USING THE COLLABORATIVE CROSS MOUSE POPULATION TO INVESTIGATE ENVIRONMENTAL AND GENETIC FACTORS THAT INFLUENCE COMPLEX BEHAVIORS

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ABSTRACT

Sarah Elizabeth Adams Schoenrock: Using the Collaborative Cross Mouse Population to Investigate Environmental and Genetic Factors That Influence Complex Behaviors
(Under the direction of Lisa Tarantino)

Affective disorders and substance abuse disorders (SUDs) are highly prevalent within the US and result in substantial burdens on the affected individual, their loved ones, and society. Relatively few effective treatments exist however, due to a lack of understanding regarding the etiology of these complex disorders. Development of affective and SUDs are due to both environmental factors, (i.e. perinatal insults, childhood maltreatment, stressful life events), genetic background and the complex interplay of the two. Using inbred strains of mice can potentially disentangle the two (environment and genetic factors), and elucidate their interactions to aid in the identification of specific mechanisms. However, traditional inbred strains pose some limitations in the variation of genetic diversity present, which limits our ability to capture a full phenotypic range that may better model disease states seen in humans. In this thesis, we use a relatively new population of recombinant inbred strains, the Collaborative Cross (CC), that were designed to have increased genetic, and therefore phenotypic, diversity over traditional inbred strains.

We used a panel of female F1 hybrids of CC strains (RIX) to investigate the effects of an environmental factor, nutritional deficiency in the perinatal period, genetic background, parent-of-origin (PO) and any interactions on stress response, anxiety-, and depressive-like behaviors in

adulthood. From this phenotypic screen, we identified two RIX lines (RIX 41/51 and 04/17) that were outliers for novelty-induced locomotion, a predictive trait for addiction-related behaviors. We characterized RIX 41/51 and 04/17 for cocaine (COC)-related behaviors and possible underlying mechanisms including COC metabolism, HPA axis dysregulation and dopamine dynamics in the striatum. We also performed QTL mapping for low initial locomotor response to COC using an F2 intercross of CC041/TauUnc and C57BL/6NJ and identified three significant QTLs on Chr 7, 11 and 14. These studies are the first to assess the CC or RIX of CC for addiction-related behaviors and provides evidence that RIX 41/51 and 04/17 are novel models with a unique genetic diversity to study the underlying mechanisms involved in COC-related behaviors.

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LIST OF ABBREVIATIONS

129 = 129S1/SvlmJ

5-HT = Serotonin

AUC = Area Under the Curve

B6J = C57BL/6J

B6N = C57BL/6NJ

BZE = Benzoylecgonine

CAST = CAST/EiJ

CC = Collaborative Cross

Chr = Chromosome

Cm = Centimorgan

COC = Cocaine

COMT = Catechol-O-methyltransferase

CORT = Corticosterone

CPP = Conditioned Place Preference

CRISPR = Clustered Regularly Interspaced Short Palindromic Repeats

CRISPR/Cas9 = CRISPR-associated protein-9 nuclease

DA = Dopamine

DBA = DBA/2J

DO = Diversity Outbred

DSM = Diagnostic and Statistical Manual of Mental Disorders

dST = Dorsal Striatum

EPM = Elevated Plus Maze

eQTL = expression Quantitative Trait Loci

FSCV = Fast-Scan Cyclic Voltammetry

FST = Forced Swim Test

GWAS = Genome-Wide Association Study

Hipp = Hippocampus

HPA = Hypothalamic-Pituitary-Adrenal (axis)

HPLC = High Performance Liquid Chromatography

IP = Intraperitoneal

IVSA = Intravenous Self Administration

LD = Light/Dark

LOD = Logarithm of Odds

Mb = Megabase

ME = Methyl Enriched

METH = Methamphetamine

MUGA = Mouse Universal Genotyping Array

NAc = Nucleus Accumbens (aka Ventral Striatum)

NE = Norepinephrine

NOD = NOD/ShiLtJ

NOR = Norcocaine

NZO = NZO/HlLtJ

OF = Open Field

PFC = Prefrontal Cortex

PD = Protein Deficient

PK = Pharmacokinetics

PND = Postnatal Day

PO = Parent-of-Origin

PWK = PWK/PhJ

QTL = Quantitative Trait Locus

RI = Recombinant Inbred

RIA = Radioimmunoassay

RIX = Recombinant Inbred Intercross

SIH = Stress-Induced Hyperthermia

SNP = Single nucleotide polymorphism

Std = Standard (diet)

SUD = Substance Use Disorder

VDD = Vitamin D Deficiency

VTA = Ventral Tegmental Area

WSB = WSB/EiJ

CHAPTER 1: GENERAL INTRODUCTION

An individual's phenotype is the result of their genetics, environment, and the interaction of the two. My research interests lie in understanding the role of genes and the environment in predisposition to develop psychiatric disorders. Psychiatric disorder is a term used to refer to a large number of disorders including: affective disorders (anxiety, depression, bipolar), eating disorders (bulimia, anorexia nervosa), dissociative disorders, schizophrenia, obsessive-compulsive disorder, personality disorders, and substance use disorders (SUDs). Within the scope of my thesis, I focus on affective disorders (anxiety and depression) and SUDs – particularly for the psychostimulant, cocaine (COC).

The lifetime prevalence of affective disorders such as anxiety and depression are approximately 29 and 21% respectively in the United States (Kessler *et al*, 2012). In 2015, approximately 20.8 million individuals met the diagnostic criteria for a SUD including 15.7 million for alcohol and 7.1 million for illicit drugs (CBHSQ, 2016). Affective and substance use disorders are highly prevalent and result in significant burdens to the affected individual, their loved ones, and society. Treatments for depression and anxiety include selective serotonin reuptake inhibitors, serotonin-norepinephrine reuptake inhibitors and benzodiazepines but there remains a high rate of treatment cessation due to adverse side effects and a high incidence of individuals who are nonresponsive to treatment (Bull *et al*, 2002; Ferguson, 2001; Trivedi *et al*, 2006). There are a few treatments for alcohol, opioid and nicotine SUDs, however there are currently no Food and Drug Administration-approved treatments for psychostimulants

(SAMHSA, 2014). The complex nature of these disorders, including the interplay of genetics and environmental factors, makes it difficult to fully understand the various underlying mechanisms and prevents development of new effective treatments. Below I will briefly describe the environmental factors and genetic variants that play a role in predisposition to develop these disorders as well as the methods used to study these including genetic reference populations, genetic mapping, and behavioral assays.

Role of Environmental Factors

There are numerous environmental exposures that have been linked to increased predisposition to develop psychiatric disorders. These include exposures that happen in the perinatal period, early childhood, adolescence and adulthood. I thoroughly reviewed the environmental factors related to increased risk for affective disorders in Schoenrock and Tarantino (2014). Briefly, these include exposure to toxins or infection, maternal stress, and nutritional deficiencies during the perinatal period; maternal care/bonding and maltreatment in childhood; and stressful life events, social stress and periods of extreme hormonal change in adulthood. In this thesis, I focused on exposure to nutritional deficiencies during the perinatal period, defined as the time immediately preceding conception, through gestation, and in infancy. The perinatal period is a critical time for brain development, therefore exposures during this time could affect brain development and result in persistent changes throughout the lifespan. Studies linking nutritional deficiencies during this critical developmental period come from exposed groups such as the Dutch population during the Hunger Winter of 1944-1945 and the Chinese during the Great Famine of 1959-1961. Both populations represent unique case studies to examine the effects of perinatal insults throughout the lifespan based on the availability of

detailed medical and food ration records and numerous longitudinal follow-up studies of the affected individuals. Studies of these cohorts in adulthood found that, compared to non-exposed individuals, those exposed to nutritional deficiencies in the perinatal had an increased risk for developing psychiatric disorders including affective disorders (Brown *et al*, 1996; Brown *et al*, 1995; Brown *et al*, 2000; Stein *et al*, 2009), addiction (Franzek *et al*, 2008), and schizophrenia (Brown and Susser, 2008; St Clair *et al*, 2005).

In addition to affective disorders, environmental factors have also been shown to affect the development and maintenance of SUDs. The development of a SUD is a multistep process including initiation of drug use, repeated drug use and the transition to development of a SUD which is characterized by a repeating cycle of three steps: binge/intoxication, withdrawal/negative affect, preoccupation/anticipation (Koob and Volkow, 2010). In this thesis, I focused on the initiation and repeated use of drug phases of the progression towards development of an SUD. Several environmental factors have been identified to play a key role in the likelihood to initiate and repeat drug use, including childhood maltreatment, peer influence or social stress in adolescence, and stressful events in adulthood (Ducci and Goldman, 2012).

Role of Genetics

Familial and twin studies have found a high heritability (h^2) for psychiatric disorders, indicating a significant role for genetics. The h^2 of SUDs range from 0.4 to 0.7 with addiction to COC ranking among the highest (Ducci *et al*, 2012; Goldman *et al*, 2005) and the h^2 for major depression is estimated at 0.37 (Sullivan *et al*, 2012). However, identification of genes that contribute to risk has been challenging due to the polygenic nature of the disorders with many causative variants including both common and rare polymorphisms with varying effect size

(Sullivan *et al*, 2012). Two common approaches for identifying genes involved in these disorders in humans are candidate gene analysis and genome-wide association studies (GWAS). Candidate gene studies are based on the known pathogenesis or pathways used in the treatment of the disorder while GWAS are hypothesis-free surveys of the entire genome.

Candidate genes identified include those that mediate the monoamines, serotonin (5-HT), dopamine (DA), norepinephrine (NE) and play a role in the etiology of affective disorders and SUDs. Catechol-O-methyltransferase (COMT) plays a key role in the metabolism of DA and NE. A common variant of the *COMT* gene, *Met158*, results in less active COMT and higher DA levels. Individuals with the *Met158* allele have decreased stress resilience and higher anxiety (Enoch *et al*, 2003). Another enzyme involved in monoamine metabolism is monoamine oxidase A (MAOA). A rare variant in the MAOA gene that results in an early stop codon and a non-functional protein was reported in patients with mental retardation and impulsive behaviors (Brunner *et al*, 1993).

Candidate genes have also been identified for drug metabolism, mainly genetic variants of enzymes involved in alcohol metabolism, alcohol dehydrogenase (*ADH1B- His48Arg*) and aldehyde dehydrogenase 2 (*ALDH2- Glu487Lys*), both of which have a protective effect on alcohol consumption and dependence (Wall, 2005; Wall *et al*, 2005). Presence of these variants are common in East Asian populations and act to increase buildup of acetaldehyde resulting in an adverse 'flushing' response to alcohol. Disulfiram is a treatment for alcohol use that blocks ALDH resulting in a build-up of acetaldehyde and the 'flushing response'.

