condition/sex for all behavioral tests and no more than 3 males or females from a litter were included in analysis. The within strain/condition/sex sample size ranged from 7 to 25.

Restraint Stress

PNS began two weeks post initial breeding setup. This corresponded to embryonic day (E) 11 through 14. PNS was induced by a repeated restraint stress protocol. The dams were taken from the vivarium into the laboratory and were restrained in 50 mL conical tubes for 1 hour periods, three times a day. Each one hour stress session was separated by one hour of home cage access. PNS continued daily until parturition. Control dams were left undisturbed in their home-cage and were not removed from the vivarium during pregnancy.

After parturition, PNS and control litters were left undisturbed with the dams. Litters were weaned at approximately 3 weeks of age and the sexes were housed separately. The weanlings were left undisturbed until behavioral testing.

Acoustic Startle and Pre-Pulse Inhibition

At 8 weeks of age, PNS and control offspring were tested for ASR and PPI. Subjects were confined to platforms equipped with accelerometers to sense movement. The platforms reside within sound-attenuated chambers equipped with speakers (San Diego Instruments, San Diego, CA, USA). The procedure consists of 6 different trials types presented in pseudo-random order, with a variable inter-trial interval between 10 and 20 seconds (average 15 seconds). Trial types include: no pulse (st0), startle pulse (110 dB/40 milliseconds; st110), low prepulse stimulus given alone (74 dB/20 milliseconds, st74), high prepulse stimulus given alone (90 dB/20 milliseconds; st90), st74 or st90 given 100 milliseconds before the onset of the st 110 startle pulse (pp74 and pp90, respectively). St110, st0, pp74 and pp90 trials were applied 10 times, st74 and st90 trials were applied five times. Data was averaged across all trials. Startle response was measured as the response amplitude after st110. PPI was calculated as (100-100*(pp74/st110)) (referred to as PPI74) and (100-100*(pp90/st110)) (referred to as PPI90).

Data Analysis

Data for ASR, PPI74 and PPI90, st0 (basal activity), st74 and st90 (reactivity to prepulse-only trials) were assessed by three way ANOVA with

strain, condition and sex as factors. Individual mice that were more than two standard deviations from the group mean (calculated within condition and sex) were excluded. Significant main effects or interactions involving sex were followed by main effects (using the grand mean error) 2 way ANOVA tests. Heritability was calculated by taking the r squared term from a one way ANOVA with strain as a factor within condition and sex. This statistic determines the proportion of variance accounted for by strain and is a measure of broad sense heritability. Two standard deviation outliers were not excluded for heritability estimates.

ASR and PPI BXD strain data from independent studies are available on genenetwork.org. Because the BXD panel is a genetic reference population, it is important to assess reliability of trait measurement between studies and laboratories. Pearson's correlations were determined between ASR and PPI control strain means of the present study and all available ASR and PPI data on genenetwork.org. Significant, positive correlations indicate good inter-study reliability.

QTL Analysis

Strain data for ASR, PPI74 and PPI90 were subjected to interval mapping for QTL discovery. Data were uploaded to genenetwork.org. This program

contains genotype data for all BXD strains and uses interval mapping by Haley-Knott regression to generate likelihood ratio statistics (LRS) across the entire genome (Haley & Knott, 1992). Significance thresholds for LRS are generated by randomly permuting the strain IDs and means 1000 times and then mapping each of those permutations (Churchill & Doerge, 1994). The peak LRS that occurs in 5% of these permutations is used as the significance threshold, this corresponds to a genome wide p-value of 0.05. Any locus with an LRS that exceeds this threshold was deemed as a significant QTL. A suggestive threshold, which corresponds to a genome-wide p-value of 0.63, was also determined. Any locus with an LRS that exceeds this threshold was determined a suggestive QTL. Previous research indicates that this threshold determines QTLs that are worth cautious consideration, but many false positives will also be generated. Outlier strain means are determined by Tukey's interquartile range with a 1.5 constant. Where outliers were identified, the values were winsorized and remapped (Shete et al., 2004)

QTL confidence intervals were determined by 2-LOD (1 LOD=4.61 LRS) drop-off intervals from the peak LRS, which is estimated to provide greater than 95% coverage (Dupuis & Siegmund, 1999; Manichaikul, Dupuis, Sen, & Broman, 2006). Where main effect QTLs where identified, the control-only means were mapped and compared to the sum score QTL. The QTL with the smaller interval was used for identification of candidate genes.

PNS X QTL Interactions

QTLs that interact with PNS were determined by subtracting the control mean from the PNS mean for each strain (difference score). The strain difference scores were subjected to interval mapping on genenetwork. QTLs produced by mapping the difference score represent the LRS for the interaction term, when genotype and an environmental factor are included in a linear model for QTL mapping (Lowry et al., 2013). Conversely, main effect QTLs were determined by adding the PNS and control strain means (sum score). Main effect QTLs represent the main effect term for genotype (Lowry et al., 2013).

Where sex was found to interact with PNS in the ANOVA results, separate means, difference and sum scores were calculated and mapped for each sex.

Prioritization of Positional Candidates

Significant QTLs were investigated by determining all genes within the 2-LOD support interval. Candidate genes were prioritized by considering those with cis-eQTL and transcript levels which covary with the behavioral phenotype or transcripts with non-synonymous SNPs (nsSNPs).

cis-eQTL

QTLminer (genenetwork.org) was used to determine all cis-eQTLs in the 2-LOD interval for the following brain regions; whole brain (UTHSC Mouse BXD Whole Brain RNA Sequence (Nov12) RPKM), amygdala (INIA Amygdala Cohort Affy MoGene 1.0 ST (Mar11) RMA), cerebellum (SJUT Cerebellum mRNA M430 (Mar05)), hippocampus (Hippocampus Consortium M430v2 (Jun06)), hypothalamus (INIA Hypothalamus Affy MoGene 1.0 ST (Nov10)), midbrain (VU BXD Midbrain Agilent SurePrint G3 Mouse GE (May12)), neocortex (HQF BXD Neocortex ILM6v1.1 (Dec10v2) RankInv), nucleus accumbens (VCU BXD NA Sal M430 2.0 (Oct07)), pituitary (INIA Pituitary Affy MoGene 1.0ST (Jun12)), prefrontal cortex (VCU BXD Prefrontal Cortex Sal M430 2.0 RMA), striatum (HQF BXD Striatum ILM6.1 (Dec10)) and ventral tegmental area (VCU BXD VTA Saline AffyM430 2.0 (Jun09)). Transcripts with significant cis-eQTLs (genome wide $p<0.05$) were then checked for genetic correlation with the behavioral phenotype by determining the Pearson's and Spearman rank order correlations. Bonferroni significance levels were determined for total number of transcripts evaluated, for a family-wise significance threshold of 0.05. Transcripts with a cis-eQTL within the 2-LOD interval and levels that correlate with the behavioral phenotype are considered top candidate genes. Transcripts were also considered in instances where correlations did not reach Bonferroni-corrected

significance, but did reach uncorrected significance ($p<0.05$) and were present in more than one region and expression assay.

Non-Synonymous SNP

The variant browser on genenetwork.org was utilized to identify all genes within the 2-LOD confidence interval with nsSNPs. Genes with nsSNPs were considered for biological relevance and implications in psychiatric disorders or relevant behavioral phenotypes.

Results

Assessment of basal and pre-pulse only reactivity

Strain differences in basal activity (st0) or startle reactivity to pre-pulse only trials (pp74 and pp90) may confound between strain differences in ASR and PPI. Therefore, data from these trials was subject to analysis. A 3-way ANOVA for st0 revealed a main effect of strain [$F(20, 1096)=2.35$, $p=0.001$], and a main effect of sex [$F(20, 1096)=8.32$, $p=0.004$]. Within sex analysis revealed a main effect of strain in males [$F(20, 1096)=2.22$, $p=0.003$] but no significant effects in females. Due to strain differences in basal activity, st74, st90 and st110 were corrected by dividing the values by the value for st0.

Reactivity at 74 dB pre-pulse only trials, corrected for basal activity (st74/st0), was assessed by 3-way ANOVA. No significant effects were detected.

Reactivity at 90dB pre-pulse only trials, corrected for basal activity (st90/st0), was assessed by 3-way ANOVA. A strain by sex by PNS interaction [$F(20, 1095)=1.65$, $p=0.036$] and a main effect of strain [$F(20, 1095)=5.76$, $p<0.001$] were detected; no other interactions of main effects reached significance. Within sex analysis revealed a strain by PNS interaction [$F(20, 1095)=1.73$, $p=0.004$] and a main effect of strain [$F(20, 1095)=6.51$, $p<0.001$] in males but no significant effects or interactions in females.

Acoustic Startle Response

A 3-way ANOVA for ASR revealed a strain by PNS by sex interaction [$F(20, 1039)=3.2$, $p<0.001$], a strain by PNS interaction [$F(20, 1039)=2.5$, $p<0.001$], a strain by sex interaction [$F(20,1039)=4.2$,$p<0.001$], a main effect of strain [$F(20, 1039)=20.9$, $p<0.001$], and a main effect of sex [$F(1,1039)=36.3$, $p<0.001$].,

A 2-way (strain by PNS) within sex ANOVA in females revealed no interaction but a main effect of strain [$F(20, 1039)=4.6$, $p<0.001$]. For males, a strain by PNS interaction [$F(20, 1039)=5.1$, $p<0.001$] and a main effect of strain [$F(20, 1039)=20.8$, $p<0.001$] were found.

Heritability estimates for control females is 0.16 and for PNS females 0.27. For control males 0.33 and PNS males 0.37.

PPI74

A 3-way ANOVA of PPI with a 74 dB prepulse revealed a main effect of strain [$F(20, 1103)=1.7$, $p=0.024$] but no interactions or other main effects reached significance.

The heritability estimate for control females is 0.08 and for PNS females 0.06. For control males 0.06 and PNS males 0.1.

PPI90

A 3-way ANOVA for PPI with a 90dB pre-pulse revealed main effect of strain [$F(20, 1055)=25.3$, $p<0.001$], a main effect of sex [$F(1, 1055)=28.2$, $p=0.026$] and a strain by sex interaction [$F(20, 1055)=2.6$, $p<0.001$].

A 2-way ANOVA within sex revealed a main effects of strain for females [$F(20, 1055)=10.6$, $p<0.001$] and for males [$F(20, 1055)=18.3$, $p<0.001$].

Because there are strain differences and strain by PNS interactions in male reactivity to st90 trials, an ANCOVA was performed for PPI90 with st90 as a covariate. Although st90 reached significance as a covariate, significance did

not change for any factor relative to the 3-way ANOVA or the within sex 2-way ANOVA results. The relationship between st90 reactivity was further assessed by determining the correlation between st90 strain means and PPI90 strain means within condition and sex. There is a positive correlation between these measures for PNS females $r=0.629$, $p=0.002$, PNS males $r=0.55$, $p=0.018$ and control males $r=0.66$, $p=0.001$. However, in males, this relationship is dependent on one outlier strain (BXD48a). The relationship of PNS effects on st90 and PNS effects on PPI90 was assessed by determining the correlation for strain difference scores for st90 and PPI90 within sex. There is a positive correlation in females $r=0.60$, $p=0.004$ and males $r=0.89$, $p<0.001$, that does not appear to rely on any one outlier strain.

The heritability estimate for control females is 0.23 and for PNS females 0.26. For control males 0.30 and for PNS males 0.34.

Correlations to Independent BXD Studies

Significant correlations for ASR were found between the present study and Philip et al., (2010) (19 overlapping strains) and Loos et. al., (2012) (11 overlapping strains). A significant correlation was found for PPI90 in the present study and Philip et. al., (2010) (19 overlapping strains). Correlations did not reach significance for PPI in Loos et. al., (2012) (10 overlapping strains) and ASR/PPI in Brigman et. al., (2011) (7 overlapping strains). See table 1.

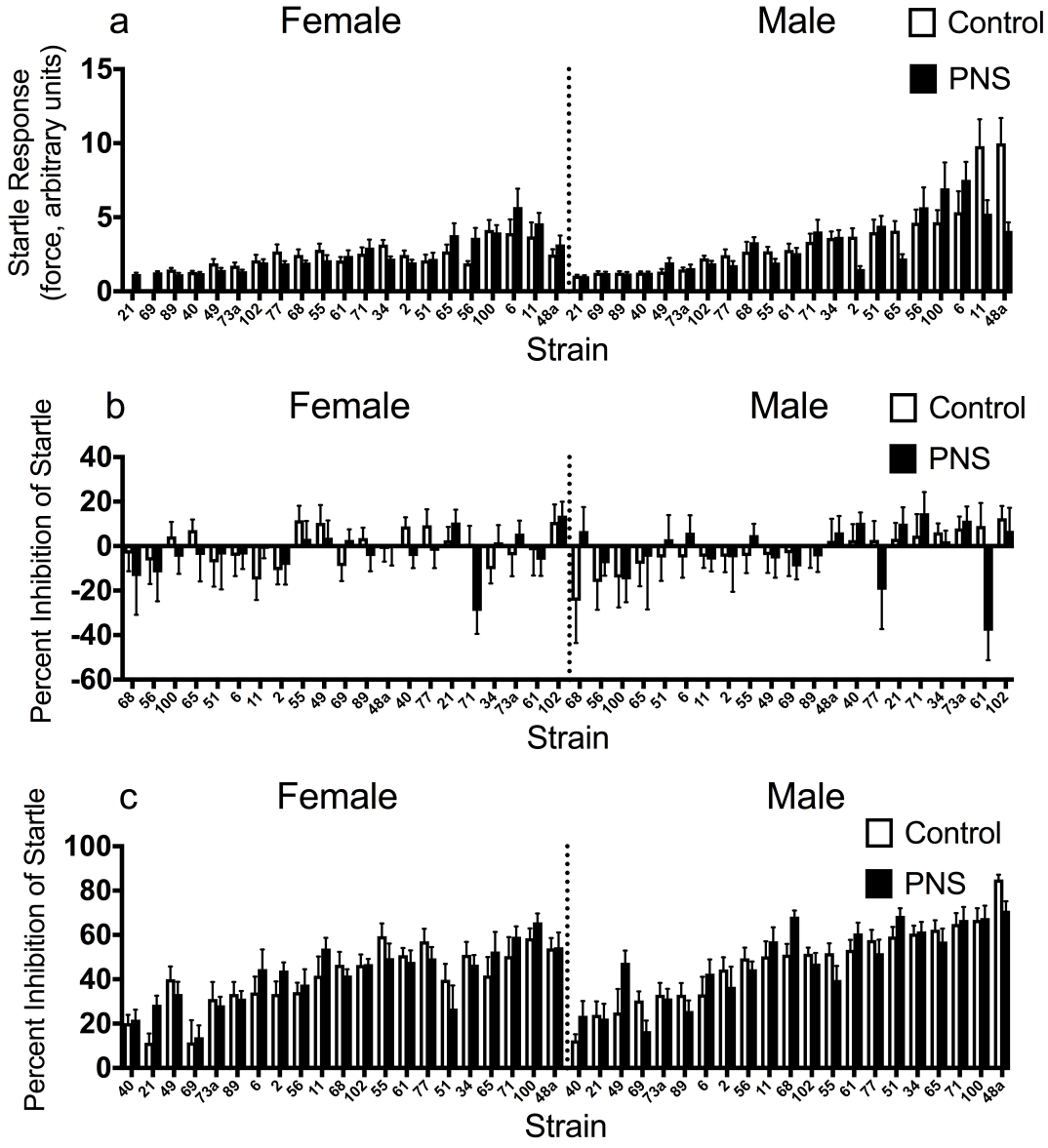

Figure 1. Effects of PNS on ASR, PPI74 and PPI90 in BXD strains. Strains ordered by male control values. a) PNS interacts with strain in males to affect ASR. b) No effect of PNS was detected for PPI74. c) No significant effect of PNS was detected for PPI90.

Table 1 Correlations of ASR/PPI to the same measures in independent studies of BXD strains

ASR (publication, r, p-value, n)	PPI (publication, r, p-value, n)
Philip et. al., 2011 0.69, 0.0007, 19	Philip et. al., 2011 0.66, 0.0015, 19
Loos et. al., 2012 0.72, 0.0102, 11	Loos et. al., 2012 0.59, 0.0728, 10
Brigman et. al., 2009 0.59, 0.174, 7	Brigman et. al., 2009 0.57, 0.194, 7

Table 2 Suggestive QTL for PPI90 and ASR (Startle)

Phenotype	Sex	Difference or Sum Score	LRS	Chromosome	Position	Marker	Additive Effect
PPI90	Female	Difference Score	12.681	6	148.827869	UNCHS019387	-5.34
			15.846	7	97.465336	rs46652854	-6.116
		Sum Score	13.188	9	64.513351	rs29942478	-17.796
	Male	Difference Score	17.93	3	107.129194	rs3702359	-8.591
			13.29	10	5.694659	rs51815932	-6.62
			12.534	12	46.816739	rs3709102	-6.482
		Sum Score	16.954	9	64.513351	rs29942478	-24.733
Startle	Female	Difference Score	13.576	19	32.04901	rs13459194	0.52
			12.515	X	153.533292	rs265619204	0.598
		Sum Score	13.638	9	65.547668	rs32892673	-1.369
			12.869	15	68.818097	rs31265809	-1.408
	Male	Sum Score	13.363	2	178.977635	rs27686798	-2.725
			15.493	4	96.896949	rs28138719	-2.868
			11.946	9	64.513351	rs29942478	-2.665
			12.705	12	34.201787	rs29220711	-2.651
			11.806	15	48.804757	rs48105593	-2.604

QTL by PNS Interactions

PNS effects on st90 associate with PNS effects on PPI90, suggesting that strain X PNS differences in reactivity to pre-pulse only trails may obscure discovery of QTLs for PPI. Therefore, adjusted means were calculated with st90 as a covariate. The difference scores were mapped for adjusted and unadjusted means. A suggestive QTL on chromosome 3 (LRS=17.5) was detected for unadjusted means (Figure 2a). This QTL became highly significant when difference scores for adjusted means were mapped (LRS=21.1) (Figure 2b). The 2-LOD interval is 107-108.6 mb. No other significant QTL X PNS interactions were detected for ASR or PPI74/90. For suggestive QTLs see table 2.

Main Effect QTL

No significant main effect QTLs were detected. For suggestive QTLs see table 2.

Prioritization of Positional Candidate Genes

Cis-eQTL

The confidence interval for the chr 3 QTL for male PPI90 difference scores contains 48 transcripts. All transcripts within the confidence interval were evaluated for cis-eQTL, and those with cis-eQTL were evaluated for

covariation with male PPI90 difference scores. Nine transcripts had genome wide significance cis-eQTL in one more regions, one of which covaried with male PPI90 difference scores at Bonferroni-corrected threshold.

The transcript AI504432 demonstrates a significant cis-eQTL in the amygdala and covaries with male PPI90 difference scores at Bonferroni corrected (0.05/9) significance level (see table 3).

The transcript Slc16a4 demonstrates a significant cis-eQTL in the midbrain and a suggestive cis-eQTL in the amygdala. Slc16a4 did not meet Bonferroni corrected significance for covariation, but did for uncorrected significance (see table 3).

Non-synonymous SNPs

Of the 48 transcripts with the 2-LOD interval, 5 contain nsSNPs (see table 4).

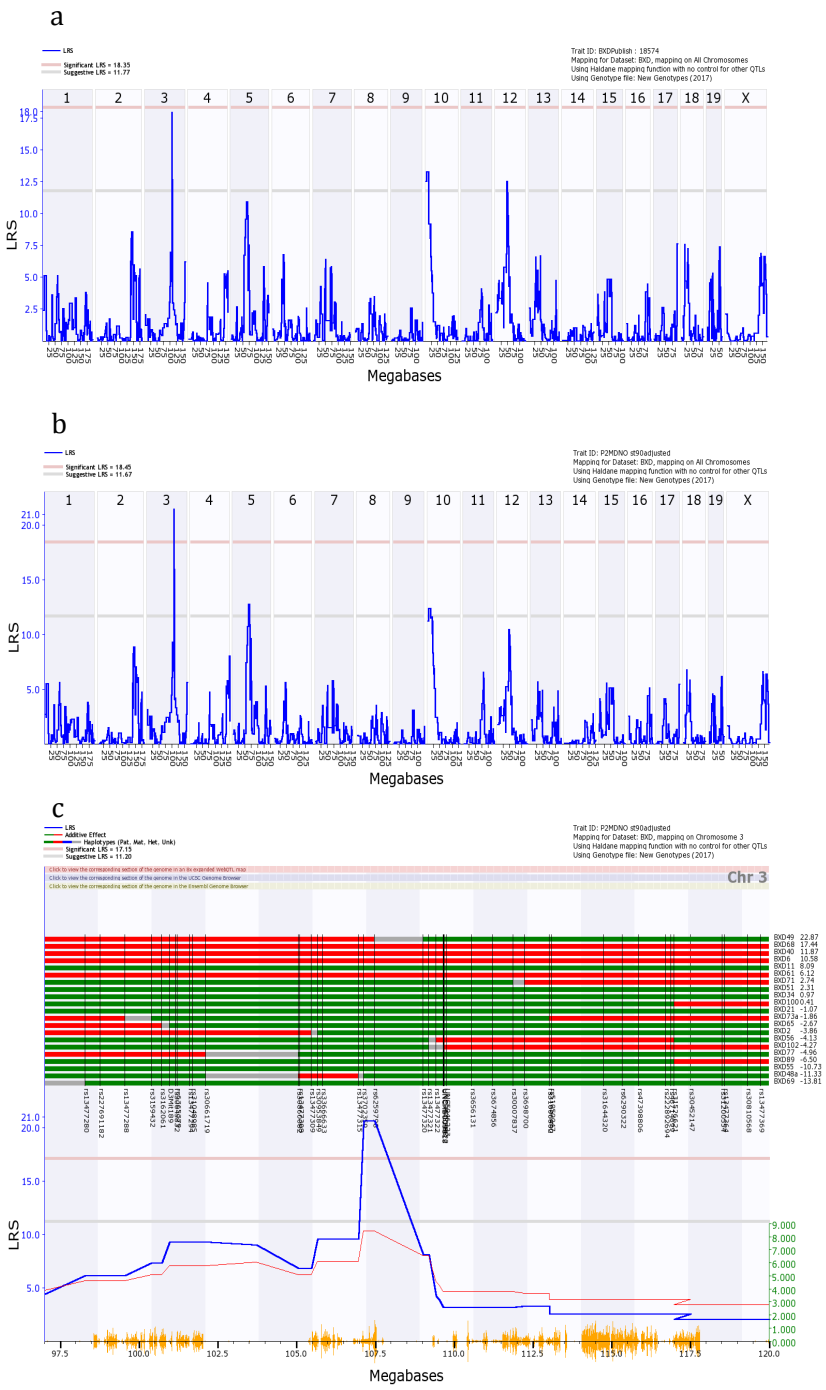

Figure 2 a) QTL map for male PPI90 difference scores. Suggestive QTL on chromosome 3. b) QTL map for male PPI90 difference scores after PPI90 strain means were adjusted for st90 reactivity. Significant QTL on chromosome 3 (107 to 108.6 mb) c) Significant QTL on chromosome 3 for male PPI90 difference scores from st0 adjusted means. Yellow lines on x-axis indicate SNP density. Red line and green numbering on right y-axis indicates the effect of the B6 allele on PPI90 difference scores (units = percent inhibition of PPI). Bars above graph indicate strain genotypes (red=B6, green=D2)

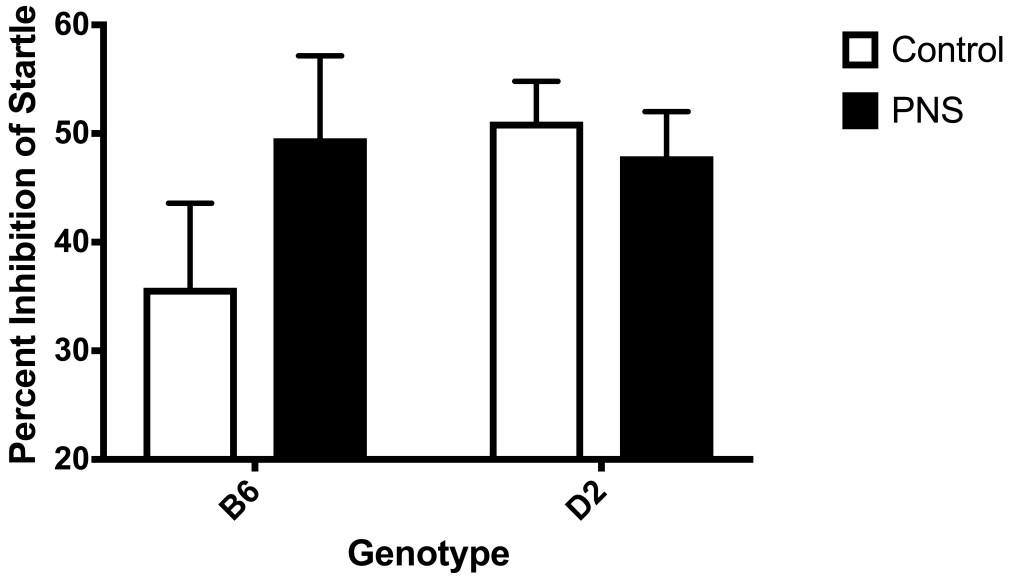

Figure 3. Male PPI90 mean and SEM, grouped by condition and genotype at the peak marker for the male PPI90 chr 3 QTL by PNS interaction

Table 3 Transcripts within the chr 3 PPI90 QTL interval with cis-eQTL and covariation with male PPI90 difference scores.

Transcript cis-eQTL							Covariation		
Gene	Probe	Position	Region	n	cis-eQTL	LRS	n	Pearson r, p-value	Spearman r, p-value
AI504432	10495183	Chr3: 107.039504	Amygdala	50	Chr3: 105.059546	43	13	0.62, 0.0219	**0.74, 0.0025**
Slc16a4	10495206	Chr3: 107.291292	Amygdala	50	Chr3: 107.326274	11	13	0.59, 0.0335	0.59, 0.0308
	A 51 P340653	Chr3: 107.311940	Midbrain	37	Chr3: 107.326274	24	10	0.65, 0.0384	0.71, 0.0192

Table 4 Genes within the chr 3 PPI90 QTL interval that contain nsSNPs

Gene	SNP ID	Chr3:Mb	Alleles (C57/DBA)
A930002I21Rik	rs30208013	107.097133	T/C
Kcna10	rs33751439	107.194802	T/C
	rs31241907	107.195489	A/C
Cym	rs33750908	107.213409	A/G
Gstm5	rs8243834	107.896457	T/A
Gstm1	rs8261761	108.015018	A/T

Discussion

The effects of PNS on ASR and PPI were assessed in 21 BXD recombinant inbred strains. Effects of PNS were found to interact with strain to alter ASR, indicating the effects of PNS on ASR are heritable in the BXD panel. No main or interaction effects for PNS were detected in either PPI measure, indicating that the effects of PNS on PPI may not be heritable or present at all in the BXD panel. However, a trend towards a strain by PNS interaction ($p=0.09$) was observed in males for PPI90. These results fit the pattern in which males are exclusively affected by PNS, as observed for ASR and multiple cocaine related phenotypes (see Chapter 3). Therefore, failure to reach significance may represent a type II error due to inadequate power.

The direction of the effect of PNS appears to be predominantly attenuation in ASR of males while an approximately equal number of strains appear attenuated and potentiated for PPI90 in males. The effects of PNS were assessed on the progenitor strains and the ASR was found to be potentiated in B6 females but not males, and potentiated in D2 males but not females. Trait distributions in an RI panel may not be constrained by the progenitor trait values. Furthermore, different alleles may affect a trait in different directions, and the direction of effect of a particular allele can even be reversed on different genetic backgrounds, indicating dramatic epistasis effects (Stephens, Sittig, & Palmer, 2015). Therefore, it is not surprising that the effects of PNS might show

strain dependent effects in both directions. There are likely to be PNS interacting alleles with effects in both directions. However, because potentiation of startle was observed in the progenitor strains, it is surprising that few if any strains were observed to be potentiated in the BXD panel. However, because the present study is a relatively small sample of the BXD panel, lack of ASR potentiation may be do to chance; i.e. missed sampling of strains with allele combinations that allow for potentiation of ASR.

A main effect of strain was detected for ASR and PPI74/90 indicating that these phenotypes are heritable in the BXD panel. These results are congruent with other studies, which found heritability of ASR and PPI in the BXD panel. The between-study reliability of ASR and PPI appears to be good, with significant correlations observed between the present study and other studies that characterized BXD strains (see table 1). Those that failed to reach significance appear to be trending towards positive correlations. The power to reach significance is likely limited by low numbers of over-lapping strains. The heritability of ASR is reported as 0.53 (females) and 0.57 (males) (Philip et al., 2010b) and 0.41 (sex collapsed) (Brigman et al., 2009). Philip et. al. (2010) report the heritability of PPI as 0.38 (female) and 0.36 (male) and Brigman et. al., (2009) report 0.32 (sex collapsed). These estimates are higher than those determined by the present study. Philip et al., (2010) characterized 60 strains and Brigman et. al., (2009) characterized 25 strains. Philip et. al., (2010) had

substantially greater power to estimate heritability and may provide a more accurate estimate. It also possible that procedural and facility differences contribute to differences in heritability between studies.

Strain differences in sensitivity to pulse intensity may act as a confound when characterizing ASR and PPI. One likely contributor is age related hearing loss. Genetically mediated differences in hearing loss may render some strains incapable of perceiving prepulses or capable but less sensitive to behavioral effects. Both the B6 and D2 progenitor strains present age-related hearing loss, with B6 beginning at 2 to 3 months of age and D2 at weaning (Willott & Turner, 1999). However, hearing loss and its relationship to ASR/PPI has been investigated in the BXD panel, and it is found that hearing loss may be a concern for pure tone pulses but did not affect white noise pulses (McCaughran et al., 1999). As the present study utilized white noise pulses, hearing loss may not be concern. Nevertheless, other factors may affect pulse sensitivity. Differences in strain startle thresholds were detected in the BXD panel when using a white noise pulse (Loos et al., 2012). Similarly, in the present study, strain differences were detected in reactivity to the 90dB prepulse-only trials. This effect was specific to males and indicated a main effect of strain and a strain by PNS interaction. Genetic correlations indicate strain means for 90 dB prepulse-only reactivity predict ASR and PPI in control and PNS males. However this relationship is dependent on an extreme outlier, indicating strain variance in

reactivity at 90 dB prepulses may be largely independent of ASR or PPI90. Correlations for strain difference scores were also assessed in order to determine associations between the effects of PNS on prepulse reactivity and ASR/PPI90. The PNS effect on 90 dB prepulse reactivity strongly associated with the effects on ASR and PPI90. As PNS effects on ASR also associate with effects on PPI90, the relationship between prepulse-only reactivity and PPI90 was assessed with the PNS effect on ASR as a controlling variable. A significant correlation remained, indicating associations between prepulse reactivity and PPI90 are not completely moderated by effects on ASR.

QTL Mapping: QTL X PNS Interactions

A suggestive sex-specific QTL X PNS interaction with a relatively high LRS score was detected on chromosome 3 for PPI90 in males (see figure 2a). Because reactivity to 90 dB prepulse-only trials associated with strain main effects and PNS by strain interactions in PPI90, the strain means for PPI90 were adjusted with 90 dB prepulse-only responses as a covariate. Difference scores were determined from adjusted means and re-mapped for males and females separately. The LRS score for the male-specific chromosome 3 QTL increased to significant with the adjusted difference scores (see figure 2b). These results suggest reactivity to the 90 dB prepulse may have obscured discovery of the chromosome 3 QTL for PPI90. However, with 90 dB prepulse-only as a covariate,

there is still not a significant main effect of PNS or strain by PNS interaction in males or females for PPI90. If these results are taken as no heritability of PNS effects on PPI, the validity of the QTL may be questionable.

Positional Candidate Genes

Discovery of the PNS interacting QTL on chromosome 3 was followed by determining cis-eQTLs for any transcripts within the 2-LOD interval, for all available brain regions in genenetwork. This yielded 9 transcripts with cis-eQTLs, one of which had strain transcript levels that met the threshold for correlation with PPI90 difference scores in males (see table 2). AI504432 maps a cis-eQTL in the amygdala and AI504432 expression levels positively correlate with PNS effects on PPI90 in males. AI504432 is a long intergenic noncoding RNA (lincRNA) with an uncharacterized function. It has expression in the developing brain but minimal adult CNS expression (Allen brain atlas). Although this transcript is not implicated in any traits or diseases, generally, GWAS frequently find disease-associated SNPs within or near lincRNAs (Cabili et al., 2011; Vinod Kumar et al., 2013). Additionally, some lincRNAs are found to be cis-regulated by disease-associated SNPs (Vinod Kumar et al., 2013). Genetic variants may act by modifying lincRNA expression and variation in lincRNA expression may have phenotypic consequences by downstream effects on gene expression. Thus, variants that affect lincRNA expression are biologically

plausible candidates for associations with psychiatric disease, but require functional characterization.

The transcript Slc16a4 did not meet the Bonferroni-corrected p-value threshold for association with PNS effects on PPI90, however this gene maps a cis-eQTL in both the midbrain and amygdala, with correlations (uncorrected $p<0.05$) to PNS effects on male PPI90 in both regions (see table 2). These results may be taken as particularly robust because expression data for midbrain and amygdala were collected independently and on different microarray platforms. Slc16a4 is a monocarboxylate transporter (MCT) isoform with expression in astrocytes (Pierre & Pellerin, 2005). MCTs transport lactate, pyruvate and ketones. Monocarboxyaltes, such as lactate, can serve as energy substrates in the CNS (Magistretti & Pellerin, 1999). It is proposed the astrocyte expression of SLC16a4 allows for a astrocyte-neuron lactate shuttling system, by which astrocytes can provide lactate for neurons in periods of hypoglycemia and hypoxia (Pierre & Pellerin, 2005). Metabolic perturbations by PNS have been reported, including elevation of lactate in the frontal cortex of PNS rats (Detka et al., 2015), however there are no reports that directly support a role for MCTs in the response to PNS.

Amino acid substitutions due to nsSNPs may alter protein function and interact with PNS to influence behavioral phenotypes. Of the transcripts within the 2-LOD interval for the chromosome 3 PPI90 QTL, 5 contain nsSNPs. The

protein coding gene GSTM1 may be considered particularly promising as candidate gene. This gene encodes for a gluthatione S-transferase isoform that catalyzes glutathione to substrates that participate in detoxification of endogenous and exogenous compounds including reactive oxygen species. GSTM1 alleles have been associated with risk for autism (Buyske et al., 2006; Ming et al., 2010). It is proposed that variants that impair GSTM1 function may interact with early life toxicant exposure to increase risk of autism. A knockout mouse for GSTM1 demonstrates interactions with early life sodium valporate exposure, a potential toxicant with damaging effects to the CNS, with increases in CNS apoptosis and decreases in play behavior (Yochum, Bhattacharya, Patti, Mirochnitchenko, & Wagner, 2010). Glutathione is down-regulated in the CNS after PNS exposure, which may compromise the neuroprotective effects of this antioxidant system (Sahu, Madhyastha, & Rao, 2012). High glucocorticoid exposure during development may increase oxidative damage by disruptions of neurotransmitter systems and consequential excitotoxicity, including oxidative damage (Song et al., 2009). Thus, a compromised glutathione system may be a mechanism by which PNS alters development by excessive oxidative stress in the CNS. These effects may be further exacerbated by alleles that also compromise the glutathione system, including variants of the GSTM1 gene. The GSTM1 nsSNP between the B6 and D2 strains (rs8261761) leads to a leucine (B6) to methionine (D2) substitution in position 128. This substitution is predicted to compromise protein function of the D2 allele (results obtained with

the POlyPhen-2 software, http://genetics.bwh.harvard.edu/pph2/). However, PNS appears to interact with the B6 allele at the chromosome 3 QTL to increase PPI, with little effect on strains carrying the D2 allele (see Figure 3). Thus, the evidence does not appear to support the possibility that compromised GSTM1 function of the D2 allele exacerbates PNS effects on PPI. Nevertheless, there is no experimental evidence to characterize differences in function between B6 and D2 alleles of the GSTM1 protein. Evaluation of PNS interactions with the GSTM1 knockout mouse may lend direct support to GSTM1 by PNS interactions and indicate that compromised GSTM1 function moderates PNS effects.

Summary

The evidence presented here indicates genetic variants in the male BXD population interact with PNS to alter ASR. PNS by strain interaction for male PPI did not reach statistical significance, but did display a trend. Furthermore, a QTL by PNS interaction was detected for this measure in males, supporting that BXD variants interact with PNS to alter PPI. Analysis of transcript expression levels within this QTL for cis-eQTL and covariance with PNS effects on PPI prioritized two candidate genes. Some genes within the QTL interval also contain snSNPs, indicating that the variant detected may act by altering protein structure. Of these genes, GSTM1 appears to be most promising, due to its implication in autism and early life GXE interactions. These candidate genes should be

investigated and validated experimentally. A gene nominated by this data can be investigated in human subjects for gene by early life interactions that confer risk for PPI-associated psychiatric disorders and simultaneously evaluated in preclinical models for effects on other phenotypes and neurobiological mechanisms. Ultimately, this approach may greatly improve our understanding of psychiatric genetics and neurobiology.

Chapter 3

Discovery of QTL by PNS interactions for cocaine reward and locomotion

Cocaine abuse disorder is considered a neuropsychiatric condition involving dysregulated and deleterious use of cocaine (Volkow et al., 2003). Cocaine abuse exacts devastating consequences on the afflicted individual and profound costs on society as a whole. The individual often experiences health, social, and legal consequences, with mortality as a far too common end point (McGinnis & Foege, 1999; Pouletty, 2002). These consequences pose a burden on society in the form of elevated health care costs, lost productivity and increased crime rates (McGinnis & Foege, 1999; Pouletty, 2002). The profound impact of cocaine abuse, and drug abuse collectively, necessitates a better understanding of etiological factors that will allow for improved treatment and preventative options. The etiology of cocaine is not completely understood, however genetics appear to have a major role. The heritability estimates for cocaine abuse range from 0.42 to 0.79 (Agrawal et al., 2012). Despite high heritability of cocaine abuse, to date, few alleles are associated with this disorder in human populations (Gelernter et al., 2014) and it is expected that many await discovery.

Preclinical models are utilized to investigate the neurobiology and genetics of cocaine abuse and have good potential for revealing genes that modulate cocaine-related behavioral phenotypes. Measurements of cocaine-induced locomotion, reward and reinforcement demonstrate heritability in rat and mouse populations. These populations have been utilized for QTL mapping studies. The bulk of these efforts have focused on locomotion; multiple QTLs

have been associated with cocaine induced locomotion, with confirmation of some QTLs by secondary mapping approaches (Boyle & Gill, 2001, 2009; Gill & Boyle, 2003; B. C. Jones et al., 1999; V. Kumar et al., 2013; Miner & Marley, 1995a, 1995b; Phillips et al., 1998; Tolliver et al., 1994; Vendruscolo et al., 2009). More recently, reward and reinforcement measures, including cocaine CPP and self-administration, have also been characterized in forward genetic approaches (Dickson et al., 2015; Philip et al., 2010a). Multiple QTLs were discovered for cocaine self-administration. Interestingly, one of these overlaps with an independently discovered cocaine locomotion sensitization QTL, suggesting these behaviors are genetically related (V. Kumar et al., 2013). Collectively, this research is making progress in identifying cocaine associated alleles and revealing the genetic relationships between the various cocaine related behaviors under study.

Environmental factors are also thought to have a role in the etiology of cocaine abuse. Early life stressors, including prenatal stress (PNS), are likely important etiological factors for many psychiatric disorders, including cocaine abuse (Enoch, 2011). Preclinical studies suggest that PNS may enhance cocaine addiction liability. PNS increases cocaine locomotion and alters self-administration parameters in rats (Kippin et al., 2008; Thomas et al., 2009). Effects on self-administration include augmented acquisition, increased intake, resistance to extinction and augmented cocaine-primed reinstatement. The

effects of PNS may be moderated by genetic variants, in gene by environment interactions that confer risk of cocaine abuse. Previous characterization of PNS effects on the B6 and D2 mouse strains revealed that PNS interacts with genotype to alter cocaine locomotion behaviors and cocaine reward (Kippin et al., 2015). These data suggest that genetic variants interact with PNS to alter cocaine responsiveness, including an increase in cocaine reward sensitivity. Identification of these variants will allow for the discovery of genes that may interact with early life stress to moderate risk of cocaine addiction. QTL mapping strategies that incorporate early life stress to search for QTL by PNS interactions are capable of genome-wide scans for PNS interacting alleles. These alleles may otherwise be undetectable in preclinical QTL studies that do not incorporate environmental factors. This approach holds promise for elucidating the early life gene by environment interactions that confer risk of cocaine abuse.

The BXD mouse panel is derived from B6 and D2 progenitors. The PNS interactions observed in the progenitor strains indicate that the BXD panel is suitable for QTL by PNS interaction mapping strategies. We sought to characterize the effects of PNS on cocaine acute locomotion, locomotion sensitization and cocaine CPP on multiple BXD strains. Cocaine CPP is a relatively high throughput procedure that simultaneously yields cocaine reward and locomotor measures. Although cocaine locomotor effects are well studied in QTL mapping experiments, reward and reinforcement measures are not.

Mapping for cocaine CPP may reveal reward related genes that are otherwise undetectable by locomotor measures. Strain variance in the effects of PNS on these measures was utilized to map for QTLs that interact with PNS to alter cocaine responsiveness. QTL discovery was followed by bioinformatics approaches to prioritize positional candidate genes. These efforts may serve as a preliminary step in identifying genes that interact with PNS to alter cocaine abuse liability.

Methods

Subjects, Breeding and PNS

Breeding and PNS was performed on BXD strains as described in chapter 2. The subjects involved in the PPI experiment (chapter 2) also received CPP/locomotion testing (following PPI).

In order to limit litter effects, a minimum of 4 litters were represented in each condition/sex for all behavioral tests and no more than 3 males or females from a litter were included in analysis. The within strain/condition/sex sample size ranged from 8 to 19.