GWAS are a powerful method to detect common alleles with large or moderate effects across the entire genome for a particular phenotype. GWAS requires a large sample size, but integration of samples is facilitated across testing sites by the use of the same microarrays. Additionally, the

cost of genotyping is declining making large scale GWAS much more feasible. The most notable GWAS finding for SUDs is the *CHRNA5-CHRNA3-CHRNB4* cluster of chromosome 15q25, that encodes for the subunits of the nicotinic acetylcholine receptors and confers risk for addiction to nicotine (Bierut *et al*, 2007; Thorgeirsson *et al*, 2008).

Role of Gene-by-Environment

Not all individuals exposed to a specific environmental factor such as perinatal nutritional deficiency or a stressful life event go on to develop a psychiatric disorder. This indicates an interplay between genetic background and environmental exposure in development of these disorders and highlights the need to consider this interaction in experimental design. Very few genetic variants that interact with environmental factors have been identified. One that is heavily studied in the context of psychiatric disorders is a variant that results in differences in copy number and transcriptional efficiency in the promotor region (*HTTLPR*) of the serotonin transporter gene (*SLC6A4*). Genotype at *HTTLPR* has been associated with development of depression following a stressful live event (Caspi *et al*, 2003). A common variant in the regulatory region of the *MAOA* gene, *MAOA-LPR* leads to differences in copy number and therefore activity of the MAOA enzyme. An interaction between the *MAOA-LPR* low activity genotype and exposure to child adversity (i.e. maltreatment or abuse) has been shown to increase risk for developing conduct disorders, alcoholism and antisocial personality disorder (Caspi *et al*, 2002; Ducci *et al*, 2008).

Using Animal Models to Study Environmental Factors, Genetics and Gene by Environment Interactions

The end goal of my research is identification of gene variants that interact with specific environmental factors to influence risk for development of psychiatric disorders. A major obstacle is disentangling these effects (gene, environment, gene-by-environment) for a given phenotype. Our ability to do this is humans is complicated due to the inability to accurately document all environmental exposures an individual has encountered throughout their lifespan (especially the perinatal period). Additionally, it is hard to properly control for the diversity of exposures in a given population.

Inbred Strains of Mice

Animal models, specifically inbred strains of mice, can be of substantial benefit in the research of psychiatric disorders. An inbred strain is generated through brother-sister mating pairs for at least 20 generations to reach homozygosity at loci in the genome. This allows for reproduction of the inbred genetic background at any time. Hundreds of inbred strains of mice currently exist, allowing for assessment of genetic background and environmental factors by comparison across or within strains, respectively. Additionally, gene-by-environment effects can be assessed using a panel of inbred strains exposed to the same environmental factor.

Advantages of using inbred strains include; 1) ability to control the environment to allow for manipulation and assessment of specific variables at certain time points, 2) access to relevant tissues (i.e. brain) needed for mechanistic studies both *in vivo* and postmortem, 3) availability of numerous stable and reproducible genetic backgrounds to assess phenotypic and genetic variation across strains, 4) access to fully sequenced genomes or dense genotyping and well-

developed tools for manipulation of the genome (i.e. clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein-9 nuclease (Cas9)), 5) short gestation periods (~21 days) and life spans with adulthood reached at ~60 days. These advantages will enable for the detection of the effects of genetics (comparison across strains), a specific environmental exposure (comparison within strain), and gene by environment interactions. Below we will discuss the methods used to assess behavioral phenotypes related to psychiatric disorders and identification of causative genes in mice.

Behavioral assays that measure aspects of psychiatric disorders

Our ability to study psychiatric disorders in mice is limited to assessment of specific aspects of these disorders. Numerous behavioral assays have been validated to have some degree of construct, face or predictive validity (reviewed in Nestler and Hyman (2010)). These assays were developed to assess one of the Diagnostic and Statistical Manual of Mental Disorders (DSM) criteria for diagnosis of that disorder, for example depression-related assays assess psychomotor agitation or retardation, anhedonia, and homeostatic symptoms (changes in sleep or eating behavior). However, there will always be certain aspects of the disorders that are impossible to assess in rodents, such as suicidal thoughts reported in patients with depression. Additional behavioral assays were developed as screens for antidepressant or anxiolytic agents, such as the forced swim test (FST), light/dark (LD) test and elevated plus maze (EPM) and, thus, show good predictive validity. Below I will focus on the assays employed in this thesis.

Depressive-like behaviors

To assess depressive-like behavior we used the FST in which a mouse is placed into a glass cylinder filled with water and the amount of time the animal exhibits escape behaviors (swimming, climbing) is monitored. This phenotype is thought to correlate with behavioral despair in humans and was developed based on the ability of antidepressants to increase the amount of time an animal exhibits escape behaviors (Porsolt *et al*, 1977a; Porsolt *et al*, 1977b).

Anxiety-like behavior

We assessed anxiety-like behavior using two common tests- the open field (OF) and LD. Both assays depend on the natural instinct of rodents to avoid open spaces and predators and the conflicting instinct to venture into riskier areas in search of possible rewards (Nestler *et al*, 2010). Time spent or entries into the center of the OF arena or the lighted side of the LD arena are used to measure anxiety-like behavior with a greater amount of time spent or entries in these areas indicating lower anxiety (Crawley, 1981; Prut and Belzung, 2003). We also used stress-induced hyperthermia (SIH) which is a physiological measure of anticipatory anxiety that is sensitive to anxiolytics (Borsini *et al*, 1989).

Assessing stress response

Exposure to stressful events and the ability to respond to a stressor are thought be precipitating factors for a variety of psychiatric disorders. Therefore, the functioning of the hypothalamic pituitary adrenal (HPA) axis is commonly assessed in rodents at baseline and after exposure to an acute or chronic stressor. Multiple stressors have been validated as a means to increase circulating corticosterone (CORT), the primary stress hormone in rodents that indicates

increased stress. These assays include restraint stress, exposure to a novel environment, chronic unpredictable mild stress, and social stress (Katz, 1981; Kudryavtseva *et al*, 1991; Piazza *et al*, 1989; Willner *et al*, 1992).

Addiction-related behaviors

Behavioral assays that assess addiction-related phenotypes, specifically psychostimulant behaviors, include non-contingent (experimenter-administered) and contingent (self-administered) drug administration. Psychomotor activation in an arena such as an OF is commonly used to assess non-contingent acute and repeated exposure to psychostimulants (van den Buuse *et al*, 2005). Behavioral sensitization, or an increase in psychomotor activation with repeated exposures to a drug, is a long-lasting phenomenon and is thought to reflect the presence of long-term neuronal changes that could underlie relapse behavior (Steketee and Kalivas, 2011). Conditioned place preference (CPP) is another test that involves repeated non-contingent drug exposure in which Pavlovian conditioning is used to train an animal to associate the subjective effects of a drug with a specific environment based on lighting, smell, and floor texture. After multiple training days, an animal's preference-for the drug paired environment is thought to indicate whether they find the subjective effects of the drug rewarding or aversive (Bardo and Bevins, 2000). Intravenous self-administration (IVSA) is a commonly used paradigm that allows for the assessment of multiple phenotypes associated with addiction including acquisition and maintenance of drug-taking behavior, motivation to obtain drug (progressive-ratio breakpoint), extinction and reinstatement behaviors (Sanchis-Segura and Spanagel, 2006; Thomsen and Caine, 2007) and it is used to validate potential treatments for SUDs (Mello and Negus, 1996).

Gene identification in mice: QTL mapping

As in humans, both candidate gene analysis and genome-wide analysis of genotype and phenotype are used in mice to identify genes that influence behavior. An approach called quantitative trait loci (QTL) mapping depends on phenotypic and genetic diversity within the mapping population. Genotyping is done using single nucleotide polymorphisms (SNPs) at places evenly distributed throughout the genome that differ between the mouse strains with millions of SNPs present among all strains. Interval mapping methods are used to infer genotypes between markers based on recombination frequency and distance between markers. A test is then performed at each locus to determine if the genotype is associated with the phenotype. Logarithm of odds (LOD) scores are used to determine genome-wide significance thresholds, corrected for multiple testing. There are programs available to aide in QTL mapping including *R/qtl* (Broman and Sen, 2009). Below we discuss the genetic reference populations used for genetic analysis with a focus on those using inbred strains and recombinant inbred (RI) populations.

Genetic Reference Populations of Mice

Intercross and backcross populations

A common approach used in QTL mapping is generating a backcross or intercross population using two inbred strains. For an intercross, two inbred strains (for example, AA and BB) are crossed to generate F1s that are heterozygous at all loci for the two parental alleles (AB). F1s are then intercrossed to generate an F2 population. Due to recombination, animals will be homozygous for either parental strain (AA or BB) or heterozygous (AB) at any given locus. A backcross, begins the same way with a generation of heterozygous F1s (AB) but the F1 is

backcrossed to one of the parental strains (for example, AA) resulting in N2 offspring that are either AB or AA at a given loci. Within each of these mapping populations, each F2 or N2 animal is genetically distinct resulting in a quantitatively distributed phenotypic range. Each animal in an intercross or backcross needs to be genotyped, although low density genotyping is sufficient due to the level of recombination present in these types of crosses. There are advantages and disadvantages to a F2 and N2 crossing approach which are discussed in Tarantino and Eisener-Dorman (2012b).

Recombinant inbred strains with two parental strains

Panels of RI strains are also used for QTL mapping. RIs are generated from crossing two inbred strains to generate an F2 and then intercrossing random pairs of F2 followed by successive brother-sister matings for at least 20 generations to reach inbred status (Bailey, 1971). Multiple RI strains derived from a cross between the same two inbred strains represent a RI panel. One of the most common RI panels is the BXD which resulted from a cross between C57BL/6J (B6J)and DBA/2J (DBA) (Peirce *et al*, 2004; Taylor *et al*, 1999) and currently has 198 available strains (http://www.genenetwork.org/mouseCross.html). Advantages of the BXD population include a publicly available database GeneNetwork.org that has gene, protein and metabolite expression data, genotypes, and phenotypic data to facilitate systems genetics (Parker *et al*, 2017). BXD strains have been used to perform QTL mapping for behaviors related to several drugs of abuse including ethanol, COC and methamphetamine (METH) (Browman and Crabbe, 2000; Bryant *et al*, 2009; Dickson *et al*, 2016; Phillips *et al*, 1998).

Limitations of commonly used inbred strains

While we have focused on the advantages and uses of traditional inbred strains thus far, there are limitations to these strains. One fundamental limitation is the lack of genetic diversity present across commonly used strains. A study by Yang *et al* (2011) found that there was limited and non-random genetic diversity present among classical inbred mouse strains with overrepresentation of genetic origin from the *Mus musculus domesticus* haplotype. Limited genetic diversity present across strains can limit the phenotypic range. Therefore, increasing genetic diversity can increase the phenotypic range and allow for identification of new phenotypic extremes that may represent disease states not seen in traditional inbred strains.