Cocaine CPP and Locomotion

At 9 weeks of age, the PNS and control offspring were subjected to a cocaine CPP protocol. This procedure allows for assessment of the rewarding efficacy of cocaine as well as exploratory and locomotion measures that include: acute and sensitized cocaine locomotion, locomotion in a novel environment, and locomotion after saline injection (Tzschentke, 2007). The CPP procedure involves a 2-compartment chamber which distinct visual and tactile cues between the compartments. Initially, mice were placed into the chamber with access to both sides and with no injections (pre-test). Time spent in each compartment and locomotion were measured by video tracking with Any-Maze software (Stoelting, Wood Dale, IL, USA). Assignment of cocaine- and saline-paired compartments was biased, with cocaine paired to the un-preferred side. Conditioning consists of four once a day saline and cocaine sessions, alternating between saline and cocaine. Mice were injected with saline (i.p., 10 mL/kg) or cocaine (i.p., 10 mg/kg, 10 mL/kg) and immediately placed into the assigned compartment for 15 minutes. After completion of conditioning the mice were placed, without injection, into the chamber with access to both compartments (post-test). CPP was measured as the shift in time spent in the cocaine-paired compartment from pre-test to post-test. Horizontal distance traveled was measured to quantify locomotion and was tracked during pre-test, post-test and all conditioning sessions. Acute cocaine locomotion was measured as the

difference between the first saline conditioning session and the first cocaine conditioning session. Cocaine locomotion sensitization was measured as the difference between the fourth and first cocaine conditioning session.

Data Analysis and QTL Mapping

Data was assessed for strain, sex and PNS effects by 3-way ANOVA, as described in chapter 2. QTL mapping was performed as described in chapter 2.

Assessment of Reliability Across Independent Studies

Control strain means for acute locomotion, sensitization and CPP were compared to independent experiments of the same measures in BXD strains. Pearson's correlations were determined with data available on genenetwork.org.

Genetic Correlations: PNS

The effects of PNS were assessed for genetic correlation between traits. The strain difference scores for acute locomotion/sensitization, CPP, acoustic startle response (ASR) and pre-pulse inhibition with 74 and 90 db prepulses (PPI74/PPI90) were assessed by Pearson's correlation. Significant correlations

suggest common alleles mediate the effects of PNS across traits (Hegmann & Possidente, 1981).

Genetic Correlations: Cocaine Behaviors

Control data for acute locomotion/sensitization, CPP and cocaine self-administration (days to acquisition and number of infusions, data collected by Dickson et. al. 2015 and available on genenetwork.org) were assessed by Pearson's correlation. Dickson et. al. 2015 assessed cocaine self-administration at eight doses. The lowest three doses contain 8 strains in common with the present study, limiting statistical power. The top five doses contain 12 strains in common. The strain means for the top five doses correlate well to each other, therefore one dose (1.0 mg/kg/infusion) was selected for assessment with the present study. Significant correlations suggest common alleles mediate these behaviors.

Results

CPP

CPP was assessed by 3-way ANOVA. A PNS by sex interaction [$F(1, 989)=4.33$, $p=0.038$], a strain by sex interaction [$F(20, 989)=2.16$, $p=0.002$], a

main effect of strain [$F(20, 989)=12.88$, $p<0.001$], and a main effect of PNS [$F(20, 989)=10.07$, $p=0.002$] were detected. Within sex analysis revealed a main effect of strain in females $F(20, 989)=7.3$, $p<0.001$. For males, a main effect of strain [$F(20, 989)=7.95$, $p<0.001$] and a main effect of PNS [$F(1, 989)=13.24$, $p<0.001$] were detected. See figure 1a.

The heritability of CPP for control females is 0.18 and PNS females 0.10. The heritability for control males is 0.19 and PNS males 0.16.

Acute Locomotion

Acute cocaine induced locomotion was assessed by 3-way ANOVA. A strain by PNS interaction [$F(20, 966)=1.82$,$p=0.015$], a strain by sex interaction [$F(20, 966)=2.74$, $p<0.001$] and a main effect of strain [$F(20, 966)=27.07$, $p<0.001$] were detected. Within sex analysis revealed a main effect of strain [$F(20, 966)=17.3$, $p<0.001$] in females. For males, a main effect of strain [$F(20, 966)=12.93$, $p<0.001$] was detected. See figure 1b.

The heritability of acute locomotion for control females is 0.30 and PNS females 0.35. The heritability for control males is 0.31 and PNS males 0.24.

Locomotion Sensitization

Sensitization of cocaine induced locomotion was assessed by 3-way ANOVA. A strain by sex interaction [$F(20, 963)=2.92$, $p<0.001$], a PNS by sex interaction [$F(1, 963)=3.85$, $p=0.05$], a main effect of strain [$F(20, 963)=9.28$, $p<0.001$] and a main effect of sex [$F(1, 963)=11.79$, $p=0.001$] were detected. Within sex analysis revealed a main effect of strain [$F(20, 963)=4.68$, $p<0.001$] for females. For males, a main effect of strain [$F(20, 963)=5.94$, $p<0.001$] and a main effect of condition [$F(1, 963)=4.9$, $p=0.027$] were detected. See figure 1c.

The heritability of sensitization for control females is 0.16 and PNS females 0.10. The heritability for control males is 0.15 and PNS males 0.14.

Locomotion Without Cocaine

Locomotion in the pre-test, first saline conditioning trial and post-test were assessed. A 3-way ANOVA for pre-test locomotion revealed a strain by PNS interaction [$F(20, 993)=2.03$, $p=0.005$], a strain by sex interaction [$F(20, 971)=2.9$, $p<0.001$] a main effect of strain [$F(20, 993)=43.79$, $p<0.001$] and a main effect of sex [$F(1, 993)=26.17$, $p<0.001$]. Within sex analysis revealed a main effect of strain for females [$F(20, 993)=26.59$, $p<0.001$]. For males, a strain by PNS interaction [$F(20, 993)=2.21$, $p=0.002$] and a main effect of strain [$F(20, 993)=21.56$, $p<0.001$] were found.

A 3-way ANOVA for locomotion in the first saline conditioning session revealed a strain by PNS by sex interaction [$F(20, 971)=1.82$, $p=0.015$], a strain by sex interaction [$F(1, 971)=2.69$, $p<0.001$], a main effect of strain [$F(20, 971)=62.98$, $p<0.001$], a main effect of PNS [$F(1, 971)=4.19$, $p=0.041$] and a main effect of sex [$F(1, 971)=19.38$, $p<0.001$]. Within sex analysis revealed a main effect of strain in females [$F(20 ,971)=37.62$, $p<0.001$]. For males, a strain by PNS interaction [$F(20 ,971)=2.66$, $p<0.001$] and a main effect of strain [$F(20, 971)=28.35$, $p<0.001$] were found.

A 3-way ANOVA for locomotion in the post-test revealed a strain by sex interaction [$F(20, 983)=2.26$, $p=0.001$], a strain by PNS interaction [$F(20, 983$, $p<0.004$], a main effect of strain [$F(20, 983)=42.43$, $p<0.001$] and a main effect of sex [$F(1, 983)=28.86$, $p<0.001$]. Within sex analysis revealed a strain by PNS interaction for females [$F(20, 983)=1.66$, $p=0.035$] and a main effect of strain [$F(20, 983)=26.12$, $p<0.001$]. For males, a strain by PNS interaction [$F(20 ,983)=1.93$, $p=0.008$] and a main effect of strain [$F(20, 983)=18.81$, $p<0.001$] were found.

Associations between the strain effects of PNS (difference scores) on locomotion without cocaine and the strain effects of PNS on acute cocaine locomotion, cocaine locomotion sensitization and CPP were assessed by Pearson's correlation. PNS effects on female pre-test locomotion associated with the effects on female CPP ($r=-0.46$, $p=0.016$).

Reliability Across Independent Studies

A significant correlation was found for acute locomotion of the present study and Philip et. al. (2010). No significant correlations were found for sensitization or CPP. See table 1.

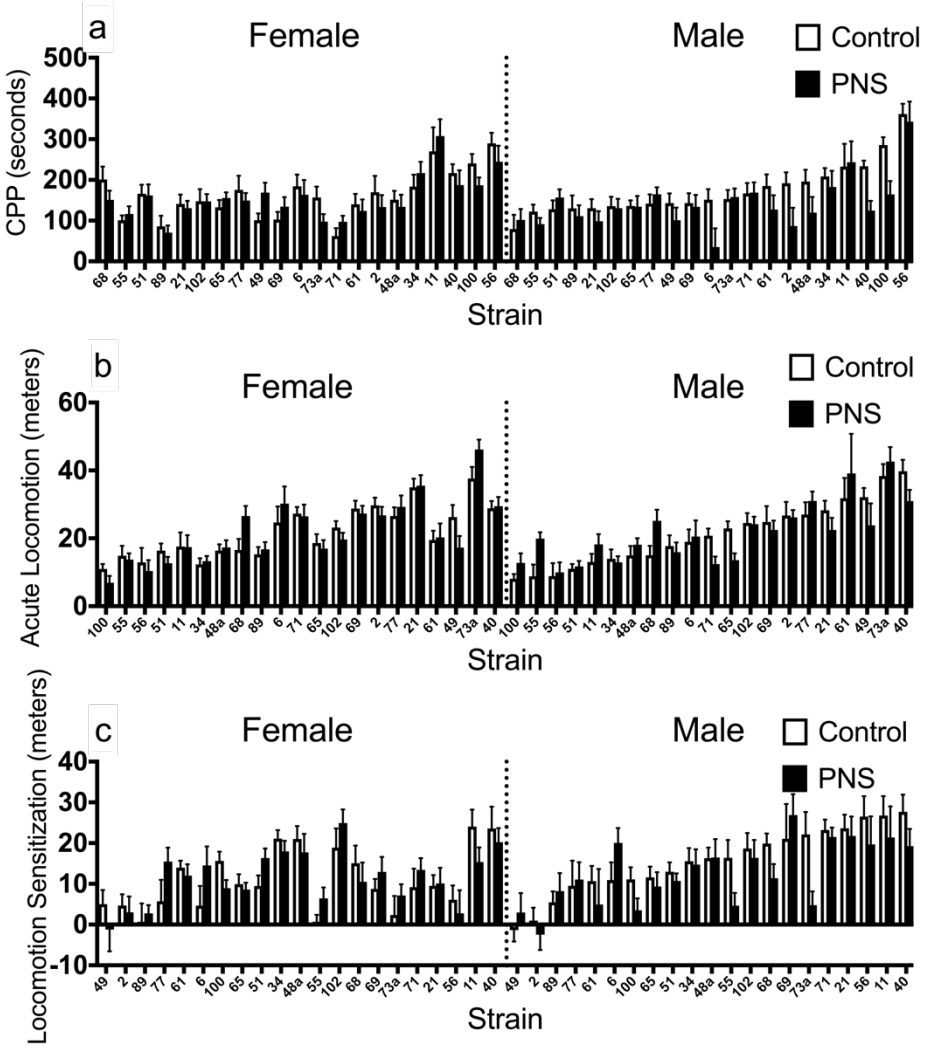

Figure 1 Effects of PNS on cocaine behaviors in BXD strains. a) PNS affects male CPP. No effects of PNS were detected for females. b) PNS interacts with strain to affect acute cocaine locomotion in both sexes. c) PNS interacts with strain to affect cocaine locomotion sensitization in males. No effects were detected in females.

Table 1 Pearson's correlations between acute cocaine locomotion/sensitization/CPP of the present study and the same measures in Philip et. al., 2010. Males and females reported separately where values differed substantially. Bold text p<0.05.

Acute Locomotion (r, p-value, n)	Sensitization (r, p-value, n)	CPP (r, p-value, n)
0.52, 0.020, 19	Female: -0.14, 0.590, 19 Male: 0.41, 0.082, 19	-0.29, 0.246, 19

Table 2 Suggestive QTLs for difference and sum scores

Phenotype	Sex	Difference or Sum Score	LRS	Chromosome	Position (mb)	Marker	Additive Effect
CPP	Female	Sum Score	11.9	4	95.744705	rs28081143	-68.013
			12.2	4	75.840751	rs28019260	-70.364
			13.6	11	68.147546	rs3677986	-78.909
	Male	Difference Score	13.4	4	74.243614	rs28098609	33.949
		Sum Score	8.5	11	74.536422	rs26920237	-77.511
Acute Locomotion	Both	Sum Score	11.8	3	90.470819	rs45891719	-10.225
			15.8	9	65.547668	rs32892673	11.455
Sensitization	Female	Sum Score	18.3	1	161.98198	rs6255075	9.896
			12.9	8	51.738632	rs33154273	8.631
			15.1	11	19.745817	D11Mit79	-9.12
			18.5	11	96.609418	rs235845435	-10.235
			12.9	15	3.236252	rs50363876	-8.624

QTL Mapping

QTL by PNS Interaction

A QTL by PNS interaction was detected for acute locomotion (alQTLXPNS) on chromosome X (LRS=17.9). The 2-LOD interval is 37.7 to 50.95 mb (see figure 2). One outlier strain was identified, however the QTL remained significant after winsorization. No other significant QTL by PNS interactions were detected. For suggestive QTLs see table 2.

Main Effect QTLs

A sex-specific main effect QTL was detected for female CPP (cppQTL), on chromosome 11 (LRS=17.9) . The 2-LOD interval is 67.5 to 81.5 mb (see figure 3). A sex-specific main effect QTL was detected for female locomotor sensitization (sensQTL), on chromosome 16 (LRS=19.7). The 2-LOD interval is 95.8 to 98.319 (see figure 4). No other significant main effect QTLs were detected. For suggestive QTLs see table 2.

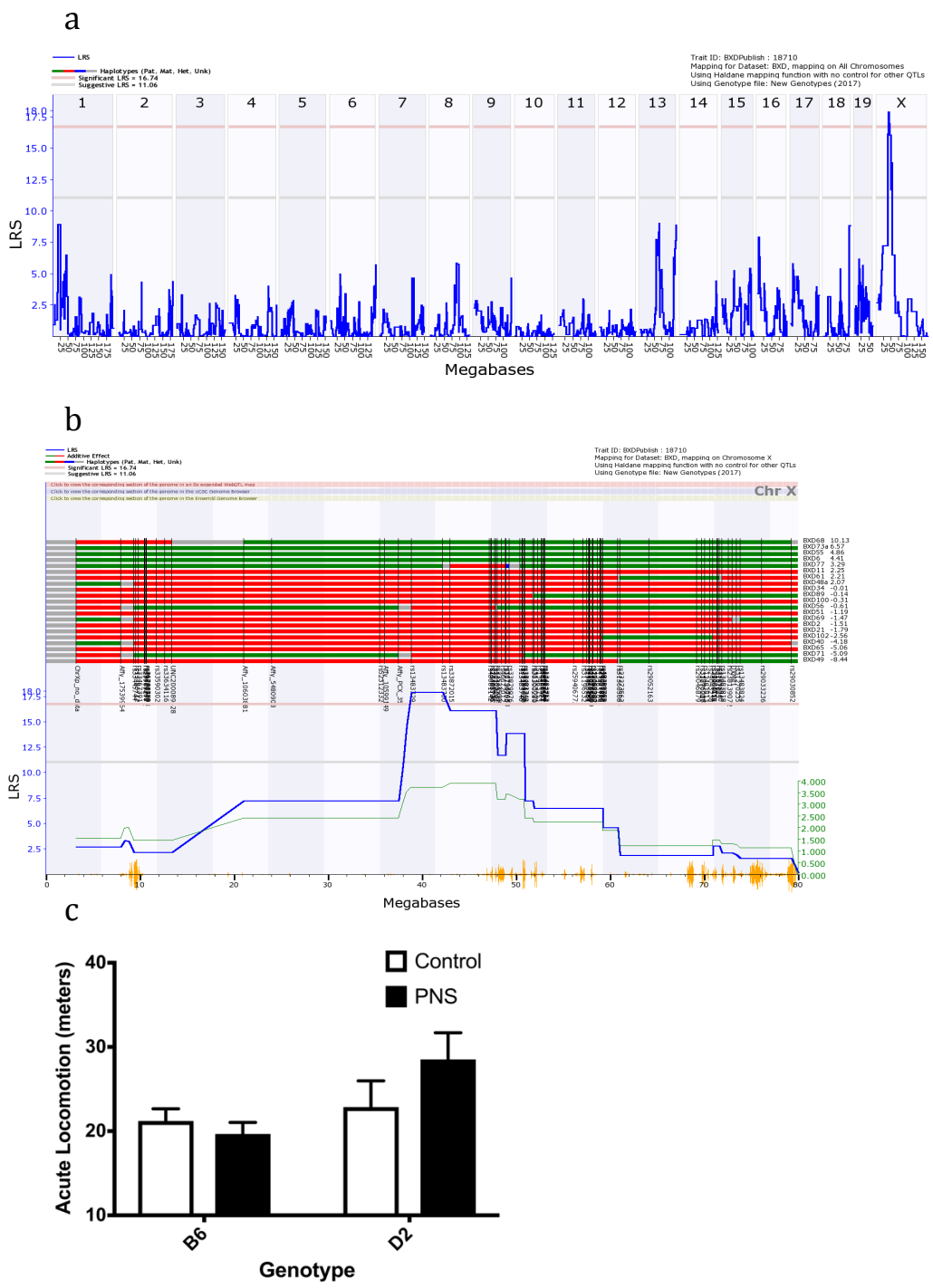

Figure 2 Acute Locomotion: QTL by PNS interaction. a) A significant QTL by PNS interaction on chromosome X. b) QTL by PNS interaction on chromosome X (green line=additive effect of B2 allele with values on right axis, red and green bars= B6 and D2 haplotypes respectively, yellow lines on x-axis=SNP density). c) Effects of genotype at the peak marker and PNS on acute locomotion.

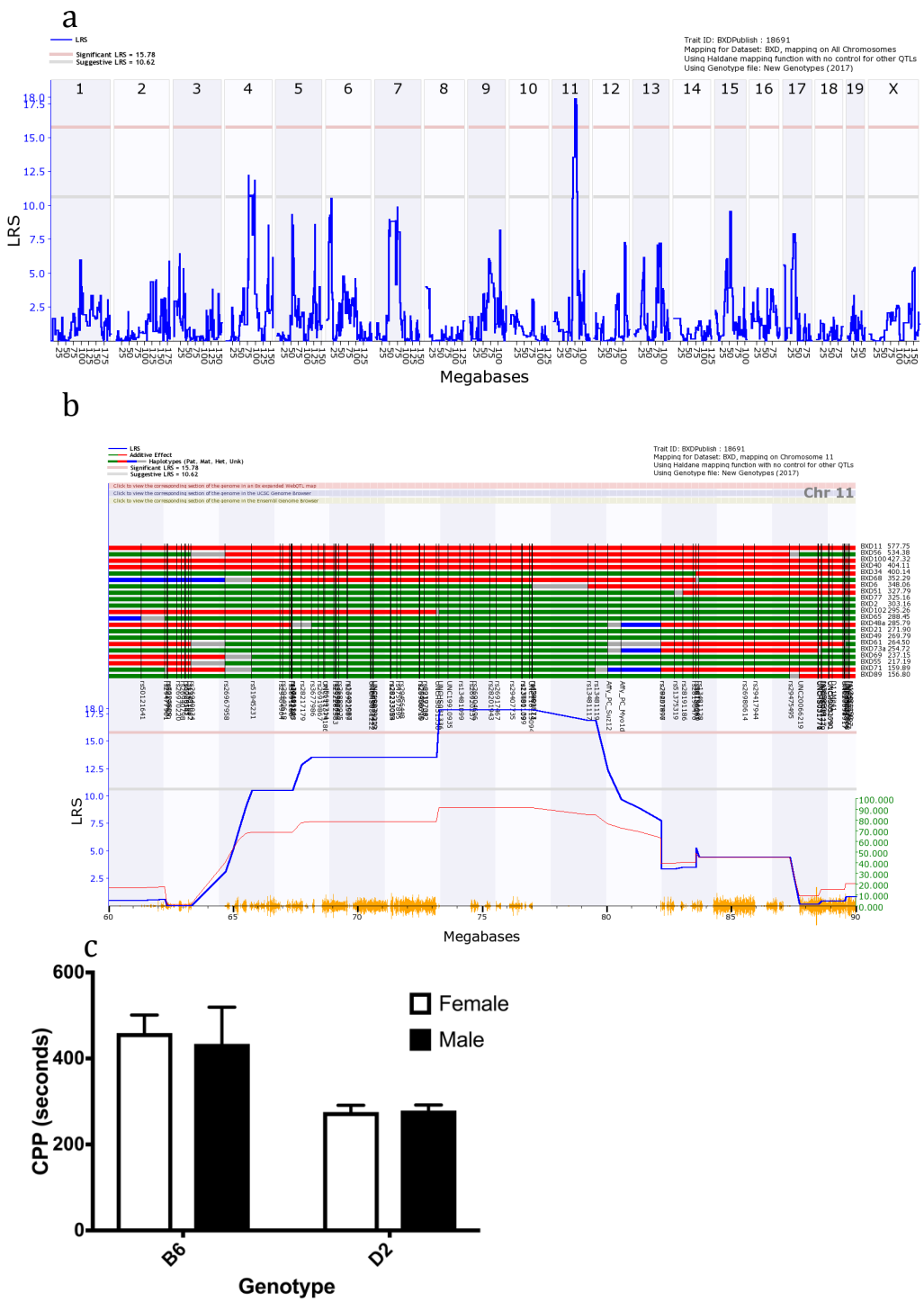

Figure 3 CPP: main effect QTL. a) A significant main effect QTL for female CPP on chromosome 11. b) Main effect QTL on chromosome 11 (red line=additive effect of B6 allele with values indicated on right y-axis, red and green bars=B6 and D2 haplotypes respectively, yellow lines on x-axis=SNP density). c) Effects of genotype at the peak marker and sex on CPP.