Collaborative Cross

The Collaborative Cross (CC) is a population of RI strains with eight parental strains that was designed by the Complex Trait Consortium to enable system genetic approaches for studying complex traits. The eight strains were carefully selected to represent three different subspecies of *Mus musculus* (*domesticus, musculus, castaneus*) and include both classical (A/J, B6J, 129S1Sv/ImJ, NOD/ShiLtJ, NZO/HlLtJ) and wild-derived (PWK/PhJ, CAST/EiJ, WSB/EiJ) strains (Churchill *et al*, 2004; Collaborative Cross, 2012; Threadgill and Churchill, 2012).

Early studies characterizing the genetic background of pre-CC strains (not fully inbred) via dense genotyping arrays have shown genome-wide distribution of the eight founder strains in relatively estimated ratios, although there are a few strains with missing contributions from one-two strains due to breeding errors. Additionally, the genetic diversity present in the CC captures ~90% of that present in the domesticated house mouse, and there is no evidence of long-range

linkage disequilibrium that can confound QTL detection (Collaborative Cross, 2012; Iraqi *et al*, 2008; Roberts *et al*, 2007; Welsh *et al*, 2012). Recent analysis of the CC genomes via genotyping microarrays and DNA sequencing has refined earlier haplotype reconstructions and reports a reduction in overall contribution from two of the wild-derived strains (CAST/EiJ and PWK/PhJ) and new genetic variants on the order of tens of thousands due to mutations and genetic drift, including large deletions (>10kb) (Srivastava *et al*, 2017). Studies using panels of pre-CC, CC or F1s have validated the increased phenotypic range present in this population over traditional experimental mouse populations for a variety of complex traits (Ferris *et al*, 2013; Graham *et al*, 2015; Gralinski *et al*, 2015; Levy *et al*, 2015; Mosedale *et al*, 2017). Additionally, due to the increased diversity, new mouse models of disease states present in humans have also been identified (Rasmussen *et al*, 2014; Rogala *et al*, 2014). These studies highlight the usefulness of the CC to overcome limitations of traditional inbred strains for the study of complex traits, although the CC have not been used until now to assess behavioral phenotypes related to affective disorders and SUDs.

Questions Addressed in This Thesis

In Chapter 2, we assessed the role of genetics and an environmental factor, nutritional deficiency during the perinatal period, on behaviors that model anxiety- and depressive-like behavior and stress response. We used 18 different CC strains to generate nine sets of female F1 hybrids, also known as recombinant inbred intercross (RIX) lines. We exposed dams from each of the CC strains to one of four experimental diets (protein deficient, vitamin D deficient, methyl enriched or standard) during the entire perinatal period. This experimental design allowed us to

assess genetic background, parent-of-origin (PO) effects, and perinatal diet exposure on behavior.

In Chapter 3, two RIX lines (41/51 and 04/17) were identified in the experiment described in Chapter 2 as phenotypic outliers for novelty-induced locomotion. These lines were characterized for their utility as a model to assess high and low susceptibility for development of a COC SUD. Initial characterization of the lines included assessment of initial locomotor sensitivity, dose response, rewarding properties and behavioral sensitization to COC. Additionally, we assessed possible underlying mechanisms for the divergent behaviors observed in the two RIX lines including the HPA axis response to stress, tissue levels of monoamines (DA, 5-HT, NE), dopamine dynamics and pharmacokinetics (PK). These studies are the first to assess CC strains and RIX lines for addiction-related behaviors and provide evidence for the utility of RIX 04/17 and 41/51 for the study of addiction.

In Chapter 4, we assessed the genetics of initial cocaine sensitivity as measured by locomotor response to an acute dose of COC. We used an F2 intercross of CC041/TauUnc x C57BL/6NJ to perform QTL mapping for low locomotor response to COC to determine the mode of inheritance and QTL regions for identification of potential candidate genes controlling this response.

CHAPTER 2: PERINATAL NUTRITION INTERACTS WITH GENETIC BACKGROUND TO ALTER BEHAVIOR IN A PARENT-OF-ORIGIN DEPENDENT MANNER IN ADULT COLLABORATIVE CROSS MICE [1,2]

INTRODUCTION

It is now well-established that the risk for developing a psychiatric disorder is influenced by a combination of genetics, the environment and gene-by-environment interactions (Lee and Avramopoulos, 2014). The complex etiology of psychiatric disease, whereby risk is likely due to the actions of hundreds or thousands of genes as well as numerous and largely undefined environmental factors, has made it difficult to pinpoint mechanisms and improve treatment and prevention strategies.

One potentially modifiable environmental factor that has been linked to increased risk of psychiatric disorders is perinatal exposure to nutritional deficiencies. Longitudinal studies of the Dutch Hunger Winter and Chinese Famine cohorts have shown that perinatal nutritional deficiency increases risk for developing schizophrenia (Brown *et al*, 2008; Hoek *et al*, 1998; Hoek *et al*, 1996; St Clair *et al*, 2005; Susser *et al*, 1996; Xu *et al*, 2009), affective disorders

[1]This chapter previously appeared as an article in the *Genes, Brains and Behavior* journal. The original citation is as follows: Schoenrock SA, Oreper D, Farrington J, McMullan RC, Ervin R, Miller DR, Pardo-Manuel de Villena F, Valdar W and Tarantino LM (2017). Perinatal nutrition interacts with genetic background to alter behavior in a parent-of-origin dependent manner in adult Collaborative Cross mice. *Genes Brain Behavior*. 2017, 1-18. https://doi.org/10.1111/gbb.12438 Reprinted with premission from Wiley.

[2]Supplemental material for this chapter is provided in Appendix A

(Brown *et al*, 1996; Brown *et al*, 1995; Brown *et al*, 2000; Stein *et al*, 2009), and addiction (Franzek *et al*, 2008) later in life. However, not all individuals exposed to perinatal nutritional deficiencies develop psychiatric disorders, suggesting potential interactions between the perinatal environment and genetic background. Published studies further suggest this interaction could be modulated by genomic imprinting, a phenomenon in which an allele is preferentially expressed depending on its parent-of-origin (PO)(Ishida and Moore, 2013); example, the expression of imprinted genes *IGF2, GNASAS,* and *MEG3* were persistently altered in exposed vs unexposed siblings from the Dutch Hunger Winter cohort (Heijmans *et al*, 2008; Tobi *et al*, 2009). Approximately 150 imprinted genes have been identified, many of which are highly expressed in the brain (Davies *et al*, 2005; Williamson *et al*, 2013). Imprinted genes are known to be key regulators in prenatal development and postnatal growth (Cleaton *et al*, 2014), and dysregulation of imprinting has been shown to result in developmental disorders such as Prader-Willi, Angelman, Beckwith-Wiedmann and Silver-Russell syndromes, all of which result in growth and behavioral alterations (Ishida *et al*, 2013).

Although the Dutch Hunger Winter and Chinese Famine allowed for observational studies of a perinatal nutrition challenge in human populations, obvious ethical issues preclude controlled studies during this key developmental period in humans. Fortunately, rodent models of psychiatric disorders have been developed and offer clear advantages for studying genetic and environmental risk factors. The advantages include: a short gestation and time to adulthood; the ability to control and manipulate the environment; access to relevant tissues (i.e., brain) for mechanistic studies; and access to well-characterized and replicable experimental populations along with advanced genetic tools.

A growing number of rodent studies demonstrate the persistent effect of perinatal nutritional deficiency on behavior in adulthood. Adult rodents exposed prenatally to vitamin D deficiency (VDD) show alterations in behaviors that model schizophrenia, including enhanced sensitivity to amphetamine (Kesby *et al*, 2010), spontaneous hyperactivity (Burne *et al*, 2004; Burne *et al*, 2006; Eyles *et al*, 2006; Harms *et al*, 2008; Kesby *et al*, 2006) and decreased sustained attentional processing (Harms *et al*, 2012b; Turner *et al*, 2013). Rodents exposed to prenatal protein deficiency (PD) exhibit alterations in behavioral models of schizophrenia including enhanced sensitivity to amphetamine (Palmer *et al*, 2008) and decreased prepulse inhibition and startle response (Palmer *et al*, 2004); behaviors that model affective disorders including increased depressive-like (Belluscio *et al*, 2016; Belluscio *et al*, 2014; de Godoy *et al*, 2013; Vucetic *et al*, 2010) and anxiety-like behaviors (Belluscio *et al*, 2014; Reyes-Castro *et al*, 2012a); and addiction-like behaviors including enhanced sensitivity to cocaine (Valdomero *et al*, 2006; Vucetic *et al*, 2010). Rodents exposed to methyl donor deficiency (i.e. choline, folate) during some part of the perinatal period display increased anxiety-like behaviors (Ferguson *et al*, 2005; Konycheva *et al*, 2011) and alterations in learning ability and memory (Berrocal-Zaragoza *et al*, 2014; Konycheva *et al*, 2011). A few studies in rodents have also reported strain differences in behavior after exposure to perinatal nutritional deficiencies (Harms *et al*, 2012a; Harms *et al*, 2008; Langley *et al*, 2015), although genetic-background dependent effects are largely unexplored in animal models. Rodent models also support the hypothesis that perinatal nutritional deficiencies lead to the dysregulation of imprinted genes and mediate behavior. Two studies using mice have reported alterations in the imprinted genes *Cdkn1c* and *Igf2* following perinatal exposure to protein deficiency (Vucetic *et al*, 2010) or methyl donor deficiency (Waterland *et al*, 2006).

In this study, we used a recently established mouse resource, the Collaborative Cross (CC) (Churchill *et al*, 2004; Srivastava *et al*, 2017). The CC is a panel of recombinant inbred strains with each strain derived from an independent cross of eight inbred founders. The eight founders, composed of five classical laboratory strains and three wild-derived strains, capture 90% of the common genetic variation present in the domesticated house mouse (Roberts *et al*, 2007). The CC was specifically designed for the study of complex phenotypes like behavior (Threadgill *et al*, 2012).

In our study, we exposed females from 20 CC strains to one of four experimental diets prior to and during gestation and throughout the postnatal period until weaning. Strains were grouped into 10 pairs, and each strain within a pair was crossed with its CC strain partner, generating 10 genetically distinct types of reciprocal F1 hybrid female offspring (also known as Recombinant Inbred Intercrosses, RIX). Based on this breeding strategy, all females within a reciprocal F1 pair were genetically identical except for the PO of the nuclear genome and, potentially, the mitochondria. These F1 females were subjected to a battery of commonly used behavioral models of psychiatric disorders (**Fig 2.1c**).