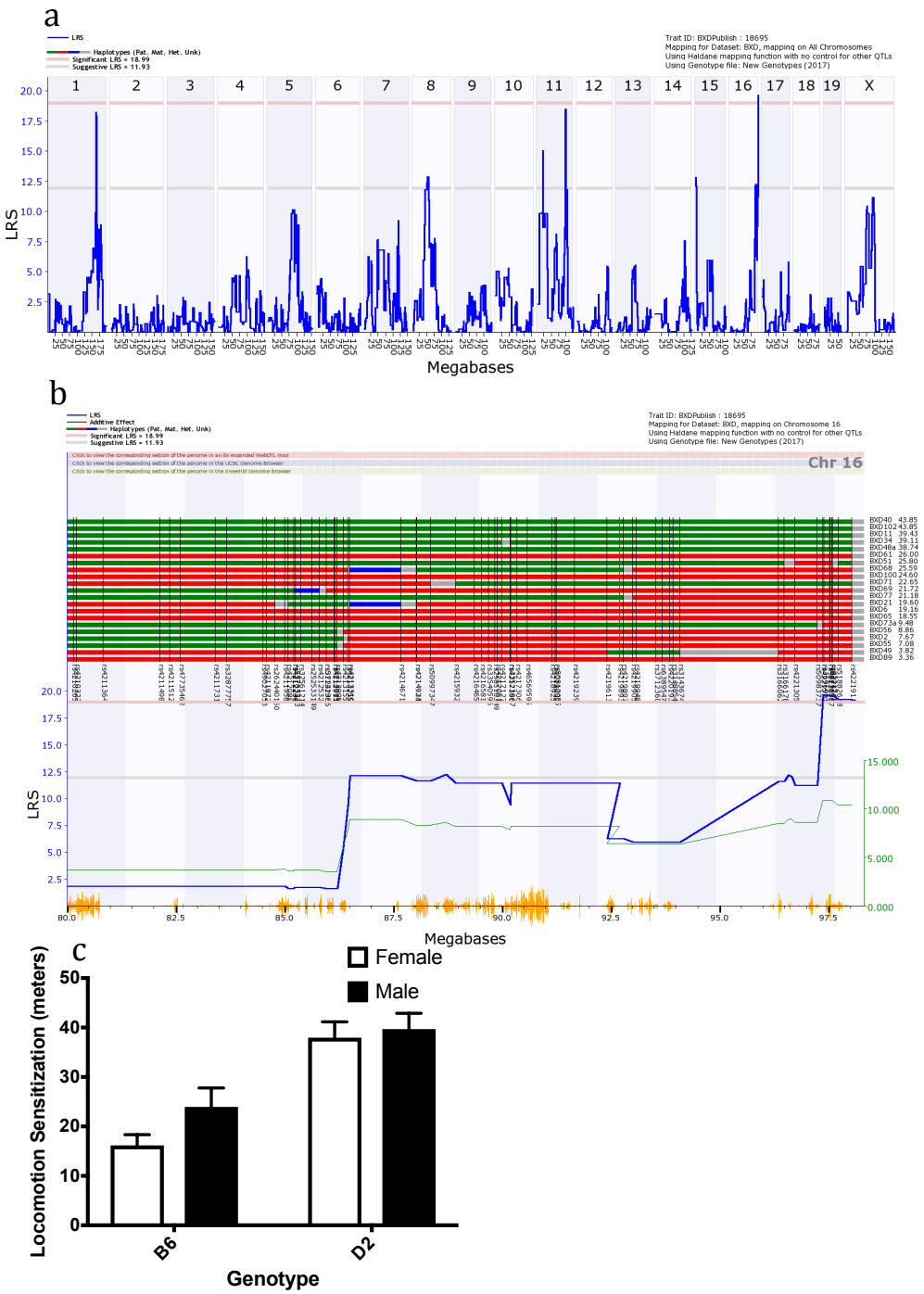

Figure 4 Sensitization: main effect QTL. a) A significant main effect QTL for female sensitization on chromosome 16. b) Main effect QTL on chromosome 16 (green line=additive effect of F2 allele with value indicated on right y-axis, red and green bars= B6 and D2 haplotypes respectively, yellow lines on x-axis=SNP density). c) Effects of genotype at the peak marker and sex on sensitization.

Positional Candidate Genes

alQTLXPNS cis-eQTL

The 2-LOD interval for alQTLXPNS contains 65 transcripts. Two of these transcripts were found to have a cis-eQTL in one or more brain regions. One of these cis-eQTL transcripts (AIFM2) has expression levels that correlate with Bonferroni-corrected (0.05/2) significance levels in the midbrain, amygdala and hypothalamus. Significant correlations were also found in the striatum and neocortex, however these regions demonstrate suggestive cis-eQTL. See table 3.

alQTLXPNS snSNP

Five genes within the alQTLXPNS 2-LOD interval contain nsSNPs. See table 4

cppQTL cis-eQTL

The 2-LOD interval for cppQTL contains 373 transcripts. 138 of these map a cis-eQTL in one or more brain regions. Of these cis-eQTL transcripts, 10 demonstrate Bonferroni-corrected (0.05/138) significant correlations to female CPP strain means. However, eight of these transcripts have one or more SNPs in

the probe target region and were excluded from consideration. These SNPs may affect probe hybridization and produce false positive cis-eQTLs. See table 3.

cppQTL snSNPs

118 genes within the cppQTL 2-LOD interval contain nsSNPs. See table 6.

sensQTL cis-eQTL

The 2-LOD interval for sensQTL contains 30 transcripts. Of these transcripts, six map a cis-eQTL in one or more brain regions. Of these cis-eQTL transcripts, one (Ripk2) demonstrates a Bonferroni-corrected (0.05/5) significant correlation to female sensitization strain means in the hippocampus. Ripk2 also correlates at uncorrected significance in the ventral tegmental area and neocortex. See table 3.

sensQTL nsSNPs

Six genes within the sensQTL 2-LOD interval contain nsSNPs. See table 5.

Table 3 Transcripts within 2-LOD QTL intervals with cis-eQTL and strain expression levels that correlate to the behavioral phenotype strain difference or sum scores. Bold text = significant at Bonferroni-corrected threshold.

		Transcript cis-eQTL						Covariation		
QTL	Gene	Probe	Position (mb)	Region	n	cis-eQTL	LRS	n	Pearson r, p-value	Spearman r, p-value
alQTLxPNS	AIFM1	10604405	ChrX: 48.474944	Amygdala	50	ChrX: 47.144891	26	13	**0.61, 0.0239**	0.56, 0.0452
		10604405	ChrX: 48.474944	Hypothalamus	50	ChrX: 48.026885	35.3	13	**0.80, 0.0006**	0.60, 0.0268
		A_55_P2002864	ChrX: 48.474961	Mid Brain	37	ChrX: 47.144891	65.5	10	**0.71, 0.019**	0.43, 0.223
		ILM2060070	ChrX: 48.504847	Neocortex	72	ChrX: 50.523592	14.1	16	**0.55, 0.0245**	0.49, 0.0561
		ILM2060070	ChrX: 48.504847	Striatum	75	ChrX: 48.504847	13.3	16	**0.69, 0.0024**	**0.61, 0.0107**
cppQTL	Pitpna	1423283_at	Chr11: 75.625491	Hippocampus	99	Chr11: 77.197052	23	16	-0.67, 0.0043	-0.71, 0.0020
		A_55_P2166773	Chr11: 75.626667	Midbrain	37	Chr11: 72.859313	27	10	**-0.95, 3.4E-5**	-0.92, 0.0005
		1423283_at	Chr11: 75.625491	NAc	36	Chr11: 76.607143	18	6	-0.93, 0.0078	-0.89, 0.0333
	Sat2	10377560	Chr11: 69.622052	Amygdala	50	Chr11: 69.788406	136	13	**0.85, 0.0002**	0.85, 0.0004
		1430318_at	Chr11: 69.623689	Hippocampus	99	Chr11: 69.788406	131	16	0.60, 0.0153	0.38, 0.1447
		10377560	Chr11: 69.622052	Hypothalamus	50	Chr11: 69.788406	142	13	0.80, 0.0010	0.75, 0.0044
		A_55_P2345425	Chr11: 69.622212	Midbrain	37	Chr11: 69.788406	133	10	0.74, 0.0154	0.64, 0.0545
		A_55_P2047345	Chr11: 69.623810	Midbrain	37	Chr11: 69.788406	143	10	0.73, 0.0165	0.40, 0.2568
		10377560	Chr11: 69.622052	Pituitary	52	Chr11: 69.788406	119	14	0.78, 0.0009	0.75, 0.0033
		ILM5860731	Chr11: 69.622901	Striatum	75	Chr11: 72.494723	29	16	0.53, 0.03367	0.59, 0.0186
sensQTL	Ripk4	1418487_at	Chr16: 97.741954	Hippocampus	99	Chr16: 97.356657	28	16	**0.65, 0.0065**	**0.68, 0.0037**
		ILM360019	Chr16: 97.742194	Neocortex	72	Chr16: 97.496522	25	16	0.44, 0.0907	0.57, 0.0249
		1418488_s_at	Chr16: 97.742021	VTA	37	Chr16: 96.501253	16	6	0.87, 0.0256	0.89, 0.0333

Table 4 nsSNPs in genes within the chr X acute locomotion QTL by PNS interaction interval.

Gene	SNP ID	ChrX:Mb	Alleles (C57/DBA)
Smarca1	wt37-X-45245444	45.24544	T/G
	wt37-X-45245464	45.24546	T/C
Arhgap36	wt37-X-46850395	46.8504	G/A
	MRS4841936	46.8504	G/A
	wt37-X-46851809	46.85181	T/A
	MRS4841937	46.85181	T/A
Olfr1320	wt37-X-47037080	47.03708	C/A
	MRS4842190	47.03708	C/A
Xpnpep2	rs8276099	48.11698	G/C
	rs8276212	48.11841	G/T
Gpc4	wt37-X-49406763	49.40676	C/T
	MRS4843276	49.40676	C/T
Arhgap36	rs31214442	49.49863	T/A
Fam122c	wt37-X-50635748	50.63575	G/A
	MRS4843712	50.63575	G/A

Table 5 nsSNPs in genes within the chr. 16 sensitization main effect QTL interval.

Gene	SNP ID	Chr16:Mb	Alleles (C57/DBA)
Itgb2l	wt37-16-96647834	96.64783	C/T
	MRS4373332	96.64838	T/C
Tmprss2	rs3692626	97.56906	T/C
Bace2	wt37-16-97578440	97.57844	C/T
	wt37-16-97578517	97.57852	C/G
Tmprss2	rs4221760	97.59675	A/G
	wt37-16-97790666	97.79067	T/C
	wt37-16-97818358	97.81836	A/G
	MRS4374560	97.81836	A/G
Prdm15	wt37-16-98072702	98.0727	T/C
	MRS4375160	98.0727	T/C
C2cd2	rs3165977	97.88621	G/T
	wt37-16-98107813	98.10781	G/T

Table 6 nsSNPs in genes within the chr 11 CPP main effect QTL interval.

Gene	SNP ID	Chr11:Mb	Alleles (C57/DBA)
Glp2r	37-11-675536	67.553625	A/C
	37-11-675536	67.553669	C/T
	37-11-675545	67.554554	A/G
	37-11-675545	67.554561	T/C
	37-11-675558	67.555805	T/C
	37-11-675843	67.584338	C/A
	rs28217203	67.740122	A/C
	rs28217197	67.741051	A/G
	rs28217187	67.742302	T/C
	rs29401189	67.770835	C/A
Odf4	37-11-687404	68.740438	T/C
Arhgef15	37-11-687583	68.758317	G/T
	37-11-687670	68.767028	C/T
	37-11-687679	68.767958	G/A
	37-11-687679	68.76799	T/A
Rangrf	37-11-687886	68.788642	G/A
Pfas	37-11-688015	68.801535	A/C
	37-11-688017	68.801726	C/A
1500010J02Rik	37-11-688345	68.83459	G/A
	37-11-688346	68.834601	G/A
	37-11-688409	68.8409	T/C
	37-11-688449	68.844943	G/T
Aurkb	37-11-688613	68.861372	T/C
9330160F10Rik	37-11-688734	68.873447	A/G
	37-11-688736	68.873671	G/A
Per1	37-11-689227	68.922718	G/A
Odf4	rs13481085	68.926935	T/C
Hes7	37-11-689364	68.936458	T/C
Arhgef15	rs26921890	68.944814	G/T
Aloxe3	37-11-689477	68.947714	T/A
Rangrf	rs29423376	68.975139	G/A
Alox12b	MRS3206382	68.982363	G/A
Pfas	rs29427934	68.988032	A/C
	rs29400296	68.988223	C/A
1500010J02Rik	rs29462951	69.021087	G/A
	rs29420274	69.021098	G/A
	rs29449054	69.027397	T/C
	rs29414447	69.03144	G/T
Aurkb	rs29417126	69.047869	T/C
Gucy2e	37-11-690494	69.049498	A/G
	37-11-690501	69.050105	C/G
Cntrob	37-11-691178	69.11781	G/A
Trappc1	37-11-691391	69.139157	C/T
Kcnab3	37-11-691403	69.140328	C/T
Chd3	37-11-691587	69.15871	C/T
	37-11-691626	69.162694	C/T
	37-11-691636	69.163687	C/T
	37-11-691644	69.16441	A/G
	37-11-691698	69.169866	A/G
	37-11-691713	69.171345	T/C
	37-11-691726	69.172694	C/T
	37-11-691737	69.173772	G/A
	37-11-691741	69.174134	G/A
	37-11-691749	69.174953	A/G
Cyb5d1	37-11-692087	69.208704	A/G
Kdm6b	37-11-692173	69.217365	G/C
	37-11-692177	69.217773	C/A
	37-11-692180	69.218079	C/A
	37-11-692211	69.221142	G/A
Gucy2e	rs26889949	69.236602	C/G
Dnahc2	37-11-692502	69.250286	G/T
	37-11-692503	69.250323	C/G
	37-11-692787	69.278785	A/G
Dnahc2	37-11-693063	69.306306	T/C
Chd3	rs29414255	69.349191	C/T
	rs29444756	69.350184	C/T
	rs29397045	69.357842	T/C
	rs26938077	69.359191	C/T
	rs29429936	69.36145	A/G
Wrap53	37-11-693752	69.375291	C/T
	37-11-693756	69.375697	C/T
	37-11-693912	69.39121	T/C
Kdm6b	rs29424868	69.403862	G/C
	rs29388988	69.40427	C/A
	rs29458579	69.407639	G/A
Dnahc2	rs26922737	69.436783	G/T
	rs29406499	69.43682	C/G
Sox15	37-11-694692	69.469278	A/G
	37-11-694700	69.470097	G/T
Cd68	37-11-694779	69.477994	T/G
Wrap53	rs29466452	69.561788	C/T
	rs26922595	69.562194	C/T
	rs26922542	69.577707	T/C
Zbtb4	37-11-695894	69.589425	A/G
	37-11-695953	69.595358	T/C
Chrnb1	37-11-695985	69.598554	C/A
	37-11-695992	69.599286	T/A
Tmem102	37-11-696186	69.618603	G/C
Spem1	37-11-696345	69.634572	T/C
	37-11-696348	69.634855	C/T
Cd68	rs26908342	69.664491	T/G
Tnk1	37-11-696656	69.665628	C/T
	37-11-696656	69.665691	G/A
	37-11-696699	69.669956	C/A

Gene	SNP ID	Chr11:Mb	Alleles (C57/DBA)
Acap1	37-11-696981	69.698133	C/T
	37-11-697033	69.703366	C/T
Neurl4	37-11-697225	69.72258	G/A
Rai12	37-11-697819	69.781959	G/T
	37-11-697819	69.781998	T/C
	37-11-697842	69.784217	C/T
Spem1	rs26908210	69.821069	T/C
	rs26908209	69.821352	C/T
Dlg4	37-11-698447	69.844732	A/G
Tnk1	rs26908182	69.852188	G/A
Dlg4	37-11-698580	69.858096	G/A
Mgl2	37-11-699485	69.948554	T/C
	MRS3212189	69.949943	G/A
	37-11-699502	69.950221	C/T
	37-11-699505	69.950559	G/C
Clec10a	37-11-699839	69.983939	C/T
	37-11-699840	69.984017	G/A
Bcl6b	37-11-700405	70.040501	C/T
	37-11-700412	70.04121	G/T
	37-11-700425	70.042597	G/A
Alox12	37-11-700679	70.067975	C/A
Alox12e	37-11-701300	70.130001	C/T
	37-11-701313	70.13137	C/A
	37-11-701342	70.134261	C/A
Alox15	37-11-701581	70.158191	G/A
	37-11-701607	70.160749	A/G
	37-11-701634	70.163426	C/T
Pelp1	37-11-702074	70.207475	G/A
	37-11-702089	70.208955	A/G
	37-11-702099	70.209914	A/T
Cxcl16	37-11-702694	70.269472	A/G
Zmynd15	37-11-702746	70.27461	G/A
Gltpd2	37-11-703336	70.333612	G/C
Pld2	37-11-703688	70.368853	C/T
Mink1	37-11-704223	70.422387	C/T
	37-11-704231	70.423124	C/T
	37-11-704231	70.423148	A/G
Chrne	37-11-704290	70.429048	G/A
	37-11-704311	70.431164	C/T
Gp1ba	37-11-704540	70.454052	C/A
	37-11-704543	70.45438	C/A
	37-11-704543	70.454389	A/C
	37-11-704545	70.454545	C/T
Camta2	37-11-704835	70.483527	A/G
Inca1	37-11-705024	70.502495	T/C
Kif1c	37-11-705421	70.542157	T/C
	37-11-705422	70.542274	A/T
Rabep1	37-11-707479	70.747914	G/A
Nup88	37-11-707832	70.783207	C/T
Dhx33	37-11-708056	70.80566	T/C
9330403K07Rik	37-11-708467	70.846738	A/G
Nlrp1a	37-11-709057	70.905745	T/C
	37-11-709059	70.905959	G/A
	37-11-709107	70.910753	C/T
	MRS3217962	70.927017	C/T
	37-11-709364	70.936475	T/C
	37-11-709370	70.937053	C/T
	37-11-709371	70.937158	C/T
	37-11-709375	70.93759	T/C
Nlrp1b	37-11-709857	70.985715	G/A
	37-11-710308	71.030853	C/A
	37-11-710309	71.030977	G/T
	37-11-710310	71.031007	C/T
	37-11-710310	71.031028	C/A
	37-11-710311	71.031151	T/A
	37-11-710311	71.031155	C/T
	37-11-710311	71.031164	C/T
	37-11-710312	71.031244	A/T
	37-11-710312	71.031251	A/C
	37-11-710312	71.031263	C/T
	37-11-710314	71.031415	C/T
	MRS3218579	71.031668	G/A
	MRS3218580	71.031692	T/C
	MRS3218581	71.03173	T/A
	37-11-710318	71.031805	C/A
	37-11-710318	71.031837	C/A
	rs28255433	71.217439	T/C
	rs28255431	71.217529	T/C
	rs28255427	71.217912	C/T
	rs28255424	71.217966	C/G
Nlrp1b	rs28255423	71.217979	G/A
Wscd1	37-11-715853	71.585386	A/G
	37-11-715853	71.585398	T/C
	37-11-715967	71.596795	C/T
	37-11-715978	71.597803	G/A
	rs29431171	71.783292	C/T
	rs26907678	71.7843	G/A
Pitpnm3	37-11-718653	71.865372	A/G
9933427D14Rik	37-11-719822	71.982261	C/T
	37-11-719920	71.992061	C/T
	37-11-719939	71.993961	T/C
	37-11-719940	71.994063	G/T
	37-11-719941	71.994126	T/C
	37-11-720031	72.003118	G/T
	37-11-720120	72.012036	C/A