Our experimental design takes advantage of this unique and powerful mouse population to detect the effects of exposure to nutritional deficiencies, genetic background, PO effects, and importantly, their interactions to alter behavior in adulthood.

METHODS AND MATERIALS

Collaborative Cross (CC) strain selection

Generation of CC strains has previously been described in detail (Collaborative Cross, 2012; Srivastava *et al*, 2017). Briefly, CC are recombinant inbred strains created from eight inbred

lines from the three major *Mus musculus* subspecies, *domesticus* (A/J, C57BL/6J (B6J), 129S1/SvImJ, NOD/ShiLtJ (NOD), NZO/H1LtJ, WSB/EiJ), *castaneus* (CAST/EiJ) and *musculus* (PWK/PhJ). CC mice were purchased from the Systems Genetics Core Facility (SGCF) at the University of North Carolina (UNC)(Welsh *et al*, 2012).

The 20 CC strains used to generate the 10 RIX lines (**S Table 1; Fig 2.1a**) were selected using Rexplorer, a program developed at UNC for reciprocal cross strain selection (Oreper, Tarantino and Valdar, personal communication). Strain-pair selection aimed to maximize several criteria. 1) The number of known brain imprinted loci, as defined from (Crowley *et al*, 2015; Williamson *et al*, 2013), that are heterozygous between two categories of haplotypes: those that are identical by descent with NOD, and those that are identical by descent with B6J. This criterion was based on pilot data suggesting that offspring from a reciprocal cross between these two strains had different behavioral responses to perinatal nutritional deficiencies (Oreper, Valdar and Tarantino personal communication). Other criteria included: 2) maximum heterozygosity at all other haplotypes; 3) linkage disequilibrium between imprinted loci of interest in the ten-RIX population; and 4) predicted reproductive success (unpublished observations; http://csbio.unc.edu).

Perinatal diet exposure and breeding RIX lines

CC mice were purchased at approximately 4-5 weeks of age and acclimated for at least one week prior to diet exposure. After acclimation, dams were placed on one of four diets (**S Table 2** and see below) for five weeks. The average age at which dams were placed on the experimental diets was 41.4 ± 7.1 days. After five weeks on the experimental diet, dams were mated with a sire from a different CC strain to generate F1 offspring (**S Table 1**, **Fig 2.1a**). The average age of

dams at mating was 76.5 ± 7.3 days. CC dams remained on experimental diets throughout gestation and until litters were weaned, ensuring that the offspring were exposed throughout the entire perinatal period (**Fig 2.1b**). Sires were removed from the breeding cage once pregnancy was confirmed by visual inspection. We maximized the number of dams used in the study to ensure that behavioral observations in the F1 offspring were not attributable to a single dam. At least 3 dams per CC strain were used (**S Table 3**). We also used only the first litter per dam to avoid differences in reproductive parity and to minimize variation in timing of exposure to experimental diets. Due to the large scale of this study, we did not cross-foster the F1 offspring; consequently, any observed PO effects could be due to genomic imprinting and/or maternal effects.

F1 offspring were weaned at postnatal day (PND) 23.1 ± 2.8 days onto standard laboratory chow (Harlan Teklad 2920; Envigo, Frederick, MD, USA). Female offspring were co-housed with reciprocals to control for cage effects. Male offspring were provided at weaning to a collaborator and used for unrelated studies (Xue *et al*, 2016).

All mice were housed in a specific pathogen free vivarium and maintained on a 12-hour light/dark cycle with lights on at 7 A.M. All procedures and animal care were approved by the UNC Institutional Animal Care and Use Committee and followed the guidelines set forth by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Nomenclature

The official name of each CC strain used in this study is provided in **S Table 1**. For simplicity, we use only the last two digits of the name when referring to F1 hybrids in the text and figures. For each reciprocal F1 line the dam is listed first and the sire is listed second; for

example CC(01x11)F1 refers to (CC001/Unc x CC011/Unc)F1. When referring to the collective group of F1 mice from a pair of reciprocal crosses, we simply list the two CC strains in numerical order separated by a forward slash; for example RIX 01/11 includes mice from both reciprocal crosses, (CC001/Unc x CC011/Unc)F1 and (CC011/Unc x CC001/Unc)F1.

Experimental Diets

Experimental diets were formulated by Dyets Inc. (Bethlehem, PA): vitamin D deficient (VDD; #119266), protein deficient (PD; 7.5% casein; #102787), methyl donor enriched (ME; #518893) or standard control (Std; #AIN-93G). **S Table 2** shows the nutrient composition for all experimental diets. The PD and VDD diets were nutritionally matched to the Std diet. ME was matched to a methyl donor deficient diet that was used in pilot studies and eliminated from these experiments due to the inability of dams to produce viable offspring when exposed to the diet. All diets were administered in pelleted form and were available *ad libitum* to the dams during the perinatal period. The amount of diet consumed by each dam was not recorded.

Behavior Assays

Only female F1 offspring were tested to ensure an identical genetic background, including the sex chromosomes, between reciprocal hybrids. All behavioral testing was performed during the light part of the light/dark cycle between 8:00 A.M. and 12:00 P.M. Animals were assessed in adulthood (average age at initiation of testing was 68.2 ± 5.3 days). Mice were screened in a two-week behavioral pipeline that consisted of (in order of testing) open field (OF), light/dark (LD), stress-induced hyperthermia (SIH), forced swim (FST), and restraint stress tests (**Fig 2.1c**). Mice were free from testing for at least 1 day between behaviors. Behavioral tests were

administered in order of lowest to highest invasiveness/stress, in order to mitigate prior testing effects. We aimed to screen at least 20 females per RIX (10 of each reciprocal) for each of the four diet exposures. Actual sample sizes are provided in **S Table 1**. Due to the large number of animals screened (N=685), behavioral testing took place in 28 batches over a two-year period. In each batch, we tested a minimum of two diet exposure groups and two RIX. Each RIX and diet exposure was distributed across at least 3 test batches to mitigate seasonal and batch effects. All testing equipment was cleaned with a 0.25% bleach solution between test subjects unless otherwise stated.

Body weight

Female F1 offspring were weighed at weaning (referred to as PND 21) and again at the initiation of behavioral testing (referred to as PND 60). We did not weigh dams so as not to induce stress associated with manipulation during the perinatal period.

Open field (OF)

The OF apparatus (ENV-515-16, Med Associates, St. Albans, VT, USA) was a 43.2x43.2x33 cm arena consisting of a white Plexiglas floor and clear Plexiglas walls with infrared detection beams at 2.54 cm intervals on the x, y, and z axes that tracked the animals' position and activity automatically during the test session. The OF apparatus was enclosed in a sound-attenuating chamber (73.5x59x59 cm) fitted with two overhead light fixtures containing 28-V lamps. Mice were placed in the OF arena for 10 minutes and scored for total distance traveled (cm), number of vertical movements (rearing), and percent time spent in the center of the arena (defined as the 22.86 cm^2 central part of the arena). Percent time spent in the center of the OF arena is commonly used as a measure of anxiety-like behavior. Data were analyzed post-session in 2-minute bins using commercially available software (Activity Monitor 5.1, Med Associates).

Light/dark (LD)

The LD apparatus consisted of a 42x42x30 cm open field arena (Versamax420 Animal Activity Monitoring System, AccuScan Instruments Inc., Columbus, OH, USA) with a white Plexiglas floor and clear Plexiglas walls. The arena was surrounded by 16 photobeams along each side that allowed for tracking of both horizontal and vertical activity. The black Plexiglas LD box (40x21x13 cm) occupied one-half of the arena and had a 10x3 cm opening to the light side and holes on all four sides that allowed detection of movement by the photobeams. Mice were placed in the lighted area immediately adjacent to and facing the entry to the dark enclosure and left to freely investigate the apparatus for 10 minutes. The amount of time (sec), number of transitions, distance moved (cm), and percent time in the dark and light zone was scored in 2-minute bins in post-session analyses using commercially available software (VersaMap, AccuScan Instruments, Inc). Transitions into and percent time spent in the light side of the arena are used as measures of anxiety-like behavior.

Stress-induced hyperthermia (SIH)

Mice were individually removed from the home cage and the initial temperature (SIH-T1) was measured by insertion of a lubricated digital thermometer probe (TH-5 Thermalert Monitoring Thermometer with RET-3 rectal probe, Physitemp Instruments, Clifton, NJ) 1-1.5 cm into the rectum for approximately 10 seconds. The animal was immediately returned to the home cage and 10 minutes later, the temperature measurement was repeated (SIH-T2). The difference in body temperature between T2 and T1 was calculated as the change in temperature (SIH-ΔT). This physiological response to a stressor is commonly used as a measure of anxiety-like behavior and is responsive to anxiolytic drugs (Adriaan Bouwknecht *et al*, 2007).

Forced swim test (FST)

The FST was conducted in a glass-polycarbonate cylinder (46cm tall x 21cm in diameter) filled with water to a depth of 15 cm and maintained at a temperature of 25-28°C. Mice were tested for six minutes. Percent immobility during the last four minutes of the test period was recorded by video and scored using Ethovision 7.0 automated tracking software (Noldus, Leesburg, VA). Immobility was defined as the mouse making no movements other than those needed to stay afloat; this phenotype is thought to capture behavioral despair or depressive-like behavior. Mice were monitored continuously and removed from the apparatus if they were unable to keep their nose or heads above water for more than 30 seconds.

Restraint stress

Restraint was used to elicit a stress response that was quantified by measurement of corticosterone (CORT) levels in the serum. A retro-orbital blood sample was taken from unanesthetized mice to assess basal CORT levels, and then mice were immediately placed into a Broome-Style restraint tube (Plas Labs, Inc., Lansing, MI, USA) for 10 minutes. Immediately upon removal from the restrainer, a second unanesthetized retro-orbital eye bleed was performed to assess stress-induced CORT levels (Stress CORT). Whole blood was centrifuged to isolate plasma and CORT levels (in ng/ml) were measured with a competitive radioimmunoassay (RIA) using the manufacturer's protocol (MP Biomedicals, Santa Ana, CA, USA). Stress CORT minus basal CORT levels (change in CORT or ΔCORT) was calculated to assess stress reactivity. CORT RIAs were only performed on RIX lines that had an N>4 for both sets of reciprocal females within a diet exposure group. Therefore, RIX 04/17 ME, RIX 23/47 Std, RIX 03/14 ME and PD and RIX 35/62 ME and PD groups were excluded from these analyses.

Missing data from the behavior pipeline

Equipment failure led to the loss of data from 14 females in the OF (three RIX 01/11 PD, four RIX 01/11 ME, three RIX 41/51 Std and four RIX 23/47 PD) and five animals in the LD (one RIX 01/11 ME and four RIX 04/17 VDD). Ten females died during restraint stress and 15 females did not have enough serum for CORT RIA analysis.