Gene	SNP ID	Chr11:Mb	Alleles (C57/DBA)
Pitpnm3	rs26902875	72.051869	A/G
Slc13a5	37-11-720726	72.072601	T/C
	37-11-720726	72.072625	C/T
Xaf1	37-11-721254	72.125494	C/G
Fbxo39	37-11-721303	72.130348	C/T
9933427D14Rik	rs29474802	72.168758	C/T
	rs29458474	72.180458	T/C
	rs29454275	72.18056	G/T
	rs29387461	72.180623	T/C
	rs26889322	72.189615	G/T
	rs26889296	72.198533	C/A
Smtnl2	37-11-722174	72.217446	A/T
Ggt6	37-11-722508	72.250853	C/G
	MRS3228294	72.251414	C/T
	37-11-722514	72.251415	G/A
	37-11-722514	72.251453	A/C
Slc13a5	rs26915262	72.259098	T/C
Mybbp1a	37-11-722644	72.264413	A/G
	37-11-722649	72.264923	C/A
Spns2	37-11-722700	72.270098	A/G
Fbxo39	rs26915101	72.316845	C/T
Spns3	37-11-723185	72.318589	C/A
	37-11-723635	72.363539	T/C
	37-11-723635	72.363593	G/A
	37-11-723636	72.363662	T/C
Smtnl2	rs29399764	72.403943	A/T
Mybbp1a	rs4228854	72.45091	A/G
	rs29470173	72.45142	C/A
Spns2	rs26900785	72.456595	A/G
Spns3	rs26900650	72.550036	T/C
Ankfy1	37-11-725629	72.562949	A/G
	37-11-725671	72.567177	G/A
Zzef1	37-11-726886	72.688661	G/C
	37-11-726886	72.688696	C/T
	37-11-726941	72.694184	A/G
	37-11-727313	72.731356	A/G
Ankfy1	rs26887875	72.749446	A/G
	rs26887860	72.753674	G/A
Camkk1	37-11-728507	72.850734	G/A
Zzef1	rs29472036	72.875158	G/C
	rs29387618	72.875193	C/T
	rs26934219	72.880681	A/G
Itgae	MRS3232352	72.917389	T/A
	37-11-729174	72.91741	G/A
	37-11-729291	72.929142	A/G
	37-11-729306	72.930677	G/A
	37-11-729306	72.930691	C/T
	37-11-729306	72.930697	T/C
	37-11-729307	72.930733	A/G
	37-11-729354	72.935433	A/T
	37-11-729367	72.936712	G/A
	37-11-729388	72.938804	C/G
	37-11-729404	72.940429	A/G
	37-11-729519	72.951973	C/T
Itgae	37-11-729591	72.95914	C/T
P2rx5	37-11-729846	72.984698	C/T
Trpv3	37-11-730831	73.083189	C/T
	37-11-730924	73.092479	G/A
	37-11-730971	73.097178	A/G
Aspa	37-11-731356	73.135689	G/T
Olfr23	37-11-737538	73.753855	C/T
E130309D14Rik	37-11-744435	74.443556	A/G
	rs29409914	74.630053	A/G
Smg6	37-11-749697	74.969768	G/T
Hic1	37-11-749810	74.981028	G/A
Rtn4rl1	37-11-750795	75.079539	C/T
Smg6	rs28232340	75.156265	G/T
Rtn4rl1	rs29461496	75.266036	C/T
Slc43a2	37-11-753859	75.385972	C/T
	rs26917433	75.572469	C/T
Rnmtl1	37-11-760575	76.057579	G/A
	MRS3235283	76.057978	A/G
	MRS3235307	76.060897	C/T
Timm22	37-11-762206	76.220619	T/G
Rnmtl1	rs13459131	76.247394	C/T
Timm22	rs28231789	76.407116	T/G
Slc6a4	37-11-768240	76.824058	G/A
	37-11-768265	76.826534	A/G
	rs13481111	77.010555	G/A
	rs29413009	77.013031	A/G
Vtn	rs4228928	78.501688	A/T
Fam58b	37-11-785649	78.564985	G/C
Nf1	37-11-793607	79.360701	C/G
	rs13481119	79.547198	C/G
Suz12	37-11-798070	79.807015	C/G
	37-11-798070	79.80703	T/G

Genetic Correlations: PNS

A significant correlation was detected between female acute locomotion difference scores and female CPP difference scores, and female PPI90 difference scores and female ASR difference scores. No other significant correlations were detected. See table 7.

Genetic Correlations: Cocaine Behaviors

A significant correlation was detected between sensitization and CPP, and sensitization and cocaine self-administration infusions (data collected by Dickson et. al. 2015). A significant correlation was detected between CPP and days to meet self-administration acquisition (data collected by Dickson et. al. 2015). See table 8.

Table 7 Genetic correlations for PNS effects on cocaine and sensorimotor behaviors. Bold text indicates significance (p<0.05).

			CPP	Acute	Sensitization	ASR	PPI74	PPI90
CPP	Female	r		**-0.51**	-0.07	-0.01	0.06	-0.02
		p-value		**0.02**	0.75	0.96	0.81	0.94
	Male	r		0.09	-0.11	-0.09	0.06	-0.02
		p-value		0.72	0.65	0.70	0.79	0.92
Acute	Female	r	**-0.51**		0.23	-0.02	0.07	-0.03
		p-value	**0.02**		0.31	0.94	0.77	0.91
	Male	r	0.09		-0.40	-0.09	-0.08	-0.10
		p-value	0.72		0.07	0.69	0.72	0.67
Sensitization	Female	r	-0.07	0.23		-0.04	-0.05	-0.24
		p-value	0.75	0.31		0.88	0.82	0.30
	Male	r	-0.11	-0.40		0.04	-0.15	0.00
		p-value	0.65	0.07		0.87	0.52	1.00
ASR	Female	r	-0.01	-0.02	-0.04		0.01	**0.54**
		p-value	0.96	0.94	0.88		0.98	**0.01**
	Male	r	-0.09	-0.09	0.04		0.11	0.38
		p-value	0.70	0.69	0.87		0.64	0.09
PPI74	Female	r	0.06	0.07	-0.05	0.01		0.07
		p-value	0.81	0.77	0.82	0.98		0.75
	Male	r	0.06	-0.08	-0.15	0.11		0.15
		p-value	0.79	0.72	0.52	0.64		0.51
PPI90	Female	r	-0.02	-0.03	-0.24	**0.54**	0.07	
		p-value	0.94	0.91	0.30	**0.01**	0.75	
	Male	r	-0.02	-0.10	0.00	0.38	0.15	
		p-value	0.92	0.67	1.00	0.09	0.51	

Table 8 Within and across study genetic correlations for cocaine behaviors. Bold text indicates significance (p<0.05). Lower left half are Pearson's correlations, upper right are Spearman's rank order correlations. IVSA measures were collected by Philip et. al. (2010). All other measures come from the present study.

		CPP	Acute Locomotion	Sensitization	IVSA Acquisition	IVSA Infusions
CPP	r		-0.181	**0.495**	**0.681**	0.165
	p-value		0.434	**0.023**	**0.01**	0.59
Acute Locomotion	r	-0.27		-0.145	-0.175	0.044
	p-value	0.237		0.529	0.569	0.887
Sensitization	r	**0.462**	-0.031		0.014	**0.808**
	p-value	**0.035**	0.896		0.964	**0.001**
IVSA Acquisition	r	**0.667**	-0.096	0.133		-0.215
	p-value	**0.013**	0.755	0.664		0.201
IVSA Infusions	r	0.133	0.15	**0.675**	-0.112	
	p-value	0.664	0.625	**0.011**	0.508	

Discussion

The present study assessed the effects of PNS on cocaine locomotion and reward in 21 BXD strains. PNS was found to interact with strain to affect acute cocaine locomotion and locomotion sensitization. A main effect of PNS was found for CPP, however no interaction was detected. These results may indicate that the effects of PNS on CPP are not heritable in the BXD panel. However, many strains appear to be unaffected while others demonstrate large PNS induced changes in CPP, and the male strain by PNS interaction trends towards significance (p-value = 0.09). The lack of significant interaction may be due to the potential complexity of environment and polygenetic interactions as well as insufficient power. Collectively, these results suggest the BXD panel is suitable for discovery of QTLs that interact with PNS to alter cocaine-related behaviors.

The direction of PNS effect on strain means appears mixed for acute locomotion. However, most affected strains appear to be attenuated for sensitization and CPP. Assessment of the progenitor strains indicated that PNS increased CPP in B6 but not D2 mice. Similarly, PNS enhanced cocaine-induced CPP in outbred rats (Pastor et al., 2016). Therefore, it is expected that some alleles should interact with PNS to enhance rewarding properties of cocaine in some mice. As with acoustic startle (see chapter 2), the relatively small sample of BXD strains in the present study may have missed strains with allele combinations that allow for strain by PNS interactions that increase CPP. This

may be especially likely if genetically mediated PNS enhancement of CPP and ASR is rare, possibly due to complex gene-gene interactions.

The effects of PNS on sensitization, CPP and locomotion without cocaine in the pre-test/first saline session appear to be exclusive to males. Similar results were found for ASR and PPI. Collectively, these results suggest that males are more sensitive to the effects of PNS. Sex effects are commonly reported in PNS studies, with sensitivity of the sexes varying depending on the phenotype under investigation. With respect to cocaine, PNS has been reported to augment locomotor sensitization in females but not males and to increase acquisition and overall cocaine self-administration intake in males but not females (Thomas et al., 2009). PNS also has sex-specific effects on hedonic sensitivity to natural rewards. PNS increased milk-chocolate CPP in males, but attenuated the same measure in females (Reynaert et al., 2015). Characterization of the B6 and D2 mouse strains revealed sex effects and sex by strain interactions. PNS augmented acute cocaine locomotion in B6 males but not females, or either sex of the D2 strain. PNS augmented acoustic startle in D2 males, and decreased startle in B6 males, but females of either strain were unaffected. And PNS decreased PPI with a 90 db prepulse (PPI90) of D2 females but not males. Considering sex-effects in the progenitor strains, it is not surprising that sex effects are observed in the BXD strains. Furthermore, where sex-effects occurred in the progenitor strains, males were more likely to be affected than females,

with the exception of PPI90. This generally fits the pattern observed in BXD strains, however CPP was only affected in BXD males, whereas both sexes of the B6 strain were affected. Although the biological mediators of these sex effects are unknown, it may be that a general sensitivity to PNS in males allows PNS interacting alleles to exert greater influence relative to females. This possibility may explain the absence of BXD female effects in CPP, despite PNS effects on B6 female CPP. The B6 strain may also be an extreme responder, considering potentiation of CPP by PNS was not observed in the BXD strains

All cocaine-related behaviors demonstrate heritability in the present study. These results are congruent with previous studies, including multiple studies that characterized BXD strains (B. C. Jones et al., 1999; Philip et al., 2010a; Phillips et al., 1998; Tolliver et al., 1994). However, three of these studies have four or less common strains with the present study, making correlational analysis inappropriate. Philip et al., (2010) contains 19 common strains with the present study. Acute locomotion as measured by Philip et. al., 2010 demonstrates a significant relationship with acute locomotion of the present study. However, locomotor sensitization and CPP do not. The procedures for these measures were substantially different from the present study. Although Philip et. al., (2010) assessed locomotion with the same dose as the present study, sensitization was measured on the second consecutive day of cocaine administration. The present study assessed sensitization after 4 cocaine

administrations and 9 days from the initial administration. Philip et al., (2010) assessed CPP with a 3 mg/kg dose, whereas the present study utilized a 10 mg/kg dose. Genetic effects can be largely dose dependent for locomotion measures, with heritability increasing with dose (B. C. Jones et al., 1999; Tolliver et al., 1994). This is likely also true for CPP. These procedural and dose differences may account for the lack of relationship between these measures. Philip et. al. (2010) report heritability of acute locomotion (not corrected for saline locomotion) as 0.41/0.33 (females/males), and Phillips et. al. (1998) report 0.28 (females) for saline corrected locomotion at a cocaine dose of 10 mg/kg. The heritability of uncorrected acute locomotion in the present study (0.42/0.38, females/males) is similar to Philip et. al. (2010) and the saline corrected heritability (0.30/0.31, female/male) is similar to Phillips et. al. (1998). For sensitization, Philip et. al. (2010) report 0.12/0.04 (females/males) and Phillips et. al. (1998) report 0.17 (females). Heritability of sensitization in the present study (0.16/0.15, females/males) is similar to Phillips et. al., (1998) but somewhat higher than Philip et. al., (2010). Greater congruence with Phillips et. al., (1998) may be due to more procedural similarities. CPP heritability in the present study (0.18/0.19, females/males) is also somewhat higher than the Philip et. al., (2010) value of 0.11/0.11 (females/males), this may be due to increased heritability with increased dose. Collectively, heritability estimates between studies are quite similar despite several procedural differences. This similarity, in addition to correlations of strain means between independent

experiments indicate the stability and reliability of genetic effects on cocaine responsiveness in the BXD strains, although dose and procedural differences may have a substantial impact on similarity.

QTl Mapping: QTL X PNS Interactions

QTL mapping for strain difference scores revealed a QTL by PNS interaction for acute cocaine locomotion (alQTLxPNS) on the X chromosome. The D2 genotype at the peak marker interacts with PNS to increase locomotion (see figure 2). The 2-LOD interval spans 13.25 mb and contains 65 genes. Positional candidate genes were prioritized by evaluation of cis-eQTL and nsSNPs.

The gene apoptosis inducing factor, mitochondrion associated 1 (aifm1) is located within the 2-LOD interval and was found to have cis-regulated transcripts in multiple brain regions, with expression levels that correlate with acute locomotion strain difference scores. The aifm1 gene encodes for a apoptosis inducing factor (aif) isoform that locates in the mitochondria and participates in metabolic reactions. Upon cellular injury, it translocates to the nucleus to initiate apoptosis (Sevrioukova, 2011). Several lines of evidence suggest that deficiencies of aif may have protective effects against neuronal insults. Down-regulation of aif increases neural progenitor survival after

hypoxia/ischemia (Y. Sun, Zhang, Wang, Blomgren, & Zhu, 2012). Similarly, sequestration of aif in the mitochondria has a neuro-protective effect against hypoxia/ischemia in neonatal mice (Matsumori et al., 2005). NMDA-mediated neuronal excitotoxicity causes aif translocation from mitochondria to nucleus (Wang et al., 2004). Inhibition of aif by short-hairpin RNA (siRNA) protects hippocampal neurons against glutamate toxicity (Öxler, Dolga, & Culmsee, 2012), suggesting a causal role for aif in excitotoxic cell death. Although there is no previous evidence to directly associate aif with PNS, increases in apoptosis may have a role in PNS programming of development (Feng et al., 2011; Fujioka et al., 1999; Huang et al., 2016; Jia, Sun, Su, Dang, & Chen, 2016; Kim et al., 2015; Kurek et al., 2016; Liaudat et al., 2015; Qulu, Daniels, & Mabandla, 2015; Tobe et al., 2005). There is a positive association between genetically mediated brain aifm1 levels and PNS effects on acute locomotion, as indicated by comparison of data from the present study to mRNA expression data on genenetwork.org. One plausible explanation is that increased aif levels sensitize mice to apoptotic consequences of PNS. The harlequin mutation is an insertion in the aifm1 gene that leads to an 80% reduction in expression of aifm1 (Klein et al., 2002) and may be a good model for validating the role of aifm1 in PNS. This mouse displays reduced susceptibility to neuronal excitotoxicity and reduced neuronal apoptosis. Our data suggests that the harlequin mouse would be less susceptible to PNS-induced increases of cocaine locomotion. However, the role of aifm1 is complicated, as the harlequin mutation is also associated with central nervous

system and behavioral phenotypes, including motor abnormalities (Bénit, Goncalves, Dassa, Brière, & Rustin, 2008). These effects may be due to a loss of aif mitochondrial function and resulting oxidative stress (Klein et al., 2002). Despite the complexity of aif function, its role in mediating apoptosis after brain insult, including early life insults, makes it a promising candidate gene.

Main Effect QTL

A main effect QTL for female CPP scores was discovered on chromosome 11. This may be the first QTL discovered for cocaine CPP. Previous cocaine QTL mapping has largely focused on locomotor effects. CPP is thought to measure the rewarding properties of cocaine (Tzschentke, 2007) and as such, QTLs discovered may implicate genes that moderate cocaine reward and have particular relevance to cocaine addiction. The present study utilized a biased design. This procedure may be a more sensitive measure of cocaine CPP as compared to unbiased designs (Nomikos & Spyraki, 1988), however the biased design allows for habituation to the un-preferred side to contribute to the CPP score. If strain differences in habituation occur, QTLs discovered may reflect the genetic substrates of habituation. Alternatively, variance in habituation may obscure discovery of reward-related QTLs. The progenitor strains displayed no habituation in saline-only conditioning (unpublished data) however these data do not preclude the possibility that strain differences occur in the BXD panel.

BXD strain differences in habituation may be corrected by inclusion of saline-only groups, however this greatly adds to the sample size requirements and may be unfeasible for a QTL study. Alternatively, experimental follow up of any candidate genes discovered can include measurements of habituation that may allow for discrimination between habituation and reward effects.

The 2-LOD interval for the CPP QTL is large and encompasses 372 genes. Furthermore, many of the microarray probes used to asses transcript expression levels in this interval appear to have target regions with SNPs between B6 and D2 mice. Two transcripts were found to have cis-eQTL (without SNPs in probe target region) and correlate with female CPP scores.

Pitpna is involved in transport of phospholipids and phospholipase C signaling (Tilley et al., 2004). Pitpna null mutants have extensive phenotypes, including CNS and behavioral abnormalities (Alb et al., 2003). However, pitpna has not been directly implicated in drug abuse phenotypes.

Sat2 is a rate limiting enzyme that converts spermidime to spermine (Pegg, Seely, Pösö, della Ragione, & Zagon, 1982). Spermine may interact with the cocaine binding site on the dopamine transporter to inhibit cocaine binding and spermine levels are increased in the cerebellum after cocaine exposure (Ritz, Mantione, & London, 1994; Shimosato, Watanabe, Marley, & Saito, 1995). Although a role for spermidime metabolism is well established for sat1 (homologous protein to sat2), sat2 may not be involved, as spermidime is a poor

substrate for sat2 (Coleman, Stanley, Jones, & Pegg, 2004; Ying et al., 2003). Furthermore, sat2 shows minimal developmental or adult brain protein expression; lack of brain expression may indicate sat2 is not likely to affect cocaine reward.

Sensitization to cocaine-induced locomotion in females was associated with a main effect QTL in which the 2-LOD interval contains 30 genes. One transcript, receptor-interacting serine-threonine kinase 4 (ripk4), from this interval demonstrates a cis-eQTL and significant correlation with female sensitization strain means. This transcript also has cis-eQTL and correlations significant at uncorrected levels in the ventral tegmental area and neocortex. Ripk4 has been implicated in keratinocyte differentiation (Holland et al., 2002), but there is no evidence to implicate ripk4 in cocaine related behaviors, or behavior/central nervous system phenotypes generally.

A suggestive QTL for female sensitization was discovered on chromosome 11 (LRS=18.5) with a 2-LOD interval of 95.6 to 98.6 mb. The B6 allele at this locus associates with increased sensitization. A QTL for methamphetamine and opioid locomotion was discovered on chromosome 11, with an interval of 84 to 96 mb (Bryant et al., 2009; Bryant, Kole, Guido, Sokoloff, & Palmer, 2012) . Here, the B6 allele increases locomotion relative to the A/J allele. Because confidence intervals overlap and the B6 allele has the same direction of effect, the QTL discovered in the present study and that discovered by Byrant et. al., (2012) may

be detecting the same variant. Byrant et al., (2012) attempted to dissect the 84 to 96 mb interval by use of congenic lines. Three lines divided this interval into three intervals that covered unique portions of the original interval. However, all three lines failed to display the effect on methamphetamine locomotion, despite the robust and replicable QTL previously detected. Byrant et al., (2012) concluded that there may be multiple variants in this interval that interact to produce the effect. Because the QTL of the present study overlaps with the tail end of the Bryant et al., (2012) interval, it is unlikely that all of the B6 epistatic variants are captured by the present interval. However, many of the BXD B6 genotype strains at this locus, with high cocaine sensitization scores, have B6 haplotypes that extend beyond the 2-LOD interval, further into the Byrant et. al. (2012) interval. It is also possible that the other interacting variants are isomorphic between B6 and D2 genotypes. Therefore, it is possible that the QTL of the present study is representative of this epistatic effect. Selection of additional BXD lines with smaller B6 haplotypes at this locus could lend support to epistasis. A congenic approach with B6 and A/J lines could also yield similar results. Confirmation would lend support to common alleles mediating cocaine, amphetamine and opioid locomotor effects.

Genetic Correlations: PNS

Common alleles may mediate the effects of PNS across phenotypes. This possibility was assessed by examining correlations between strain difference scores for cocaine and sensorimotor phenotypes. Few genetic correlations were found for the effects of PNS across these traits. Two correlations were found; between PNS effects on female acute cocaine locomotion and PNS effects on CPP, and between PNS effects on female ASR and PNS effects on PPI90. However, PNS effects on female CPP, ASR or PPI were not detected by ANOVA, suggesting that these traits are not affected by PNS across females within the BXD panel. Therefore, these correlations may be spurious. Overall, this evidence does not support the existence of common PNS mediating alleles between the traits under study. Similarly, there were no common QTLs discovered for QTL by PNS interactions between traits.

Genetic Correlations: Cocaine Behaviors

Cocaine locomotion, CPP and self administration behaviors are often studied with the intent of elucidating the neurobiology of cocaine abuse disorder. It is important to consider the relationships between these behaviors, including genetic relationships, in order to evaluate the implications of data produced from these varied phenotypes. Dickson et al., 2015 has reported a

genetic correlation of cocaine locomotion sensitization with cocaine self-administration infusions. Furthermore, a self-administration QTL overlaps with a cocaine sensitization QTL on chromosome 11 (Dickson et al., 2015; V. Kumar et al., 2013). Both studies also hit on the same candidate gene (cyfip2) by different methods, and Kumar et. al. (2013) experimentally validated the role of this gene in cocaine locomotion sensitization. The present study has further supported the relationship between sensitization and self-administration, with locomotor sensitization in the present study demonstrating a robust correlation to the self-administration infusions, data collected by Dickson et. al. 2015.

Sensitization also associates with CPP. However, CPP does not associate with self-administration infusions, suggesting unique shared variance between these measures and sensitization. CPP does associate with days to acquire self-administration. This relationship indicates that strains with higher CPP scores require more days to acquire self-administration and unexpectedly suggests alleles that confer higher cocaine reward sensitivity may also delay acquisition of self-administration. Dickson et al. (2015) report that cocaine CPP collected by Philip et al., (2011) does not associate with self administration acquisition, however this CPP was induced by a 3 mg/kg dose. Furthermore, the Phillip et al. (2011) CPP data does not correlate to CPP (10 mg/kg) data from the present study, suggesting different alleles may be involved in mediating the effects of disparate doses on CPP.

The genetic relationship between sensitization and both self-administration and CPP suggest common alleles mediate sensitization and reward/reinforcement. Furthermore, common alleles suggest that the neurobiology involved in these behaviors is similar. Locomotor sensitization is proposed to be related to a similar process by which sensitization to the motivational valence of cocaine and cocaine related cues underlies the escalation of cocaine intake and ultimately cocaine addiction (Berridge, 2006). Future research should seek to identify the genetic relationship between locomotor sensitization and other addiction related self-administration behaviors, including long-access escalation and reinstatement, as well as reward related cue motivational valence measures such as sign vs. goal tracking. These procedures are thought to reflect addiction-relevant cocaine behaviors and evidence indicates a potential relationship to locomotor sensitization (De Vries, Schoffelmeer, Binnekade, Raasø, & Vanderschuren, 2002; Ferrario et al., 2005; Flagel, Watson, Akil, & Robinson, 2008). A genetic association to sensitization would further suggest that sensitization relevant alleles also confer risk for cocaine abuse. Identifying these relationships will have particularly pragmatic implications. Genetic studies often involve large scale phenotyping of many subjects. Locomotor sensitization is a relatively rapid and feasible test that may be used to screen genetic models for more cumbersome self-administration procedures.

Summary

This experiment has confirmed that PNS affects cocaine locomotion and reward in the BXD panel, indicating BXD strains are a suitable model for locating PNS interacting alleles. A QTL by PNS interaction for acute cocaine locomotion was detected on the X chromosome. Prioritization of positional candidate genes revealed that the gene aifm1 is cis-regulated in multiple brain regions and transcript levels associate with variance in the strain effects of PNS on acute locomotion. This gene may be particularly promising, due to its involvement in mediating apoptosis after brain insults.

Main effect QTLs were discovered for female CPP and sensitization. There are no previous reports of cocaine CPP QTLs, therefore this may be the first discovered for cocaine CPP. The interval for this QTL is large and encompasses many genes. Furthermore, many microarray probes have target regions with B6/D2 SNPs, indicating transcript expression data may not be reliable for the D2 strains, limiting the value of prioritizing candidate genes by expression data. Future work should seek to confirm this QTL and narrow the confidence interval. This may be accomplished by selecting additional BXD strains with haplotype breaks in the interval or congenic methods.

In addition to QTLs, genetic relationships between cocaine behaviors were detected. These data suggest common alleles may mediate locomotor sensitization and self-administration and CPP. Common alleles may also mediate

acquisition of self-administration and CPP. These relationships may be exploited for screening procedures. Additionally, shared variance may be utilized in future studies for principal component analysis that can be utilized for QTL mapping with improved power (Kwak, Moore, Spalding, & Broman, 2015).

Chapter 4

Characterization of stress effects on dam corticosterone and dam-pup contact

The developmental affects of PNS are mediated by the maternal stress response, with physiological and behavioral stress reactions of the mother as potential modes of transmission to the offspring. It is likely that differential maternal stress sensitivity moderates the impact of prenatal stress on offspring development. Genetic variants may be a source of differences in the maternal stress response, including BXD strain differences. In mapping for QTL by PNS interactions, genetically mediated differences in maternal stress sensitivity should be considered. Differences in stress sensitivity may have developmental consequences that are revealed in cocaine and sensorimotor phenotypes. In this case, the offspring data will retain information of the genetic effects on maternal stress responsivity. QTLs derived from this data may indicate loci that influence maternal stress response and do not interact with PNS exposure to moderate development. Understanding the nature of genotype-phenotype associations is important for follow-up investigations of candidate genes. Alternately, strain differences in maternal stress response may obscure discovery of QTL by PNS interactions. This may occur when genetic variants that influence maternal stress response are in linkage disequilibrium with variants that interact with PNS exposure to moderate development.

Glucocorticoids are a likely mechanism of stress transmission from mother to fetus. Environmental stressors cause a response of the HPA axis that ultimately results in an increase of plasma glucocorticoid levels (Herman &

Cullinan, 1997). Corticosterone (CORT) is the primary glucocorticoid in rodents. PNS procedures are repeatedly shown to cause increases in maternal plasma CORT levels (Barbazanges, Piazza, Le Moal, & Maccari, 1996; Montano, Wang, Even, & vom Saal, 1991a; Mueller & Bale, 2007; Takahashi, 1998; Zagron & Weinstock, 2006). Although the placenta may act as a metabolic barrier to glucocorticoids, fetal CORT levels rise after maternal stress exposure (Montano et al., 1991a; Takahashi, 1998). It is this in-utero exposure to elevated glucocorticoids that may explain the PNS phenotype in adult offspring. Inhibiting the dam CORT response by adrenalectomy attenuates or eliminates many of the effects of PNS on the offspring (Diaz, Ogren, Blum, & Fuxe, 1995; Salomon, Bejar, Schorer-Apelbaum, & Weinstock, 2011; Zagron & Weinstock, 2006). And exogenous administration of glucocorticoids during pregnancy produces similar effects to PNS, including altered responses to psychostimulants, morphine and ethanol (Diaz et al., 1995; Rodrigues et al., 2012; Salomon et al., 2011). Comparison of CORT levels in pregnant B6, D2 or BXD mice following stress have not been reported; there are reported differences in stress response in un-pregnant mice in the progenitor and BXD strains (Doering, Shire, Kessler, & Clayton, 1972; Freund, Martin, Jungschaffer, Ullman, & Collins, 1988; Roberts, Phillips, Belknap, Finn, & Keith, 1995). Considering this evidence, the maternal CORT response to stress may differ in the BXD strains, and this difference may account for the strain differences in the effect of PNS on cocaine CPP. However, in-utero exposure to elevated glucocorticoids may not be the only mechanism by

which PNS affects development. PNS procedures are also known to alter the maternal behavior of the stressed dam, which alters the postnatal environment of the offspring, and potentially contributes to the PNS phenotype.

Multiple lines of research indicate that PNS can alter postnatal maternal behavior. Most widely reported is a reduction in licking and grooming of the pups (S. Baker et al., 2008; Champagne & Meaney, 2006; Patin et al., 2002; Power & Moore, 1986; Smith, Seckl, Evans, Costall, & Smythe, 2004). There is also a report of reduced time nursing (Smith et al., 2004). And an experiment employing a pup retrieval task reported increased latency to retrieve and lower retrieval rates in PNS dams (Patin et al., 2002). This research has largely used rats as subjects; a literature search yields two studies involving mice. PNS was only found to affect maternal behavior when stressed pups are cross-fostered to a control dam or control pups to a stressed dam, indicating a PNS by cross fostering interaction (Meek, Burda, & Paster, 2000). Similarly, PNS had no effect on pup retrieval by the biological dam in another study, but did cause impairments in maternal aggression towards an intruder (Pardon, Gérardin, Joubert, Pérez-Diaz, & Cohen-Salmon, 2000). Although evidence for mice is lacking, there is sufficient evidence to indicate that PNS does affect some maternal behaviors in the rat. Both mouse and rat pups are dependent on maternal care, and the development of pups can be altered by variation in maternal behavior. Licking and grooming by rat dams negatively correlates with

HPA axis reactivity in adult offspring, suggesting the importance of maternal contact for HPA axis regulation later in life (Francis, Diorio, Liu, & Meaney, 1999; D. Liu et al., 1997). Maternal behavior may also affect cocaine responsiveness. Manipulations that increase licking/grooming frequency, including temporary maternal separation and litter size reduction, cause decreased locomotion sensitization and CPP in male but not female rats and decreased cocaine self-administration (only males tested) (Francis & Kuhar, 2008; Y.-Q. Li et al., 2008; Moffett et al., 2006). PNS may alter maternal behavior, and variation in maternal behavior can have long standing consequences for the offspring. Therefore, it is plausible that differential stress effects on maternal behavior in B6 and D2 strains may be a mechanism by which PNS differentially affects the phenotype of the offspring.

In order to assess strain differences in the maternal CORT response and maternal behavior in the B6, D2 and BXD strains, plasma CORT levels were measured pre and post stress and the frequency of dam-pup contact was assessed in the postnatal period. Strain differences in the CORT response to stress and strain differences in the effects of stress on dam-pup contact were tested for association with PNS effects on offspring sensorimotor (chapter 2) and cocaine (chapter 3) phenotypes.

Methods

Subjects, Breeding and Prenatal Stress

BXD strains and the C57/6J (B6) and DBA/2J (D2) progenitor strains were bred and subjected to PNS as described in chapter 2.

Corticosterone

On stress day 1 and 5, blood was collected by puncturing the submandibular vein with a lancet, once immediately before the first restraint session and once immediately after the first session. Approximately 50 microliters was collected into lithium heparin coated tubes and then centrifuged for collection of plasma. Samples were stored at -80 degrees until ready for processing.

Plasma CORT concentration was measured with the use of the DetectX® CORT Enzyme Immunoassay kit (ArborAssays K014-H5, Ann Arbor, MI, USA). Room temperature plasma samples were diluted 1:450 in assay buffer and processed according to the provided protocol. A microplate reader (Elx800, BioTEK, Highland Park, VT, USA) was used to measure optical density at 450 nm and plasma concentration was interpolated from a standard curve. Stress day 1

and 5 samples were assessed for the progenitor strains, and stress day 1 was assessed for the BXD strains. All samples were run in duplicate within a plate and the intra-assay reliability was assessed by the coefficient of variation for the duplicates. Ten samples were assessed on two independent plates to determine the inter-assay coefficient of variation. Pre- and post-stress samples for a subject were always run on the same plate and strains were balanced between plates.

Maternal-Pup Contact

After parturition, dams and their litters were observed from postnatal day (PND) 1 through 10. Observations consisted of 4 sessions daily, at times 0930, 1300, 1700 and 2000 (lights on at 0700, lights off at 1900). Night vision goggles were used during the 2000 session so that the dark phase was not disrupted. Each session consisted of one observation every 3 minutes for a total of 5 observations. At each observation, dams were noted to be in contact or not in contact with the pups. The mice were observed in their home cage and in their home rack position. Care was taken not to move the cage and to reduce any noise made by the observer. In total, dams received 200 observations. The number of in-contact observations was divided by total observations received to quantify maternal-pup contact.

Inter-observer reliability was assessed by performing simultaneous observations with pairs of raters blinded to the other observer's observation.

Statistics and QTL Mapping

Data are presented as mean ±SEM. Analysis of variance (ANOVA) was used for analysis of stress and strain effects. Heritability was calculated as described in chapter 2. QTL mapping was performed as described in chapters 2 and 3. The level of significance was set at $p<0.05$.

Results

Corticosterone

A mixed factorial 2-way ANOVA for the B6 (n=12) and D2 (n=9) strains, with CORT change from baseline to post-test as a within factor and strain as a between factor, revealed a stress by strain interaction $F(1, 19)=11.3$, $p=0.003$], a main effect of stress on stress day 1 [$F(1, 44)=79.0$, $p<0.001$], and a main effect of strain [$F(1, 19)=6.4$, $p=0.021$]. For day 5, a stress by strain interaction [$F(1, 19)=6.7$, $p=0.018$] and a main effect of stress [$F(1, 19)=167.4$, $p<0.001$] were found.

Simple main effects tests for the shift from baseline to post-stress was assessed within strain for stress day 1 and 5. Both B6 and D2 strains increase CORT from baseline on day 1, [$F(1, 19)=44.8$, $p<0.001$] and [$F(1, 19)=29.5$, $p<0.001$] respectively, and on day 5, [$F(1, 19)=109.2$, $p<0.001$] and [$F(1, 19)=44.8$, $p<0.001$] respectively. The magnitude of the CORT change was greater in D2 mice on day 1 [$F(1, 19)=11.3$, $p=0.003$] and day 5 [$F(1, 19)=6.7$, $p=0.017$].

Habituation to stress in the B6 and D2 strains was assessed by a mixed 2-way ANOVA, with the magnitude of CORT change on day 1 and 5 as within subjects factors and strain as a between factor. A main effect of strain was [$F(1, 19)=13.1$, $p=0.002$] was found (D2 greater), but no effect of stress day or strain by stress day interaction were found, indicating no habituation or sensitization to restraint stress from day 1 to 5.

The stability of the baseline CORT levels in B6 and D2 mice were assessed by mixed 2-way ANOVA, with baseline on day 1 and 5 as within factor and strain as a between factor. A stress day by strain interaction [$F(1, 19)=15.6$, $p=0.001$], a main effect of stress day [$F(1, 19)=87.5$, $p<0.001$] and a main effect of strain [$F(1, 19)=16.4$, $p=0.001$] were found. Simple main effects for stress day, within strain, revealed that both B6 and D2 strains increase baseline CORT levels from day 1 to 5 [$F(1, 19)=106.1$, $P<0.001$] and [$F(1, 19)=12.4$, $p=0.002$] respectively. The magnitude of this shift was greater in B6 mice [$F(1, 19)=15.5$, $p=0.001$]. See table 1 for baseline and post-stress strain values on day 1 and 5.

Table 1 Plasma corticosterone levels (ng/mL) of the B6 and D2 strains at baseline and post-stress on stress day 1 and 5.

Strain	Stress Day	Timepoint	Mean	SEM
B6	1	Baseline	139.63	16.63
		Post-Stress	583.09	46.38
	5	Baseline	523.80	44.33
		Post-Stress	1160.65	78.85
D2	1	Baseline	94.51	26.14
		Post-Stress	1076.23	183.69
	5	Baseline	251.30	40.61
		Post-Stress	1272.91	184.26

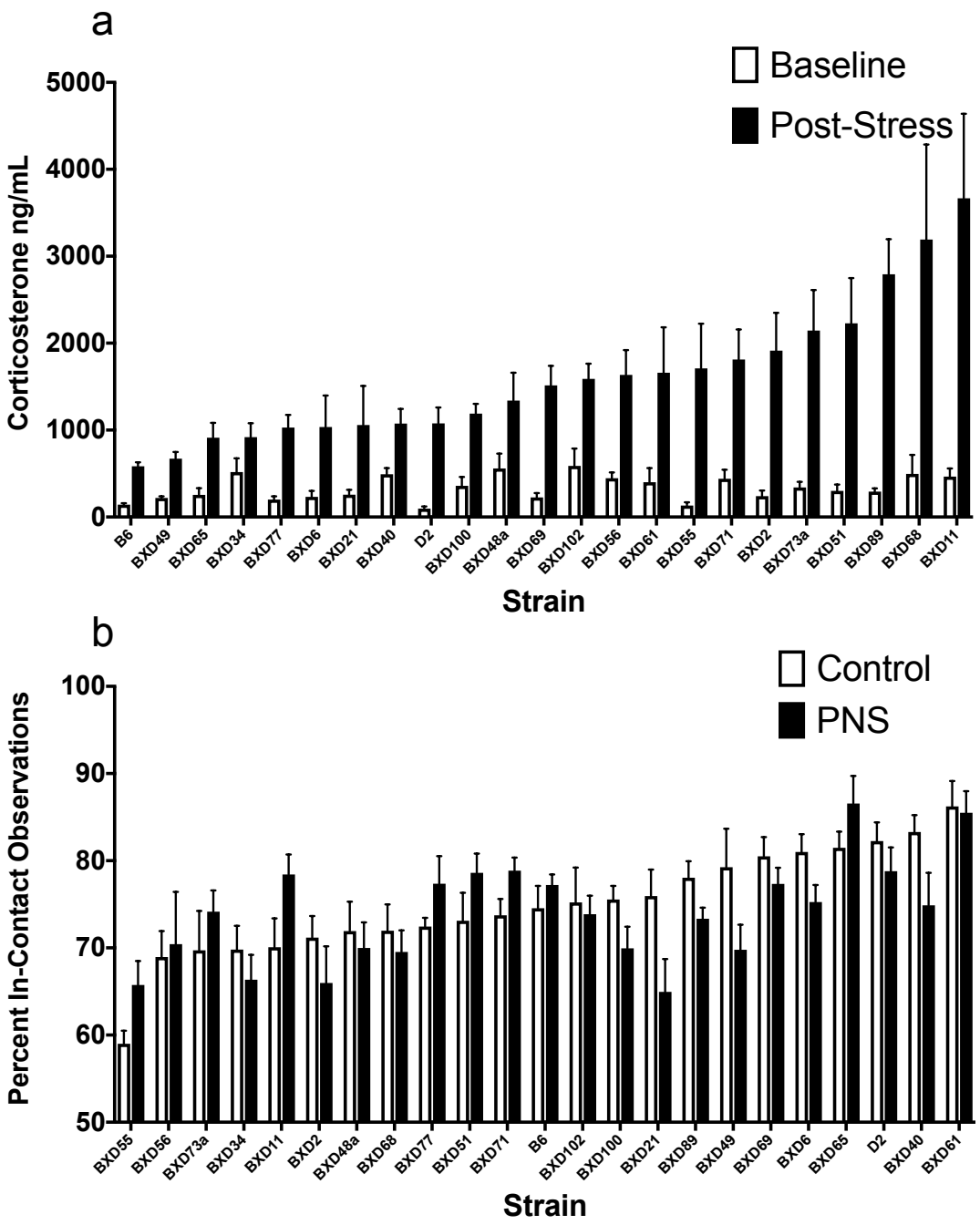

Figure 1 Effects of restraint stress on maternal corticosterone and dam-pup contact. a) A significant strain effect for the CORT response to restraint stress. b) A strain by stress interaction on maternal-pup contact.

The BXD strains were assessed by mixed 2-way ANOVA, with the baseline and post-stress CORT levels as a within factor and strain as a between factor. A stress by strain interaction [$F(20, 109)=3.9$, $p<0.001$], a main effect of stress [$F(1, 109)=254.6$, $p<0.001$] and a main effect of strain [$F(1, 109)=3.0$, $p<0.001$] were found. See figure 1a.

A 1-way ANOVA for the magnitude of the CORT change from baseline to post-stress revealed a strain effect [$F(20, 109)=9$, $p<0.001$], indicating heritability of the CORT stress response. The heritability estimate is 0.42.

The average intra-assay coefficient of variation was 6.1% and the average inter-assay coefficient of variation was 15.1%.

Maternal Behavior

A 2-way ANOVA for dam-pup contact in the progenitor strains revealed a main effect of strain $F(1, 24) = 4.5$, $p<0.044$, with D2 mice observed to have more contact with pups (see figure 1b). No significant main effect of PNS or significant interactions were found.

A 2–way ANOVA for BXD strains revealed a strain by PNS interaction [$F(20, 240)=1.7$, $p=0.03$] and a main effect of strain [$F(20, 240)=5.0$, $p<0.001$] (see figure 1b). The heritability for control condition is 0.2 and for PNS is 0.27.

The intra-class correlation for assessment of reliability was ICC(1, 2) = 0.98, 0.92 and 1.0 for the 930, 1300 and 1700 observation session, respectively. These results indicate high inter-observer reliability.

Main Effect QTL and QTL by PNS Interactions for the Maternal Stress Response

No significant QTLs were discovered for the CORT response or dam-pup contact. A suggestive QTL was discovered for CORT response on chr 13 (LRS=12.8, peak marker rs13482018, location 112.636265 mb) and on chr 19 (LRS=13.2, peak marker rs30505802, location 27.531578 mb). A suggestive main effect QTL for maternal-pup contact was discovered on chr 6 (LRS=12.3, peak marker rs30611941, location 126.790724 mb) and on chr 13 (LRS=15.8, peak marker rs3664096, location 75.764157 mb).

Associations of Maternal Stress Response to Offspring Phenotype

The strain CORT response scores (difference from baseline to post-stress) were assessed for correlations to strain difference scores for acute locomotion/sensitization, CPP, ASR, and PPI90. For males, a significant, positive correlation was detected for acute locomotion (r=0.49, p=0.025) (see figure 2a) and CPP (r=0.45, p=0.043) (see figure 2b) difference scores. No other significant correlations were detected.

The strain difference scores for maternal-pup contact were assessed for correlations to strain difference scores for acute locomotion/sensitization, CPP, ASR, PPI90. A significant, positive correlation was found between maternal-pup contact and CPP difference scores in males (r=0.60, p=0.004) (see figure 2c). No other correlations were detected.