The entire dataset from this study will be deposited and made available in the Mouse Phenome Database (MPD; RRID:SCR_003212) for use in follow-up studies.

Statistical Analysis

All statistical analyses were performed using R Studio 1.0.136 or SPSS v24 for Mac OS X 10.6+. Graphs were generated using Graphpad Prism 7.0c for Mac OS X.

Analysis of breeding performance

Percent productive matings = ((# litters born/# dams mated) x 100), average litter size at birth, % survival to weaning = ((# pups at weaning/# pups born) x 100), and sex ratio = (# F pups weaning/total pups at weaning) were calculated for each CC reciprocal cross/diet combination. For each of these four measures, an ANOVA was performed to assess effects of PO, diet and diet-by-PO effects within each RIX. Overall effects of genetic background (RIX), diet and diet-by-RIX across all 20 CC reciprocal crosses was also analyzed by ANOVA.

Correlation of behavioral phenotypes

The Hmisc 4.0-2 package in R Studio was used to generate coefficient and *p* values using Spearman correlation (Harrell *et al*, 2017). The *p* value threshold for significance was adjusted to $p<0.00026$ to correct for 190 correlations. Correlation plots were generated using Corrplot 0.77 package (Wei and Simko, 2016).

Analysis of effects of RIX, diet and diet-by-RIX

To assess the overall effect of genetic background (also referred to as RIX effect or RIX in the text), perinatal diet and perinatal diet-by-RIX interactions, we fit linear mixed models to the behavior of all mice. Model fitting was performed using the lmer function from the lme4 1.1-12 R package (Bates *et al*, 2015). Our 'base' model had fixed effects of RIX, Diet, and Diet-by-RIX, and random effects of Dam, Sire, and Behavior Batch (**Eq 1**; lmer notation, where "(1|term)" indicates a random effect).

(Eq 1) *phenotype ~ RIX + Diet + Diet:RIX + (1|Dam) + (1|Sire) + (1|BehaviorBatch)*
(Eq 2) *phenotype ~ RIX + Diet + Diet:RIX + (1|Dam) + (1|Sire) + (1|Testing Chamber) + (1|BehaviorBatch)*
(Eq 3) *phenotype ~ RIX + Diet + Diet:RIX + (1|Dam) + (1|Sire) + (1|BehaviorBatch) + (1|TestOrder)*
(Eq 4) *phenotype ~ RIX + Diet + Diet:RIX + (1|Dam) + (1|Sire) + (1|BehaviorBatch) + (1|TestOrder) + (1|Experimenter)*

For behavioral tests, additional random effects were added to the base model (**Eq 1**) as needed. For the OF, LD and FST phenotypes, test chamber was also included (**Eq 2**). For the SIH phenotypes, test order was added (**Eq 3**) whereas for restraint test phenotypes both test order and experimenter were added (**Eq 4**), as two people were required to conduct that test. The same experimenter conducted all other behavioral assays across all batches.

P-values for fixed effects were calculated by Type I ANOVA (sequential) tests, using the lmerTest 2.0-33 package (Kuznetsova *et al*, 2016) (see **Table 2.1** for a list of all phenotypes). To account for multiple testing, *p* values within each type of test (eg, tests of diet) were subject to false discovery rate correction, with *q* values generated using the Shiny implementation of the qvalue R package (Bass *et al*, 2015; Storey, 2002). When a significant diet effect was observed

(p<0.05), between-diet differences were examined by a Tukey's HSD post-hoc tests, with p values reported.

Analysis of PO, diet and diet-by-PO effects within strain

PO, diet and diet-by-PO effects were assessed within each RIX, using linear mixed models based on **Eq 5**:

(Eq 5) *phenotype ~ Reciprocal + Diet + Diet:Reciprocal + (1|Dam) + (1|Sire) + (1|BehaviorBatch)*
(Eq 6) *phenotype ~ Reciprocal + Diet + Diet:Reciprocal + (1|Dam) + (1|Sire) + (1|Testing Chamber) + (1|BehaviorBatch)*
(Eq 7) *phenotype ~ Reciprocal + Diet + Diet:Reciprocal + (1|Dam) + (1|Sire) + (1|BehaviorBatch) + (1|TestOrder)*
(Eq 8) *phenotype ~ Reciprocal + Diet + Diet:Reciprocal + (1|Dam) + (1|Sire) + (1|BehaviorBatch) + (1|TestOrder) + (1|Experimenter)*

where a 'Reciprocal' fixed effect term models PO effects and a 'Diet:Reciprocal' fixed interaction effect models diet-by-PO. **Eq 6** was used for OF, LD and FST phenotypes, **Eq 7** was used for SIH phenotypes and **Eq 8** was used for restraint stress phenotypes. P and q values as well as Tukey's HSD post-hocs for significant diet effects were generated in the same manner described above.

Analysis of variance explained by PO, diet and diet-by-PO effects within strain

The models from **Eq 5-8** include random effects, complicating a typical percent variance explained computation. To overcome this difficulty, the random effects in **Eq 5-8** were regressed out and a new simple linear model with only the effects of interest was fit, namely PO, diet and diet-by-PO effects. The percent variance explained was then calculated for each effect using the ratios of fitted sums-of-squares to total sums-of-squares from a sequential (ie, type 1 sums-of-squares) ANOVA table.

RESULTS

Effects of perinatal diet exposure on reproductive fitness

Number of dams, percent productive matings and survival of pups to weaning, number of pups born, average litter size and sex ratio for each of the 20 reciprocal crosses are provided in **S Table 3**. CC014/Unc and CC062/Unc became unavailable during the study (CC014/Unc is now extinct; SGCF personal communication) (Shorter *et al*, 2017) and we were unable to produce a sufficient number of RIX 03/14 and RIX 35/62 F1 females exposed to PD and ME diets. Therefore, RIX 05/40 was added to the study and perinatal exposure was limited to PD and ME diets.

Genetic background affected success of matings, average litter size and survival to weaning but not sex ratio (**S Table 4**). Exposure to ME lead to decreased mating productivity in comparison with the Std diet (p=0.05). We also assessed the effects of diet exposure and PO on breeding productivity between reciprocal crosses of each RIX line (p values are provided in **S Table 4**). There was a PO effect on % productive matings and average litter size for six of the RIX lines. PO differences for % survival to weaning were observed for four of the RIX lines. Only one RIX differed by PO for sex ratio (RIX 01/11).

In five of the reciprocal crosses PO affected multiple measures of breeding success (**S Table 3 & 4**) but many of these effects seem to be diet-dependent. In RIX 01/11, CC001/Unc dams had more productive matings but fewer pups survived until weaning than CC011/Unc dams. Additionally, CC001/Unc dams had litters with a skewed sex ratio in favor of females compared to litters from CC011/Unc dams, although this finding appears to be specific to dams on the ME diet. In RIX 04/17, CC004/TauUnc dams had larger litter size and more pups that survived to weaning compared to CC017/Unc dams. However, reduced survival of pups from CC017/Unc

dams was primarily observed in dams given a ME diet. In RIX 32/42, CC032/GeniUnc dams had more productive matings, larger litters and more pups that survived to weaning compared to CC042/GeniUnc dams. However, decreased survival of pups from CC042/GeniUnc dams was primarily observed in dams exposed to PD diet. There was also a significant difference of sex ratio, dependent on diet exposure in RIX 32/42. CC042/GeniUnc dams exposed to ME diet had litters with more males and dams on VDD diet had litters with increased numbers of females compared to CC032/GeniUnc dams.

Correlation of behavioral phenotypes

Spearman correlations between the 20 phenotypes measured in this study are shown in **S Fig 1**. We observed the highest correlations among phenotypes measured in a single behavioral assay. However, we also observed significant correlations across behavioral assays. Locomotor phenotypes were positively correlated in the OF and LD assays (r(188)=0.47-0.75; $p<1x10^{-14}$). Percent center time in the OF was significantly and positively correlated with percent time in the light (r(188)=0.19; $p=1.2x10^{-6}$) and transitions (r(188)=0.60; $p<1x10^{-14}$) in the LD test. Phenotypes from the two stress-based behavioral assays, SIH and restraint stress, were also significantly correlated. SIH basal temperature was negatively correlated with stress-induced CORT (r(188)=-0.17; $p=2.8x10^{-5}$) and change in CORT (r(188)=-0.21; $p=1.5x10^{-7}$) while change in body temperature was positively correlated with change in CORT (r(188)=0.16; $p=5.8x10^{-5}$).

Overall effects of genetic background, perinatal diet and diet-by-RIX

A linear mixed effects model was used to assess the overall effects of genetic background (RIX), perinatal diet, and diet-by-RIX interactions. **Table 2.1** presents the *p* and *q* values for each of the 20 phenotypes assessed in the five behavioral tests.

Genetic background affects all phenotypes

We assessed effects of genetic background across the nine RIX lines, collapsed across diets, and found significant effects of RIX on all behavioral phenotypes (**Table 2.1**).

Perinatal exposure to protein deficiency and methyl enrichment alters body weight, body temperature and behavior in adulthood

Body weight. Overall effects of perinatal diet exposure were assessed across the four diet groups, collapsed across the nine RIX lines. Females exposed to PD and ME weighed significantly less than either VDD or Std exposed females at weaning (p=2.2x10^{-16}, q=2.2x10^{-15}; **S Fig 2b; S Table 5**) and at the onset of behavioral testing (p=2.2x10^{-16}, q=2.2x10^{-15}; **S Fig 2d; S Table 5**). These data indicate that the effects of perinatal diet on body weight persisted into adulthood, well after exposure to PD and ME had ceased.

Body temperature. ME exposed females had a lower basal temperature relative to all other groups (p=0.0002, q=0.001; **Fig 2.2b**; **S Table 5**). Basal temperature in PD exposed females did not differ from the other three groups, suggesting that basal temperature is not necessarily associated with the body weight changes described above (i.e. lower body weight = normal basal temperature for PD and decreased basal temperature for ME).

Anxiety-like behavior. Perinatal diet affected two measures of anxiety-like behavior: percent time in the light compartment in the LD test (p=0.048, q=0.096) and stress-induced temperature in the SIH test (SIH-T2; p=0.0002, q=0.001). ME exposed females spent less time in the lighted compartment compared to all other groups although this difference was only significant in comparison with VDD exposed females (**S Table 5**). ME exposed females also had a lower SIH-T2 temperature compared to all other groups, although this difference was only significant in comparison with females exposed to PD (**S Table 5**). However, ME exposed females were not significantly different from other diet groups for SIH change in temperature (**Fig 2.2e**). Therefore, the significant difference in SIH-T2 in the ME exposed group may simply reflect differences in body temperature rather than response to the stressor.