Re-mapping QTLs with Adjusted Means

The estimated marginal means (EMMs) for acute locomotion and CPP in PNS subjects were calculated with maternal CORT response as a covariate. EMMs were also calculated for CPP with the dam-pup contact scores as a covariate for both control and PNS subjects. The difference scores for these means were determined and then subjected to QTL mapping. A significant QTL on chromosome X (LRS=16.6, peak marker=rs13483729, location 38.899709) was detected for acute cocaine locomotion with CORT adjusted means in the same location as the QTL detected for unadjusted means (see chapter 3). No other significant QTLs were detected.

In order to assess differences in CORT and maternal contact exposure between the two genotype groups at the acute locomotion QTL, a t-test was performed between the two genotype groups for strain maternal CORT response and dam-pup contact strain differences scores (CORT mean ± SEM, B6 allele=

1244.5 ± 191.4, D2 allele= 1623.6 ± 423.7; maternal-pup contact mean ± SEM, B6 allele= -1.8 ± 1.4, D2 allele= 1.6 ± 2.4). No significant effects were detected for CORT [t(19)=0.9, p=0.688] or maternal-pup contact [t(19)=1.2, p=0.999].

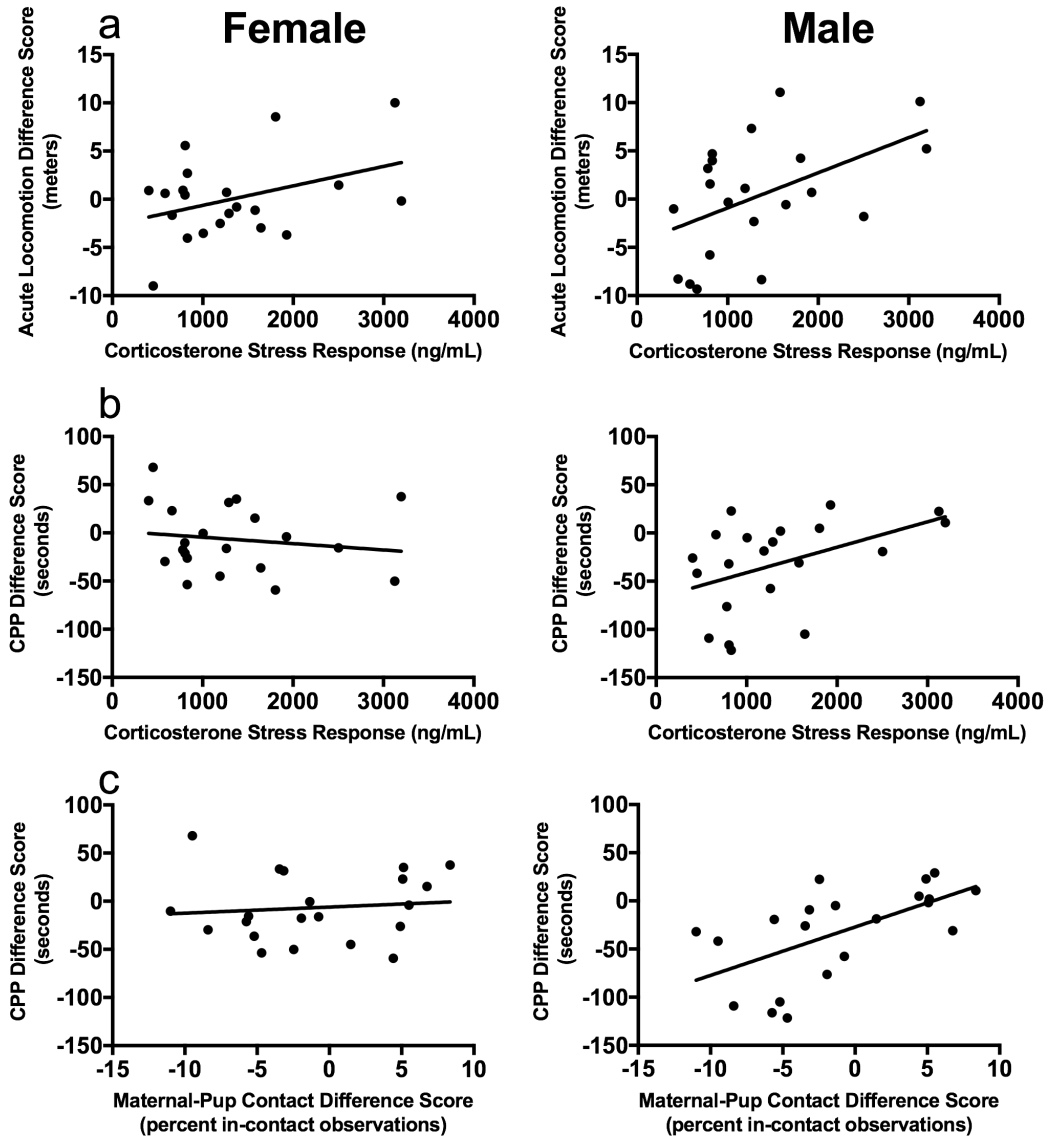

Figure 2 Associations between maternal stress response and PNS effects on offspring cocaine phenotypes. a) Significant correlation between maternal CORT response and male acute locomotion difference scores. b) Significant correlation between maternal CORT response and male CPP difference scores. c) Significant correlation between maternal-pup contact difference scores and male CPP.

Discussion

The present experiment indicates that restraint stress interacts with strain to alter maternal-pup contact in the postnatal period. Furthermore, the CORT response to restrain stress was found to be a heritable trait, with strain differences in the magnitude of the CORT response. These data collectively reveal BXD strain differences in the dam response to restraint stress that may have consequences for prenatal and postnatal development of the offspring. The strain effects of restraint stress on dam-pup contact associate with the strain effects of PNS on offspring CPP. Additionally, the strain maternal CORT response associates with the strain effects of PNS on acute locomotion and CPP. These data suggest that strain variability in the maternal stress response may have an effect on the offspring cocaine phenotypes.

In determining QTL by PNS interactions, strain variance of the maternal stress response may act as a confound. Ideally, when searching for a genotype by environment interaction, exposure of the treatment groups to the environmental factor is constant. In the present study, offspring exposure to maternal stress response—both in terms of CORT and maternal contact--varied in a strain dependent manner and that variable exposure associates with enduring consequences for behavior. In mapping for a QTL by PNS interaction, variable exposure to PNS-induced CORT elevations or variable alterations in maternal behavior may obscure the discovery of QTLs for responsiveness to these factors

as well as how PNS-programmed alterations impact brain function. For example, a strain may possess a variant that interacts with PNS to moderate the developmental consequences. However, that same strain may also harbor alleles that suppress the maternal stress response. Suppressed exposure to PNS may obscure the QTL by PNS interaction. However, if these alleles are not in linkage disequilibrium, the average exposure of the two genotype groups at the PNS interacting QTL should not differ. The greatest confounds may occur when maternal stress response mediating alleles are in linkage disequilibrium with PNS interacting alleles. Variable stress exposure may also produce QTL by PNS interactions that are actually representative of alleles that moderate the maternal stress response because influential variance in the maternal stress response may be represented in the offspring difference scores. These variants indicate important gene by stressor interactions that mediate adult stress responsivity. However, in determining the detailed biological effects of candidate genes, it will be important to discriminate between QTLs that moderate a developmental effect of stress exposure and those that moderate the adult stress response. This knowledge will guide prioritization of candidate genes and hypothesis formation. In order to account for these possibilities, the strain offspring means were adjusted with the maternal stress response as a covariate and re-mapped. These efforts resulted in no new QTL discoveries, therefore we do not have evidence to support that strain variance in the maternal stress obscured discovery of QTLs. Importantly, the QTL by PNS interaction for acute

locomotion remained, indicating that variance in the maternal stress response was not likley the source of this QTL. This is further supported by the lack of difference in mean CORT and contact exposure between the two genotype groups at the QTL by PNS interaction for acute locomotion. However, one potential caveat is the unbalanced sizes of the genotype groups (5 in D2 group, 16 in B6 group). Unbalanced groups may reduce power to detect differences. The D2 group CORT mean is 33% higher than the B6 group mean, and the D2 group has higher acute locomotion difference scores; this association is in line with the positive correlation between maternal CORT response and acute locomotion difference scores. Characterization of additional BXD strains, with biased selection by genotype at this QTL to balance the size of groups, would further clarify this issue.

Associations between maternal CORT response and cocaine locomotion/CPP suggest that the effects of stress induced CORT on these phenotypes may be plasma concentration dependent. Some effects of PNS are dependent on stress induced surges in CORT levels. Most studies utilize a single dose in an effort to simulate endogenous surges in stress- induced CORT. These data are sufficient to implicate glucocorticoids in fetal programming and PNS effects, but do not examine the implications for varying stress-induced CORT levels. One study did examine the effects of PNS in a knock-out (KO) strain of mice known to be more sensitive to stressors than the wild type (WT) strain.

Both the KO and WT strains demonstrate increases in CORT due to stress, however the KO has a greater CORT response The offspring of the PNS KO mice had lower bodyweights relative to PNS WT mice, and this effect persisted into adulthood (Mueller & Bale, 2006). This evidence indicates maternal stress sensitivity may have an enduring impact on the phenotype of PNS offspring. A dose-response relationship with the synthetic glucocorticoid dexamethasone was detected for hippocampal degeneration in primates, with greater degeneration occurring with greater dose (LaBorde, Hansen, Young, Sheehan, & Holson, 1992). Similarly, reduction in fetal growth by dexamethasone administration to rats was found to be dose-dependent (LaBorde et al., 1992). These studies indicate that the dose-response of deleterious glucocorticoid effects can be graded, with a threshold dose for harmful effects, and increasing severity with increasing dose. A similar dose-response relationship of CORT may cause differential consequences for fetal development and indicate the importance of the maternal stress CORT response variability. More extensive dose-response assessment of prenatal CORT exposure for effects on cocaine responsiveness may clarify this potential relationship.

The effect of PNS on dam-pup contact was found to associate with the effect of PNS on CPP. These results suggest that genetically mediated changes in maternal behavior may be a mechanism by which PNS differentially alters cocaine responsiveness in BXD strains. Variation in maternal behavior are

known to be a determinant of physiological and behavioral outcomes in the offspring, with associations to HPA axis function being most commonly presented (Champagne, 2011). The effects of maternal care may also be implicated in cocaine responsiveness, as licking/grooming negatively associated with cocaine self-administration (Francis & Kuhar, 2008). Furthermore, reductions in litter size, a manipulation that increases licking/grooming, decrease cocaine locomotion sensitization and CPP in males but not females (Y.-Q. Li et al., 2008). The opposite pattern was found in the present study; reduced dam-pup contact was associated with decreased CPP. However, we did not measure licking/grooming and cannot assess its relationship to PNS and cocaine CPP. Dam-pup contact and licking/grooming are not necessarily associated; in one instance a prenatal manipulation increased licking/grooming with no change in total contact and natural variations in rat licking/grooming do not associate with total contact (Menard & Hakvoort, 2007; Palanza, Howdeshell, Parmigiani, & vom Saal, 2002).

Although maternal behavior is an important driver of pup development, pup physiology and behavior are known to be determinants of maternal behavior. This is demonstrated by a study in which BXD pups were cross-fostered to B6 dams. Alterations in the B6 dam maternal behaviors are dependent on the strain of the pups (Ashbrook, Gini, & Hager, 2015a). PNS-induced changes in rat pups also alter maternal behavior. Stressed pups under

care of unstressed dams elicit less licking/grooming, contact time and retrieval rates relative to controls (Moore & Power, 1986; Pérez-Laso et al., 2013; Power & Moore, 1986). This effect may be specific to male pups and mediated by changes in urine composition, as urine of stressed male pups elicited less investigation by dams relative to controls (Power & Moore, 1986). Considering that PNS induced changes in pup physiology and behavior may alter maternal behavior, such changes in BXD strains may fully or partially explain the association between PNS alterations in maternal behavior and PNS changes in CPP in the present study. PNS exposed BXD pups may demonstrate strain dependent neonatal changes that alter maternal behavior. These changes may be associated with PNS induced changes to cocaine responsiveness. Therefore, a causal role for altered maternal behavior is especially ambiguous and requires further investigation. These relationships may be explored by cross-fostering designs and subsequent characterization of cocaine responsiveness in the adult offspring.

The results of the present experiment indicate a strain effect on maternal behavior in the progenitor strains. D2 mice were found to have more contact with their pups than B6 mice. A similar strain effect was also identified by a study in which B6 and D2 maternal behavior were compared. D2 mice were found to score higher on multiple measures of maternal behavior including nursing, nest building and contact rest (Shoji & Kato, 2006). These similarities

may indicate congruence of the dam-pup interaction traits measured across this study and the present study. No effects of PNS were found on progenitor dam-pup contact, despite strain by PNS interactions in the BXD strains. Although differences between the progenitor strains indicate heritability in the BXD panel, in some cases genetic effects are only present in the BXD strains, due to unique allele combinations in BXD strains that allow for the emergence of strain differences.

Both B6 and D2 dams demonstrated an increase in CORT levels after restraint stress, indicating that the restraint procedure is an effective stressor in both strains. The magnitude of the CORT response differed between strains, as observed in the BXD strains, and the progenitor strains did not display habituation to restraint stress. However, the baseline CORT levels increased from day 1 to day 5 in both strains. This may be explained by HPA activity associated with pregnancy. Pregnancy causes changes in CORT levels that fluctuate predictably throughout pregnancy, with a sharp increase shortly before parturition, which may explain higher baseline levels on day 5 (Barlow, Morrison, & Sullivan, 1974; Montano, Wang, Even, & vom Saal, 1991b). The magnitude of this shift was greater in the B6 mouse, indicating heritability of this trait, however implications for baseline shifts were not evaluated in the BXD strains.

No main effect or QTL by PNS interactions were discovered for maternal behavior and no QTLs were discovered for the CORT stress response, despite both measures demonstrating heritability as well as significant interaction with strain. QTL discovery for maternal behavior in a previous study produced QTLs for variants that associate with the pup influence on maternal behavior (Ashbrook, Gini, & Hager, 2015b). This experiment more extensively characterized parameters of maternal behavior. A QTL was discovered for nest building, and another QTL was discovered for the sum of nest building, suckling and nursing. Inclusion and distinction of different maternal behaviors may have improved QTL detection in the present study. QTLs have also been discovered for the CORT stress response in rat and mouse populations (Finlay et al., 2010; Gonik et al., 2012; Llamas et al., 2005; Marissal-Arvy, Heliès, Tridon, Moisan, & Mormède, 2014; Potenza et al., 2004; Redina, Smolenskaya, Maslova, & Markel, 2010; Solberg et al., 2006; Vendruscolo, Vendruscolo, et al., 2006). The present study may have missed QTLs due to inadequate power. Characterization of additional BXD strains may improve QTL detection for both CORT and maternal behavior. Genes that mediate the CORT and maternal behavior response to stressors may have significant relevance, considering these genetic variants may moderate the effects of PNS on the offspring.

Summary

Strain variance in the maternal CORT response to stress and stress-induced changes to maternal-pup contact associate with PNS-induced changes in offspring cocaine phenotypes, suggesting genetically mediated variance in the maternal stress response may account for effects of PNS on the offspring. However, when strain means were adjusted for the maternal stress response the PNS interacting QTL for cocaine locomotion remained, suggesting that shared variance between maternal stress response and PNS effects on cocaine locomotion is not the source of this QTL. Furthermore, no QTL was discovered for the maternal CORT response at this locus and CORT/dam-pup contact exposure did not differ between the genotype groups at this locus. These data suggest that the QTL discovered represents a QTL by PNS interaction for one or more variants that moderate the developmental impact of PNS on the offspring. No new QTLs were discovered with adjusted means, suggesting variance in the maternal stress response did not obscure discovery of PNS interacting QTLs.

The CORT response and changes in maternal behavior are likely modes of stress transmission from dam to fetal and neonatal pups, however, other factors may be influential. Therefore, it is difficult to exclude all sources of maternal stress variance. Follow up validation for genes nominated by QTL by PNS interactions should consider the possibility that the gene influences adult stress responsivity and does not interact with PNS to moderate offspring development.

For postnatal factors, cross fostering designs can account for maternal stress responsiveness. Prenatal factors are more difficult although possible to control for by in vitro fertilization and embryo transfer to dams with uniform genetic backgrounds. If this is unfeasible, the effects of the candidate gene on prenatal maternal stress responsiveness should still be assessed, prioritized by factors most likely to mediate effects of PNS on the offspring.

Chapter 5

Concluding Remarks

The preceding experiments characterized the effects of prenatal stress (PNS) on sensorimotor and cocaine phenotypes in 21 strains of the BXD recombinant inbred panel. These behaviors were selected as models of psychiatric disorders, including the multiple PPI-associated psychiatric conditions and cocaine abuse (Kohl et al., 2013). These disorders are heritable traits that are also affected by early life stress exposure (Enoch, 2012; Goldman et al., 2005; Kendler, 2001; Khashan et al., 2008; Kinney et al., 2008). Early life stress and genetics may intersect, in gene by environment interactions that render exposed individuals particularly vulnerable to psychiatric illness. Identifying and characterizing genes that interact with early life stress to confer risk of psychiatric illness will greatly improve understanding of the etiology and stimulate new avenues of neurobiological research.

The BXD recombinant inbred panel was selected for discovery of QTL by prenatal stress (PNS) interactions due to interactions previously observed in the progenitor strains (Kippin et al., 2015). In the BXD panel, PNS was found to affect male acoustic startle, prepulse inhibition (PPI), sensitization to cocaine induced locomotion and cocaine conditioned place preference (CPP). Sex effects were not detected in the effects of PNS on acute cocaine induced locomotion, indicating PNS may affect both sexes. Collectively, these results indicate that the BXD panel harbors genetic variants that mediate the effects of PNS, in gene by environment (GXE) interactions. Main PNS effects, but not significant strain by PNS

interactions, were detected for PPI and CPP. Here, the evidence for GXE interactions is lacking, although a trend toward a strain interaction was observed in males. Collectively, the BXD panel does appear suitable for discovery of genes that interact with PNS to alter these psychiatric disorder relevant traits.

The primary goal of the experiments presented was to utilize the BXD panel to discover quantitative trait loci (QTLs) that interact with PNS to alter sensorimotor and cocaine phenotypes. This approach is an unbiased, genome-wide scan to associate genetic variants in the BXD panel with PNS interactions. Detection of PNS interacting QTLs serves as a preliminary step in identifying genes that interact with PNS to alter these phenotypes. A QTL by PNS interaction for male PPI was detected on chromosome 3 and for acute cocaine locomotion in both sexes on chromosome X. The intervals defined by these QTLs may contain one or more genetic variants that cause gene by PNS interactions. Additionally, main effect QTLs were detected for cocaine sensitization on chromosome 16 and for CPP on chromosome 11. Main effect QTLs suggest one or more variants in these QTL intervals alter cocaine responsiveness, irrespective of PNS exposure.

The discovery of QTLs in laboratory animal populations is appealing for multiple reasons. First, as with human GWAS, these techniques allow for unbiased and genome wide associations. Therefore, detection of relevant genes does not depend on selection of genetic targets based on prior knowledge, which is often lacking, especially with respect to psychiatric disorders. Second, the

rigorous control possible in lab experiments has potential to limit environmental effects on a phenotype and maximize the signal to noise ratio of any associated alleles. This may be contrasted to human genetic studies, in which substantial heterogeneity of environmental factors can be expected. The benefits of controlled environments may be particularly relevant to GXE approaches, as detection of an interaction may be obscured by variable environmental exposure. In addition to rigorous control, experiments with lab animals expand available traits for characterization relative to human studies, which may be of great importance in optimizing detection of QTLs and in the follow up of candidate genes identified by QTL studies, which require causal experimental approaches and benefit from invasive physiological techniques.

From QTL to Gene

Despite the benefits of forward genetic screens, there are substantial challenges. Most significant is the low resolution of many QTLs. The interval on the genome that can be defined by QTL is limited by the average length of haplotypes in the population under study. Many populations utilized to date, including BXD strains, often produce QTL confidence intervals that contain dozens or hundreds of genes (Milner & Buck, 2010; Zeng, Xu, Memg, Wamg, & Hu, 2006). A large collection of positional candidate genes can be difficult to investigate for identification of the quantitative trait gene. This is demonstrated

by a preponderance of QTLs but a lack of validated quantitative trait genes in the literature (Milner & Buck, 2010; Zeng et al., 2006). The confidence intervals for QTL by PNS interactions identified in the present studies contain 48 and 65 genes for PPI and acute locomotion respectively. All of these genes are considered positional candidates. Experimentally characterizing each of these genes would be a daunting task. Therefore, following detection of a QTL, methods of prioritizing positional candidate genes were utilized.

Many SNPs associated with complex traits are located in non-coding regions (Nicolae et al., 2010). Therefore, it is expected that trait-associated variants often act by modifying gene expression. Ultimately these alleles lead to varying levels of protein expression that has consequences for the phenotype. Allele influence on gene expression can be exploited to prioritize QTL-nominated candidate genes. Heritable variation of mRNA levels is often observed in genetically diverse populations. This genetic mediation of mRNA expression can be utilized to map for cis-regulated expression QTLs (cis-eQTLs) (Kadarmideen, von Rohr, & Janss, 2006). Cis-regulation indicates that expression is modulated by a variant located in or near the parent gene of the mRNA.