Basal stress and stress reactivity. Perinatal diet altered basal CORT (p=0.038, q=0.085; **Fig 2.3b**), stress-induced CORT (p=0.003, q=0.010) and change in CORT (p=2.1x10^{-5}, q=1.4x10^{-4}; **Fig 2.3e**). PD exposed females show significantly greater stress-induced CORT and change in CORT (**Fig 2.3e**) in comparison with both ME and VDD exposed females (**S Table 5**). *Post hoc* comparisons did not identify any significant differences in basal CORT among the experimental diet groups.

Genetic background interacts with perinatal diet to alter body weight, stress response and anxiety-like behavior

Fourteen of the 20 phenotypes exhibited significant diet-by-RIX effects based on p value, whereas 13 had a significant q value. Body weight, basal temperature, anxiety-like behavior and stress response all show a diet-by-RIX effect (see **Table 2.1**). In order to investigate these interactions, we analyzed each RIX independently to examine which genetic backgrounds were

altered by perinatal diet and to determine if the diet-induced changes in a RIX matched the overall diet effects reported above. P and *q* values along with Tukey's post hoc analyses of diet effects within a RIX are reported in **S Table 6**.

Body weight. There was a significant diet-by-RIX effect on body weight at weaning (p=1.5x10^{-7}, q=7.4x10^{-7}; **S Fig 2a**) and in adulthood (p=0.002, q=0.004; **S Fig 2c**). For six of the RIX lines (RIXs 01/11, 41/51, 04/17, 23/47, 06/26 and 03/14), diet-specific differences in body weight at weaning persisted into adulthood. RIX lines 35/62 and 32/42 both exhibited diet-specific differences in body weight at only one of the time points – RIX 35/62 in adulthood and RIX 32/42 at weaning. RIX 05/40 was the only line for which perinatal diet did not differentially affect body weight, although only two diets were examined in this line.

Stress-induced hyperthermia. All three measures from the SIH assay showed a diet-by-RIX effect; basal temperature (p=3.6x10^{-7}, q=1.4x10^{-6}; **Fig 2.2a**), stress-induced temperature (p=0.008, q=0.018) and change in temperature (p=2.6x10^{-4}, q=0.001; **Fig 2.2d**). Five of the RIX lines showed no effect of diet on basal temperature (RIXs 41/51, 04/17, 23/47, 35/62, 05/40) and six showed no effect of diet exposure on change in temperature following a stressor (RIXs 41/51, 04/17, 23/47, 03/14, 35/62, 32/42). To highlight a diet-by-RIX effect, the opposing effects of the PD compared to Std diet observed in RIX 01/11 and RIX 06/26 females is shown in **Figs 2.2c** and **2.2f**. RIX 01/11 females exposed to PD showed increased basal temperature and a decreased change in temperature in response to stress while RIX 06/26 females exposed to a PD diet had a lower basal temperature and an increased change in temperature in response to stress. These data show that the effects of perinatal diet on basal temperature and stress-induced temperature change, a physiological measure of anxiety, are dependent on genetic background.

Basal stress and stress reactivity. Basal CORT (p=1.5x10^{-10}, q=1.5x10^{-9}; **Fig 2.3a**), stress-induced CORT (p=2.7x10^{-13}, q=5.4x10^{-12}) and change in CORT (p=1.1x10^{-8}, q=7.6x10^{-8}; **Fig 2.3d**) all showed significant diet-by-RIX effects. Diet had no effect on change in CORT in four lines (RIXs 01/11, 06/26, 03/14, 35/62). Interestingly, RIX 32/42 and RIX 04/17 females exposed to VDD showed diametrically opposed change in CORT levels. RIX 32/42 VDD exposed females showed increased change in CORT while VDD exposed RIX 04/17 females showed decreased change in CORT (**Fig 2.3f**). A similar opposing effect of exposure to VDD compared to Std is seen in basal CORT levels for RIX 06/26 and RIX 03/14, although this effect did not reach significance. VDD exposure in RIX 06/26 resulted in decreased basal CORT while the same dietary exposure in RIX 03/14 resulted in increased CORT (**Fig 2.3c**). Collectively, these data show that the effects of perinatal diet on basal stress and response to a stressor are also dependent on genetic background.

Parent-of-origin effects on behavior

A linear mixed effects model was used to assess the effects of PO, by comparing reciprocal females within each RIX line. We report p and q values for PO in **Table 2.2**. RIX 32/42 was the only line that showed no effect of PO on behavioral phenotypes.

PO effects for body weight were observed in six of the nine RIX (RIXs 01/11, 41/51, 04/17, 23/47, 06/26, 05/40). Five of these six lines (RIXs 01/11, 41/51, 04/17, 23/47, 06/26) also had a PO effect for vertical counts, or rearing, in the OF. In each case, the reciprocal group that weighed more was also the one that made more vertical movements in the OF. This might indicate that body weight influenced detection of vertical movements by the infrared sensors in

the OF arena. However, we observed no significant correlation between weight at testing and OF vertical counts (r(188)=0.06, p>0.05; **S Fig 1**).

Several RIX displayed multiple PO effects across behavioral tests and are highlighted below. For example CC(03x14)F1 females showed increased locomotion in the OF (**Fig 2.4a**), increased time spent in the center of the OF (**Fig 2.4b**), decreased immobility in the FST (**Fig 2.4c**) and increased transitions (**Fig 2.4d**), distance moved (**Fig 2.4e**), and time spent in the light side of the LD arena (**Fig 2.4f**) in comparison to CC(14x03)F1 females. These data indicate that female offspring from CC003/Unc dams have decreased anxiety- and depressive-like behavior compared to female offspring from CC014/Unc dams.

CC(62x35)F1 females showed increased locomotion (**Fig 2.5a**), transitions (**Fig 2.5b**), distance moved (**Fig 2.5c**), and time spent in the light side (**Fig 2.5d**) of the LD test compared to CC(35x62)F1 females. CC(62x35)F1 females also showed decreased CORT in response to a restraint stress (**Fig 2.5f**). These results indicate that female offspring from CC062/Unc dams have decreased anxiety-like behavior and are more resilient to stress compared to female offspring from CC035/Unc dams.

CC(06x26)F1 females showed increased locomotion (**S Fig 3a**) and vertical counts in the OF (**S Fig 3b**) and increased basal (**S Fig 3c**) and stress-induced CORT levels (**S Fig 3d** & **S Fig 6a**) in comparison with CC(26x06)F1 females, but no reciprocal difference in change in CORT (**S Fig 3e**). Collectively, these data indicate that female offspring from CC006/TauUnc dams have increased locomotion and exploratory behavior and a dysregulated hypothalamic-pituitary-adrenal (HPA) axis as reflected by increased basal CORT levels.

Female offspring from CC005/TauUnc mothers exhibited increased locomotor activity as measured in both the OF and LD tests (**S Figs 4a-e**) in comparison with female offspring from CC040/TauUnc mothers.

Perinatal diet interacts with parent-of-origin in certain genetic backgrounds to alter body weight and behavior

We were particularly interested in assessing whether perinatal diet would induce behavioral differences between reciprocal females within a RIX. We found 18 significant diet-by-PO effects based on *p* value, although none were significant after multiple testing corrections (based on the *q* value; **Table 2.3**).

A significant PO effect for stress-induced CORT was observed in RIX 06/26 (**Fig 2.6a**). The PO effect is driven by a significant difference between CC(06x26)F1 females exposed to ME relative to other diets (posthoc $p \leq 0.001$; **Fig 2.6b**); by contrast, stress induced CORT in CC(26x06)F1 females was unaffected by diet.

Eight of the significant diet-by-PO effects involved body weight at weaning or in adulthood. For five of the RIX (RIXs 41/51, 04/17, 03/14, 32/42, 05/40), there was a significant diet-by-PO effect on body weight at weaning. For example, the PO effect on weaning weight in RIX 04/17 (**Fig 2.7a**) was due to increased weight in VDD and Std exposed CC(04x17)F1 females compared to ME and Std (posthoc $p \leq 0.01$), a diet effect not observed in CC(17x04)F1 females (**Fig 2.7b**). Only one RIX showed a significant diet-by-PO effect on adult body weight (**Fig 2.7c-d**) only. VDD and Std exposed CC(11x01)F1 females weighed more than CC(01x11)F1 females (posthoc $p \leq 0.02$; **Fig 2.7d**). Only one RIX showed a significant diet-by-PO effect on body weight at both weaning and in adulthood. CC(06x26)F1 females weighed more than

CC(26x06)F1 females at weaning (**Fig 2.7e**). This effect was due to a difference in the ME exposed groups across reciprocals (posthoc $p \leq 0.01$; **Fig 2.7f**). In adulthood, CC(06x26)F1 females still weighed more than CC(26x06)F1 females although this effect was no longer significant (**Fig 2.7g**). However, there is still a significant diet-by-PO effect, and the distribution of diet effects on adult weight is similar to the distribution at weaning (**Fig 2.7h**). Collectively, these data demonstrate that body weight is strongly influenced by perinatal diet in a PO-specific manner across different genetic backgrounds.

Variance explained by PO, diet and diet-by-PO effects

Within each RIX line and for all 20 phenotypes measured, we assessed the percent variance explained by the effect of PO, diet and diet-by-PO (**S Table 7**). Of particular interest in this study was the variance due to PO effects as PO effects on behavioral phenotypes are seldom reported. For phenotypes that exhibited a significant PO effect, variance explained ranged from 4-59%. Body weight at weaning in RIX 23/47 had the highest variance explained by PO while basal CORT in RIX 04/17 had the lowest percent variance explained by PO. For any given phenotype, however, variance explained depended on genetic background (**S Table 7**).

DISCUSSION

Overall conclusions

We used reciprocal F1 females derived from ten pairs of CC strains to investigate the effects of perinatal diet exposure, genetic background, PO and their interactions on 20 phenotypes. Genetic background significantly affected all phenotypes, whereas perinatal diet exposure affected fewer phenotypes – namely, body weight, basal body temperature, anxiety-like behavior

and stress response – but all in a manner dependent on genetic background. We also found significant PO effects in eight of the nine RIX lines and for a variety of behavioral phenotypes. To further investigate PO effects, we examined the interaction of PO with diet, identifying a small number of diet-by-PO effects; notably, body weight showed consistent diet-by-PO effects across seven of the nine RIX lines. Our data shows that rodent behaviors that model psychiatric disorders are affected by genetic background, parent-of-origin, and perinatal diet, as well as by interactions among these factors. Below we compare our main findings with the current literature and highlight several interesting results that we believe warrant further investigation.

Genetic background effects

Significant effects of RIX were observed on all 20 phenotypes. This result was not surprising given that these phenotypes have a genetic component and the genetic backgrounds we tested covered a broad range of genetic diversity. The CC was designed to maximize genetic diversity and our choice of CC strain pairs in the 10 RIX lines was intended to maximize genetic heterozygosity so as to include novel combinations of alleles from the three-major subspecies of *Mus musculus*. The genetic diversity of the CC population resulted in an expanded phenotypic range relative to that observed in standard inbred strains (i.e. Mouse Diversity Panel) (Gralinski *et al*, 2015; Levy *et al*, 2015; Mosedale *et al*, 2017; Venkatratnam *et al*, 2017; Vered *et al*, 2014).

The wide range of phenotypes in the CC has also led to the development of stable models of human disease not previously observed in traditional inbred strains (Rogala *et al*, 2014). Our RIX lines also included clear phenotypic outliers. For example, RIX 41/51 and RIX 04/17 F1 mice were outliers for novelty-induced locomotion as measured in the OF and LD tests (**S Fig 5**).

Based on the literature linking novelty-induced locomotion with addiction-related behaviors (Piazza *et al*, 1989), we are currently investigating these two lines in rodent models of addiction.

Diet effects

Although we chose perinatal diet as our environmental challenge based on existing literature in humans from the Dutch Hunger Winter and Chinese Famine (Brown *et al*, 2008; Brown *et al*, 2000; St Clair *et al*, 2005) we acknowledge that we are not modeling human perinatal nutritional exposures *per se*. Rather, we are using these experimental diets as tools to induce behavioral changes based on evidence from published data in animal models (Burne *et al*, 2004; Glenn *et al*, 2012; Harms *et al*, 2008; Plyusnina *et al*, 2007; Vucetic *et al*, 2010), as well as data from our laboratory (Oreper, Valdar and Tarantino personal communication). Our study was designed to compare phenotypes across diets and across genetic background rather than comparing each diet to a standard or treated animals to controls.

Effects of methyl enrichment

DNA methylation is an important mechanism for regulation of gene expression and is dependent on the availability of methyl donors mainly from diet (Niculescu and Zeisel, 2002). Disruption in DNA methylation has been implicated as a mechanism for increased risk for complex diseases, including psychiatric disorders (Grayson and Guidotti, 2013). In our study, we exposed animals to methyl donor (choline) enrichment (**S Table 2**). Perinatal exposure to ME resulted in lower body weight at weaning and adulthood, decreased basal body temperature and increased anxiety-like behavior in the LD test.

Choline supplementation during development is thought to be neuroprotective (Bekdash, 2016) and is commonly used in animal models to 'rescue' the adverse effects of perinatal

challenges such as iron deficiency (Kennedy *et al*, 2014), stress (Schulz *et al*, 2014) and alcohol exposure (Thomas *et al*, 2010). Prenatal choline supplementation has also been shown to improve negative behavioral phenotypes in mouse models of neurodevelopmental disorders such as Rett Syndrome (*Mecp2*; (Nag and Berger-Sweeney, 2007)), autism (BTBR T+ltpr3tf/J; (Langley *et al*, 2015)) and Down Syndrome (Ts65Dn; (Moon *et al*, 2010)).

We found that exposure to perinatal ME increased anxiety-like behavior in the LD test. Our findings are in contrast to studies in rats in which choline supplementation decreased anxiety in the LD (Plyusnina *et al*, 2007) and OF tests (Glenn *et al*, 2012). However, the increased anxiety-like behavior we observed in ME exposed females was specific to certain RIX lines, or genetic backgrounds (**S Table 5**). Very few studies have examined the effect of methyl supplementation in different genetic backgrounds. Notably, one study comparing BTBR T+ltpr3tf/J (a mouse model of autism) to B6J mice did report differences in these two strains in response to perinatal choline supplementation on anxiety-like behavior in the EPM (Langley *et al*, 2015).

Effects of protein deficiency

Proteins play a key role in brain development as they serve as key neurotransmitters and hormones. Protein deficiency during the perinatal period has been used to induce intrauterine growth restriction and alter behaviors that model psychiatric disorders (Tarantino *et al*, 2012a). In our study, perinatal PD led to decreased body weight at weaning and in adulthood. The findings of decreased weight at weaning are consistent with previous reports in rats (Palmer *et al*, 2008) and mice (Belluscio *et al*, 2016; Belluscio *et al*, 2014; Vucetic *et al*, 2010). However, the persistence of weight deficits into adulthood has not been consistent across studies (Palmer *et al*, 2008; Vucetic *et al*, 2010).

We also observed increased stress reactivity in response to perinatal PD – a finding that is consistent with previous studies in rats exposed to PD *in utero (Reyes-Castro et al, 2012a; Reyes-Castro et al, 2012b)*. Previous studies have also reported increased depressive-like behaviors in response to perinatal PD (Belluscio *et al*, 2016; Belluscio *et al*, 2014; de Godoy *et al*, 2013; Vucetic *et al*, 2010). We did not observe any changes in depressive-like behavior in the FST in response to PD exposure. Nor did PD exposure alter anxiety-like behavior in the OF, LD and SIH assays. This finding is not particularly surprising given that the effects of exposure to PD on anxiety-like behavior have been equivocal across published studies (Almeida *et al*, 1996; Belluscio *et al*, 2014; Francolin-Silva *et al*, 2006; Furuse *et al*, 2017; Reyes-Castro *et al*, 2012a; Reyes-Castro *et al*, 2012b). We did observe a significant diet-by-RIX effect on all three measures of anxiety-like behavior and stress response (**Table 2.1**). These data in combination with conflicting results in the literature support a hypothesis that the effects of exposure to PD on anxiety and stress response are dependent on genetic background.

Effects of vitamin D deficiency

Vitamin D is well known for its role in calcium homeostasis and bone formation, but it is also a neuroactive steroid involved in brain development and function (Garcion *et al*, 2002). Exposure to VDD during development is hypothesized to increase risk for schizophrenia (McGrath, 2001). As such, the effects of VDD during the perinatal period have been studied in rodents using behavioral models of schizophrenia.

In the present study, we observed very few overall effects of exposure to perinatal VDD. We considered the possibility that VDD diet exposure was not reducing endogenous vitamin D by the amount expected. It has been shown that exposure to unfiltered fluorescent lighting can induce production of endogenous Vitamin D_3 in laboratory animals (McDowell, 1989) and we

did not filter the lighting in our vivarium. However, we did observe significant decreases in serum vitamin D levels in VDD exposed dams from several of the CC strains used in this study (Xue *et al*, 2016).

Previous studies have assessed the role of VDD exposure on the stress axis in rodents and have reported either no effects (Eyles *et al*, 2006) or increased CORT levels (Tesic *et al*, 2015). In our study, exposure to VDD induced RIX-specific alterations in basal and stress-induced CORT (**Fig 2.3c & 2.3f**) supporting a role for vitamin D in moderating the stress system.

We also considered the possibility that the behaviors examined in this study are not particularly sensitive to perinatal VDD exposure. However, the literature does report hyperlocomotion in rats exposed to VDD (Burne *et al*, 2004; Burne *et al*, 2006; Eyles *et al*, 2006; Kesby *et al*, 2006) and we did not observe an overall or RIX-specific effect of VDD exposure on locomotion in either the OF or LD test.

Perinatal diet effects depend on genetic background

Our data highlight the importance of genetic background on the expression of body weight, basal temperature, anxiety-like behavior, and stress response following exposure to perinatal dietary manipulations. We acknowledge that our assessment was limited to a small number of behaviors and did not include several that have been previously reported to be altered by perinatal diet (i.e. learning and memory tests, response to psychostimulants, sensorimotor gating). Regardless of the limitations in behavioral assays employed, our data highlight the need to consider genetic background when investigating the role of perinatal diet exposure.

Parent-of-origin effects on behavior and physiology

We found PO effects in eight of the nine RIX lines for a number of behavioral phenotypes, body weight, and basal body temperature. Although there are many studies that utilize F1 mice, few have used reciprocal F1 hybrids and even fewer have assessed the effects of PO on behavioral phenotypes. There is, however, a vast literature assessing the role of known imprinted genes on growth, physiology and behavior via reverse genetic approaches such as overexpression or gene knockouts (KO) (for a thorough review see (Cleaton *et al*, 2014)). Relevant findings from these studies will be discussed in relation to PO effects we observed.

Body weight

PO effects were observed for body weight in six RIX lines and in four of these lines, body weight differences persisted from weaning into adulthood. This result is not surprising, given the vast amount of literature implicating imprinted genes on growth (Cleaton *et al*, 2014). Additional studies are necessary to determine whether the specific genes responsible for body weight PO effects in our study are known imprinted genes or new genes. Of note, in this study we assessed body weight but no other measures of growth (i.e. body length). Follow-up studies using both body mass and body size measurements are necessary to determine whether strain, diet, or PO effects on body weight were due to the changes in the actual size of the mice (i.e. length) or reflect only weight differences (i.e. overweight or underweight).

Locomotor behavior

We found PO effects on locomotor behavior in four of the RIX lines. In two of these RIXs, we also observed PO effects on body weight, although the relationship between body weight and locomotion is opposite in the two RIX lines (RIX 06/26 vs RIX 05/40, **Table 2.2**). Hyperactivity, body weight and metabolism have been linked previously in studies manipulating the imprinted

genes *Asb4* (Li *et al*, 2010) and *Kcnq1* (Boini *et al*, 2009; Casimiro *et al*, 2001). These RIX lines can be examined in follow up studies to determine whether locomotor activity and body weight PO effects share a common genetic basis.

Anxiety- and depressive-like behaviors and stress response

We found a consistent PO effect among RIX 03/14 reciprocal females on anxiety-like and depressive-like behavior (**Fig 2.4**). Interestingly, these behavioral phenotypes are very similar to that observed in KO mice for the imprinted gene, *Sgce*. *Sgce* KO mice display increased anxiety-like and exploratory behavior in the OF and increased depressive-like behavior in the FST (Yokoi *et al*, 2006). There is also an abundance of evidence from the literature that establishes a role for imprinted genes such as *Sgce*, *Nesp55*, *Htr2a*, *Peg3*, and *Snord116* in mediating anxiety-like behavior (Champagne *et al*, 2009; Ding *et al*, 2008; Plagge *et al*, 2005; Weisstaub *et al*, 2006). Studies also show a role of *Nesp55* in mediating addiction-related behaviors (Dent *et al*, 2016; Plagge *et al*, 2005). It would be interesting to assess PO effects on addiction-related behaviors in RIX 03/14 females.

RIX 35/62 reciprocal females displayed PO effects on anxiety-like behavior and stress response. Two other RIX also displayed PO effects on measures of stress response. A previous study using F1 reciprocals of spontaneously hypertensive rat and Wistar-Kyoto rats reported a PO effect on the cardiovascular activity in response to an acute stressor (Woodworth *et al*, 1990). A recent study also reported PO effects on stress reactivity using reciprocal F1 of B6J and 129S1/SvlmJ mice (Chan *et al*, 2017). These studies, together with our findings, indicate that further work is needed to examine the HPA axis in regards to imprinted gene functions and other effects due to PO.