The QTL confidence intervals for the QTL by PNS interactions and main effect QTLs discovered were scanned for cis-eQTLs. As mRNA expression can vary significantly between tissue, regions of interest must be selected. Data sets that characterized mRNA expression in various brain regions were selected

because psychiatric pathophysiology is likely to involve the central nervous system. In some studies of neurobiologically relevant traits, a select few brain regions are assessed; selected based on likely relevance to the trait. In the present studies, relevant brain regions included those that are likely to interact and moderate the stress response (hippocampus, amygdala, hypothalamus, pituitary and prefrontal cortex (PFC)), those associated with PPI (brain stem, midbrain, striatum and PFC) and those associated with cocaine abuse (midbrain, striatum, amygdala, hippocampus and PFC). Due to this expansive list, the liberal approach of assessing all available regions was utilized. These efforts identified multiple cis-regulated transcripts, in one or more brain regions, for each of the QTLs. Following these efforts, the BXD strain transcript expression levels were assessed for correlation to the behavioral phenotype. A genetic correlation is predicted if cis-regulation of gene expression affects the phenotype (Kadarmideen et al., 2006). These efforts reduced the list to one or a few genes in each of the QTLs. The gene apoptosis inducing factor 1 (aifm1) was prioritized for the acute locomotion QTL by PNS interaction, and may be promising due to mediation of apoptosis after brain insult, including early life insults. Other genes prioritized have varying levels of biological characterization that attest to functions that may implicate them in the effects of PNS, or sensorimotor and cocaine phenotypes. However, because QTL mapping is an unbiased approach, it is expected the genes may be discovered that have no previously known role in the phenotype and little functional characterization. Exploiting mRNA

expression to prioritize candidate genes is largely an unbiased approach that can aide in validation of quantitative trait genes but will likely yield candidates that require extensive characterization.

Despite the benefits of mRNA expression analysis for prioritizing candidate genes, several limitations in the present study should be considered. Assessment of cis-eQTL involves QTL mapping for tens of thousands of probes per mRNA expression data set, each with a genome wide significance threshold of 0.05. This presents a multiple testing problem that was uncorrected. It is likely that many false positives-eQTLs are discovered by this approach. However, by testing multiple brain regions, often collected by independent studies and on different microarray platforms, cis-eQTLs for a given gene can be identified more than once, across regions and platforms. In these cases, the likelihood of a false discovery is reduced. The gene aifm1 demonstrates cis-eQTL and correlation to the effects of PNS on acute locomotion in five regions. This is an example of multiple, independent results that may enhance confidence. Other genes that were prioritized only demonstrated cis-eQTL and correlation in one region and may be viewed more cautiously. Despite the likelihood of false cis-eQTLs, uncorrected testing avoids the large reduction in power that comes when correcting for a large number of tests. Considering that this method of prioritizing candidate genes should be viewed as exploratory, and that

uncorrected testing generally yields a small number of prioritized candidate genes that can then be validated, uncorrected testing is a beneficial approach.

Associations between cis-eQTL transcript levels and the behavioral phenotype can only be assessed by the number of strains in common between the data sets. In the present studies, the average number of strains in common with mRNA expression data sets was 12, and could not exceed 21. These sample sizes are estimated to generally provide modest statistical power for genetic correlations (Crabbe, Phillips, Kosobud, & Belknap, 1990). Therefore, the phenotype to transcript expression correlations assessed may have been underpowered and true associations could have been missed.

A potentially useful approach for future research may be to characterize the effects of PNS on gene expression in BXD strains. PNS has been shown to affect gene expression profiles (Kinnunen, Koenig, & Bilbe, 2003; Strata et al., 2015; D. L. A. Van den Hove et al., 2013). The effects of adult stress in the BXD panel or progenitor strains are demonstrated to have heritable influences on gene expression in various brain regions (J. A. Baker et al., 2017; Kerns et al., 2005; van der Vaart et al., 2017; Wolen et al., 2012; Ziebarth et al., 2010) Assuming the effect of PNS on mRNA expression is heritable in at least a subset of affected genes, inter-strain variance in the effects of PNS on gene expression may be exploited to prioritize candidate genes. This may be accomplished by searching for cis-regulating variants for the mRNA expression difference scores

and for correlation to the behavioral difference scores. In comparison to assessing cis-regulation of basal expression levels, as performed in the present study, assessing cis-regulation of PNS induced expression may reveal relevant genes that are not detectable under PNS naive conditions. Strain variance in PNS induced gene expression across all transcripts may also be assessed for correlation to PNS effects on behavior. Similar to QTL analysis, this may reveal candidate genes that mediate the effects of PNS. Furthermore, multiple associated genes can be checked for covariance and utilized as seeds to reveal gene networks involved in the response to PNS (J. A. Baker et al., 2017; Dai et al., 2009). Associated gene networks may provide insight into the neurobiological effects of PNS and a rich number of gene targets to assess experimentally.

Non-synonymous polymorphisms lead to amino acid substitutions that may have consequences for protein function. All genes within the QTL confidence intervals were assessed for non-synonymous SNPs (nsSNPs). The number of genes that can be eliminated by assessment of non-synonymous polymorphisms varied between QTLs, likely due to genomic variations in SNP density. For example, 92% of genes for the acute locomotion QTL by PNS interaction were eliminated and only 68% were eliminated for the CPP QTL. This approach may exclude genes that cannot be affected by amino acid substitutions, but does not eliminate those genes in which expression is regulated by a polymorphism in regulatory, intron or intergenic regions. By assessing

association with cis-regulated transcripts and identifying nsSNPs, two sets of prioritized candidate genes are produced that represent each scenario. However, considering that many complex trait related SNPs likely regulate gene expression (Nicolae et al., 2010), it may be prudent to give priority to those nominated by association with cis-regulated transcripts.

Missing QTLs

The number of strains is the most influential determinant of statistical power when QTL mapping with inbred strains (Belknap, Mitchell, O'Toole, Helms, & Crabbe, 1996). The present study characterized 21 strains, which provides 80 percent power for a QTL with an effect size of 66%. However, the genetic determinants of a complex traits are likely numerous; each with an effect size of less than 10% (B. Bennett & Carosone-Link, 2006). Therefore, it is likely that many QTL by PNS interactions remain undiscovered. Large samples sizes are particularly challenging in QTL by environment interaction studies, due to multiple conditions required within strain. The present study characterized both sexes, further increasing the burden. Additionally, PNS requires timed breeding and many BXD strains demonstrated very poor breeding under these conditions. Considering these challenges, future forward genetics studies involving PNS may benefit from some alterations in design. One consideration is the number of mice characterized per strain. The present study attempted to account for litter

effects and included an average of six litters per condition per strain. This resulted in a relatively high number of mice produced for characterization per strain (approximately 12 per sex/condition, per strain). Although within strain numbers affect power, benefits generally diminish markedly as n increases (Belknap, 1998; Crusio, 2004). Considering the present study involves manipulation of the dams, accounting for litter effects may be prudent. However, reductions in litters/mice produced per strain could increase the feasibility of characterizing large numbers of strains. Any reductions in accuracy of the strain mean are buffered by large strain numbers. QTL mapping procedures involve testing the effect of genotype and not strain. In a bi-allelic population, such as the BXD panel, all strains are grouped into one of two genotypes at a locus. Therefore, each strain mean is akin to an individual value that is grouped to calculate a genotype mean, and consequently it is the number of strains that has most influence on power.

QTL by Stress Interactions in the Literature

To our knowledge, this is the first attempt at QTL mapping for early life stress by gene interactions. Three studies have mapped for adult stress by gene interactions. QTLs have been discovered for the effects of stress on ethanol-induced locomotion (Cook et al., 2015), fear conditioning (Carhuatanta et al., 2014) and spatial learning (Shea et al., 2015). There are no overlapping stress-

associated QTLs between these studies and the present study. However, it may be assumed that the genetic mediators of early life stress exposure are distinct from those that mediate the response to adult stress exposure. Furthermore, tests for genetic correlation of the PNS effects across traits in the present study found only two associations. These results do not support the possibility that common variants mediate the effects of PNS across cocaine and sensorimotor phenotypes. Similar heterogeneity may exist for genetic variants that mediate effects of adult stress across phenotypes; there are no adult stress-related QTLs that converge between these studies. Despite lack of convergence, future research involving stress by QTL mapping should assess genetic correlations for the effects of stress across traits. Where strain variance is shared across traits, principal component analysis may be utilized. This is thought to increase mapping power by extracting informative variance from genetically related phenotypes and reducing the various and unique sources of environmental and measurement variance associated with individual phenotypes, thus increasing the genetic signal to noise ratio (Dickson et al., 2015). This may be prudent for QTL by stress interaction studies. Carhuatanta et. al., (2015) and Shea et. al. (2015) selected matched littermates and assigned one to control and one to stress. They calculated the difference scores for each of these pairs and were able to determine a stress effect mean and variance for each strain. These data allowed for heritability calculations, and it was found that stress effect heritability was low (0.09 to 0.12). Although heritability for PNS effects cannot

be calculated by this method for the present study, comparisons of trait heritability between control and PNS groups reveal some interesting patterns. For cocaine-related measures, heritability tends to be lower in the PNS group relative to the control groups. This may suggest that the PNS procedure introduces increased environmental variance that could interfere with QTL by PNS mapping. The opposite pattern is observed for sensorimotor behaviors and maternal dam-pup contact; the PNS groups tend to have higher heritability relative the control groups. This suggests that the relative magnitude of the gene by environment interactions effects are high for these traits. However, if the heritability of PNS effects on adult phenotypes is relatively low, QTL mapping efforts may require high powered studies. In addition to adding strains, principal component analysis may greatly benefit these efforts. However, principal component analysis is limited by the occurrence of genetic correlations. It may be that there are many unique variants that mediate stress by gene interactions across traits. The probability of shared variants should increase between related behavioral tests that may measure the same construct (Philip et al., 2010a). For example, Morris water maze and radial arm maze are different tests that both may measure spatial memory. Future studies of QTL by PNS interactions should include batteries of related phenotyping tests that may allow for principal component analysis.

PNS Neurobiology

Neurobiological changes have been attributed to PNS and are likely mediators of the observed behavioral effects. In outbred rats, alterations of dopamine function under basal conditions and in response to drugs of abuse are described and may account for changes in the effects of drugs of abuse, including cocaine. PNS increases basal dopamine concentration and stimulated dopamine release by cocaine and amphetamine in the shell of nucleus accumbens (NAc) (Kippin et al., 2008; Silvagni, Barros, Mura, Antonelli, & Carboni, 2008). Increased dopamine levels may be the result of increased release by hyper-excitable ventral tegmental area (VTA) dopamine neurons (Oosterhof, El Mansari, Merali, & Blier, 2016; Silvagni et al., 2008). PNS also reduces dopamine transporter (DAT) expression, suggesting increased basal and cocaine-induced synaptic dopamine levels could be due to reduced reuptake (Son et al., 2007). DAT density in the striatum is found to be a heritable trait in the BXD panel (Janowsky et al., 2001). DAT density is genetically correlated with acute cocaine locomotion, with increasing locomotion at lower DAT density, as would be expected (Janowsky et al., 2001). However, DAT density was not found to be genetically correlated with locomotor sensitization or CPP. The effects of PNS on DAT expression may be mediated by genetic variants in the BXD panel, with consequences for cocaine locomotion. An interesting approach may be to characterize the effects of PNS on these likely physiological mediators in BXD

strains. Physiological traits may have greater heritability relative to behavioral traits, making them more amenable to QTL discovery. Therefore, genetically mediated PNS alterations to dopamine related physiology may improve discovery of QTLs that interact with PNS to moderate cocaine related behaviors.

Increases in striatal dopamine activity is a likely explanation for the PNS induced increases of cocaine locomotion and reward. However, BXD strains affected by PNS in the present study displayed a striking reduction in cocaine CPP that was specific to males. There is one report of PNS reduction in milk chocolate induced CPP; females displayed reduced CPP and males displayed enhanced CPP (Reynaert et al., 2015). In contrast, PNS enhancement of CPP is reported for nicotine, cocaine, diazepam and morphine (Kippin et al., 2015; Lakehayli et al., 2015; Said, Lakehayli, El Khachibi, et al., 2015; Yang, Li, et al., 2006). PNS augments cocaine CPP in B6 but not D2 mice, indicating variants in the BXD panel should interact with PNS to increase cocaine CPP (Kippin et al., 2015). However, the progenitor strain results do not preclude the possibility that alleles with the opposite effect are also present. There is no direct evidence to indicate the mechanism by which PNS reduces cocaine CPP. One possibility is a PNS induced anhedonic state, as suggested by reduced natural reward CPP in female mice (Reynaert et al., 2015). Reduced cocaine reward sensitivity may also be explained by PNS alterations to the dopamine receptor D2. PNS was found to increase D2 expression in the NAc (Berger, Barros, Sarchi, Tarazi, & Antonelli,

2002; Henry et al., 1995); D2 NAc receptors may attenuate cocaine reward (Calipari et al., 2016). D2 transcript expression and protein density in the NAc are found to be heritable traits in the BXD panel and transcript expression is genetically correlated with ethanol place preference (Hitzemann et al., 2003; B. C. Jones et al., 1999). However, this is a positive relationship, suggesting increased D2 expression does not attenuate ethanol reward. One caveat is a lack of correlation between D2 transcript levels and protein concentration, although both are heritable, the latter may be more indicative of functional outcomes. Strain comparisons may reveal genetically mediated PNS affects on D2 expression that associate with moderation of cocaine reward. Such data would indicate D2 expression to be a physiological mediator of PNS effects on cocaine responsiveness and a valuable phenotype for QTL by PNS mapping.

Alternately, PNS induced deficits to spatial learning may explain decreased CPP. PNS is known to impair spatial learning (Modir et al., 2014). These deficits are likely due to PNS effects on hippocampus development (Weinstock, 2011). CPP may rely, at least partially, on spatial learning (Cunningham, Patel, & Milner, 2006). PNS induced deficits to spatial learning may prevent the acquisition of CPP. These possibilities can be assessed by testing of extreme responders. The strain with the largest reduction in CPP could be compared to a non-responder, in tests of spatial learning and reward sensitivity to natural stimuli and cocaine. Reductions in spatial learning or

reward sensitivity in the responder strain would suggest a genetic relationship. Generally, careful scrutiny of complex behavioral traits may be required to clarify the role of any QTLs discovered. A behavior such as CPP is likely under the influence of multiple, dissociable heritable constructs that present a complex genetic landscape. This complexity can also be assessed in the follow up investigation of candidate genes. When characterizing a candidate gene, it may beneficial to perform extensive phenotyping in order to discern the true role.

Neurobiological effects of PNS are described that may account for effects on PPI. The neural circuitry mediating PPI involves brain stem and midbrain structures that can operate without input of forebrain structures (Koch & Schnitzler, 1997; Swerdlow, Geyer, & Braff, 2001). However, PPI can be modulated by cortical and subcortical forebrain regions. A hyper-dopaminergic state in the NAc reduces PPI (Powell et al., 2003). This may be mediated by D2 receptors in the medium spiny neurons of the NAc (Weber et al., 2010). As PNS causes a hyper-dopaminergic state and increased D2 expression in the NAc, these effects my account for PNS induced deficits of PPI (Kippin et al., 2008; Silvagni et al., 2008). As previously discussed, BXD strains exhibit heritability of NAc D2 receptor density. Assuming that genetic regulators of D2 expression may interact with PNS, characterization of PNS effects on D2 expression may yield QTLs with implications for PNS effects on PPI.

PNS also has effects on neurotransmitter systems in the prefrontal cortex (PFC) that may have implications for PPI. PNS causes a reduction in PFC dopamine and methylphenidate recovers PPI deficits, raising the possibility that deficits in PFC dopamine signaling may also contribute to PPI deficits (Zubedat et al., 2015). PNS induced changes to PFC glutamate physiology have also been described, with reductions in metabotropic glutamate 2 (mGlu2) and mGlu3 in the PFC (Matrisciano et al., 2012). A mGlu2/3 agonist recovers PNS induced deficits to PPI, suggesting a functional role for loss of mGlu2/3 expression (Matrisciano et al., 2012). GABA physiology in the PFC has also been implicated, here it is proposed that up-regulation of DNA methyltransferase causes DNA methylation patterns that suppress expression of GABA-related proteins and ultimately suppresses the function of GABAergic cells in the PFC (Matrisciano et al., 2013). DNA methylation is a good candidate for the enduring effects of early life stress due to the stability of methyl-DNA bonds. For example, low levels of maternal care induce methylation changes to glucocorticoid receptor gene that have been associated with dysregulated HPA activity in adult life (Maccari, Krugers, Morley-Fletcher, Szyf, & Brunton, 2014). Furthermore, QTLs for developmental methylation patterns map to many loci associated with risk of schizophrenia, suggesting heritable early life methylation patterns may contribute to this PPI-associated psychiatric condition (Hannon et al., 2016; Hoffmann, Ziller, & Spengler, 2016). Therefore, it may be beneficial to assess PNS induced methylation patterns across BXD strains. Genome-wide or targeted

genes could be assessed for methylation in brain regions of interest, such as the PFC. These data could be used to map QTLs for variants that moderate PNS phenotypes by mediating PNS induced methylation.

Sex Effects

The present study revealed sex to be an important determinant of PNS effects in the BXD panel. Males appear to be more sensitive to PNS, with significant effects of PNS only detected in males for acoustic startle, PPI, cocaine locomotor sensitization and cocaine CPP. The only measure in which PNS was found not to interact with sex is acute cocaine locomotion. As discussed in chapter 3, where sex effects were found in the progenitor strains, males were also more likely to be affected, although the pattern of sex effects differed somewhat between progenitor and BXD strains. Sex effects are also reported in other studies of PNS effects and prenatal synthetic glucocorticoid exposure for cocaine and sensorimotor phenotypes. Males are more sensitive to effects of PNS on cocaine self-administration and to effects of prenatal dexamethasone exposure on PPI (Hauser, Feldon, & Pryce, 2006; Thomas et al., 2009). Associations between maternal CORT stress levels and the effects of PNS on BXD strains in the present study were exclusive to males, suggesting that males may be more sensitive to the fetal programming effects of CORT on cocaine responsiveness. Similarly, PNS effects on maternal behavior associated with PNS

effects on male but not female CPP. This effect may be explained by differential effects of PNS on maternal interactions with male and female pups, as PNS induced reductions in licking/grooming were found to be exclusive to males (Power & Moore, 1986).

Although the source of sex differences in the effects of PNS is not entirely clear, interruptions of sex hormones is a likely contributor. PNS interferes with sex differentiation processes to produce partial demasculinization and feminization of male behavior, particularly sexual behavior. This effect is likely due to PNS suppression of fetal androgen activity, which prevents masculinization of the male brain and may lead to decreased circulating testosterone levels in adulthood (Ward et al., 2003). The effects of androgen disruption may extend beyond sexual behaviors. Postnatal testosterone treatment to PNS exposed male guinea pigs reverses PPI deficits (Kapoor & Matthews, 2011). PNS reductions to open field locomotion were also reversed by testosterone treatment. Interestingly, testosterone replacement was performed in early adulthood (post-natal day 75), suggesting that disruption to activational effects of testosterone mediate the PNS disruptions of PPI and locomotion. There is no direct evidence to indicate a similar effects in male mice of BXD strains; a study to examine adult plasma testosterone levels in an extreme responder strain compared to a non-responder could indicate differences in PNS induced disruptions to testosterone levels. If adult testosterone disruption is found to be

a likely mediator of male sensitivity to PNS in the BXD strains, the genetic implications need to be considered. One possibility to explaining strain differences in male sensitivity to PNS could be variants that interact with PNS to mediate the effects on male fetal androgen disruption or variants that mediate the effects of suppressed androgen exposure on adult testosterone. Both scenarios could lead to heritable PNS effects on adult testosterone levels. However, the effects of PNS across cocaine and sensorimotor phenotypes did not display genetic correlations, indicating that it is unlikely that a common disruption to adult testosterone production would explain the behavioral effects observed across phenotypes. Another possibility is that independent, trait-specific variants interact with suppressed adult testosterone levels. This possibility would be amenable to experimentation, as adult testosterone is amenable to manipulation. Although investigating a possible role for androgens in the gene by PNS interactions observed in BXD strain may be a significant challenge, exploring this mechanism may be important for elucidating male-specific gene by PNS interactions.

Conclusion

The preceding experiments revealed QTL by PNS interactions for cocaine locomotion and PPI. Gene by environment interactions may be critical determinants of risk for psychiatric disorders, yet few genes have been

identified and consequently there is a limited understanding of these interactions. The research presented here is a critical step in identifying genes that interact with early life stress exposure to produce enduring effects on offspring behavior. Although genetic variants that interact with stress are likely complex and numerous, the unbiased approach taken here has the potential to identify any variants involved. Continued efforts with similar approaches will eventually lead to an extensive understanding of gene function in health and disease. This knowledge will greatly contribute to a comprehensive understanding of psychiatric disorder etiology and allow for improved treatment and preventative options.

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