Perinatal diet interaction with PO

We identified perinatal diet-by-PO effects on body weight, exploratory behavior, basal temperature, anxiety- and depressive-like behavior, and stress response. PO effects on body weight were the most severely affected by perinatal diet, as evidenced by our observation of diet-by-PO effects in seven of the RIX lines (**Table 2.2, Fig 2.7**).

Although we cannot directly compare our diet-by-PO findings on behavior with previously published studies due to methodological differences, there are a few studies that have reported changes in imprinted gene expression due to perinatal diet alterations, which might indicate a mechanism for these effects (Vucetic *et al*, 2010; Waterland *et al*, 2006). Vucetic *et al* (2010) found that perinatal PD caused a reduction in the methylation at the promotor of the imprinted gene *Cdkn1c,* along with a correlated increase in its expression in the prefrontal cortex, nucleus accumbens, and hypothalamus. *Cdkn1c* is involved in differentiation and specification of dopamine neurons and the authors also reported an alteration in dopamine-mediated behaviors in these mice. The link between exposure to PD, imprinted gene expression differences and dopamine-mediated behaviors provides a potential avenue for future mechanistic studies in our RIX lines.

Collectively, our findings support the role of PO on body weight and behavioral phenotypes that model psychiatric disorders. It is interesting that RIX 32/42 was the only RIX that showed no PO effects on any behavioral phenotype measured. One possible explanation for this result is lower genetic diversity at imprinted loci in the CC strains used to generate RIX 32/42 mice. Importantly, the PO effects we observed are dependent on genetic background. Of note, our use of the term PO effect, by definition, assumes an interaction with genetic background. We assessed the variance explained due to PO to get a sense of the importance of these effects on the

phenotypes measured. On average, variance explained by PO was less than that explained by strain. In fact, variance explained by PO effects depended on both strain and the specific behavioral assay, ranging from 4 to 59 percent (with the highest value for body weight). Nonetheless, it is important to note that within RIX comparisons describe differences between two experimental groups that are genetically identical and differ only in the parental origin of the genome and the maternal environment; therefore, even small effects can be biologically relevant and useful for developing interventions and treatments.

Although we focused our discussion on imprinted genes, we acknowledge that they are likely not the sole source of the observed PO effects. Future studies examining gene expression in these RIX lines will likely help identify specific genes responsible. We expect that some of these genes will be at known imprinted loci, while others will be novel in the context of behavioral effects. Additionally, PO effects can be due to maternal effects independent of imprinting (Weaver *et al*, 2004), and further studies will be necessary to disentangle their respective contributions to the PO effects observed in this study. Lastly, our study illustrates how the CC and RIX lines derived from the CC can provide an ideal experimental model for jointly examining genetic background and PO, being reproducible, genetically diverse and uniquely designed to support systems genetics studies.

Table 2.1. Effect of genetic background (RIX), perinatal diet and diet-by-RIX on behavioral phenotypes

Behavior Test	Phenotype	RIX		Diet		Diet-by-RIX	
		***p*-value**	***q*-value**	***p*-value**	***q*-value**	***p*-value**	***q*-value**
Body Weight	Weaning (PND 21)	**1.4E-05**	**1.5E-05**	**2.2E-16**	**2.2E-15**	**1.5E-07**	**7.4E-07**
	Adulthood (PND 60)	**2.3E-15**	**2.9E-15**	**2.2E-16**	**2.2E-15**	**0.002**	**0.004**
Open Field (OF)	Total Distance Moved	**2.0E-16**	**3.4E-16**	0.772	0.881	0.209	0.209
	% Center Time	**2.0E-16**	**3.4E-16**	0.807	0.881	**0.041**	0.058
	Vertical Counts	**2.0E-16**	**3.4E-16**	0.998	0.998	0.072	0.089
	OF Boli	**6.3E-14**	**7.4E-14**	0.192	0.350	**0.010**	**0.021**
Light/Dark (LD)	Total Distance Moved	**2.0E-16**	**3.4E-16**	0.228	0.381	0.061	0.082
	Distance Dark	**2.0E-16**	**3.4E-16**	**0.023**	0.058	**0.021**	**0.034**
	Distance Light	**2.0E-16**	**3.4E-16**	0.805	0.881	0.097	0.114
	Total Transitions	**2.0E-16**	**3.4E-16**	0.837	0.881	0.130	0.136
	% Time Light	**1.3E-04**	**1.4E-04**	**0.048**	0.096	**0.025**	**0.039**
	LD Boli	**2.2E-16**	**3.4E-16**	0.352	0.502	**0.001**	**0.002**
Stress-Induced Hyperthermia (SIH)	SIH-T1	**4.4E-16**	**6.3E-16**	**2.0E-04**	**0.001**	**3.6E-07**	**1.4E-06**
	SIH-T2	**2.2E-16**	**3.4E-16**	**1.9E-04**	**0.001**	**0.008**	**0.018**
	deltaSIH	**0.006**	**0.006**	0.342	0.502	**2.6E-04**	**0.001**
Forced Swim (FST)	% Immobile	**2.0E-16**	**3.4E-16**	0.377	0.502	0.122	0.135
	FST Boli	**6.7E-16**	**8.9E-16**	**0.023**	0.058	**0.013**	**0.023**
Restraint Stress	Basal CORT	**2.2E-16**	**3.4E-16**	**0.038**	0.085	**1.5E-10**	**1.5E-09**
	Stress CORT	**2.2E-16**	**3.4E-16**	**0.003**	**0.010**	**2.7E-13**	**5.4E-12**
	deltaCORT	**2.2E-16**	**3.4E-16**	**2.1E-05**	**1.4E-04**	**1.1E-08**	**7.6E-08**

Significant values are shaded and **bolded**. CORT = corticosterone; PND = postnatal day; SIH-T1 = basal temperature; SIH-T2 = post-stress temperature; deltaSIH = (T2-T1); deltaCORT = (Stress CORT – Basal CORT)

Table 2.2. Parent-of-origin effects on behavior

	RIX 01/11		RIX 41/51		RIX 04/17		RIX 23/47		RIX 06/26		RIX 03/14		RIX 35/62		RIX 32/42		RIX 05/40	
Phenotype	***p***	***q***	***p***	***q***	***p***	***q***	***p***	***q***	***p***	***q***	***p***	***q***	***p***	***q***	***p***	***q***	***p***	***q***
WeightPND21	0.80	0.94	**0.01**	0.10	**2E-05**	**3E-04**	**6E-08**	**1E-06**	**0.03**	0.12	0.55	0.78	0.47	0.56	0.20	0.83	**0.05**	0.14
WeightPND60	**1E-02**	0.14	**0.01**	0.10	**1E-03**	**7E-03**	**2E-06**	**2E-05**	0.11	0.27	0.86	0.89	0.47	0.56	0.32	0.83	**2E-03**	**0.01**
OF TotalDist	0.34	0.62	0.76	0.94	0.34	0.45	0.80	0.84	**1E-03**	**0.01**	**0.03**	0.12	0.08	0.20	0.63	0.83	**0.04**	0.14
OF %Center	0.71	0.91	0.43	0.78	0.21	0.35	0.26	0.64	0.070	0.20	**0.05**	0.15	0.12	0.28	0.40	0.83	0.14	0.27
OF VertCount	**0.02**	0.15	**0.02**	0.10	**5E-03**	**0.02**	**0.04**	0.18	**2E-04**	**4E-03**	0.15	0.38	0.59	0.65	0.78	0.83	0.36	0.48
OF Boli	0.61	0.91	0.27	0.68	**0.03**	0.07	0.65	0.81	0.38	0.55	0.49	0.75	0.47	0.56	0.85	0.85	0.15	0.28
LD TotalDist	0.10	0.29	0.27	0.68	0.62	0.68	0.71	0.83	0.20	0.37	0.08	0.24	**0.04**	0.16	0.54	0.83	**2E-04**	**3E-03**
LD DistDark	0.15	0.34	0.56	0.79	0.41	0.51	0.37	0.65	0.39	0.55	0.63	0.79	0.39	0.56	0.16	0.83	**2E-03**	**0.01**
LD DistLight	0.12	0.31	0.14	0.57	0.88	0.88	0.65	0.81	0.13	0.29	**5E-03**	0.10	**0.03**	0.15	0.79	0.83	**0.05**	0.14
LD Transition	0.32	0.62	0.59	0.79	0.26	0.40	0.39	0.65	0.44	0.59	**0.03**	0.12	**0.03**	0.15	0.35	0.83	**6E-03**	**0.03**
LD %Light	0.98	0.98	0.41	0.78	0.32	0.45	0.30	0.65	0.18	0.36	**0.01**	0.12	**0.03**	0.15	0.73	0.83	0.79	0.84
LD Boli	0.66	0.91	0.95	0.95	0.55	0.65	0.61	0.81	0.52	0.65	0.31	0.69	0.87	0.87	0.66	0.83	0.12	0.26
SIH-T1	0.73	0.91	0.25	0.68	**0.02**	0.06	0.90	0.90	0.84	0.84	0.89	0.89	0.26	0.44	0.24	0.83	0.65	0.82
SIH-T2	0.72	0.91	0.07	0.34	**0.04**	0.09	0.20	0.64	0.79	0.83	0.64	0.79	0.30	0.47	0.68	0.83	0.80	0.84
deltaSIH	0.86	0.96	0.91	0.95	**3E-03**	**0.01**	0.44	0.68	0.66	0.73	0.73	0.86	0.22	0.41	0.52	0.83	0.23	0.33
FST %Imb	**0.05**	0.29	0.83	0.94	0.19	0.35	**4E-03**	**0.03**	0.36	0.55	**0.02**	0.12	0.18	0.36	0.56	0.83	0.10	0.26
FST Boli	0.10	0.29	0.36	0.78	0.69	0.73	0.33	0.65	**0.04**	0.12	0.35	0.69	0.08	0.20	0.12	0.83	0.22	0.33
BasalCORT	0.95	0.98	0.84	0.94	**0.04**	0.09	0.78	0.84	**4E-03**	**0.02**	0.86	0.89	0.82	0.86	0.68	0.83	0.17	0.29
StressCORT	0.08	0.29	0.47	0.79	**4E-05**	**4E-04**	0.09	0.38	**3E-03**	**0.02**	0.45	0.75	**0.02**	0.15	0.17	0.83	0.90	0.90
deltaCORT	0.08	0.29	0.59	0.79	**1E-03**	**5E-03**	0.25	0.64	0.66	0.73	0.49	0.75	0.07	0.20	0.36	0.83	0.77	0.84

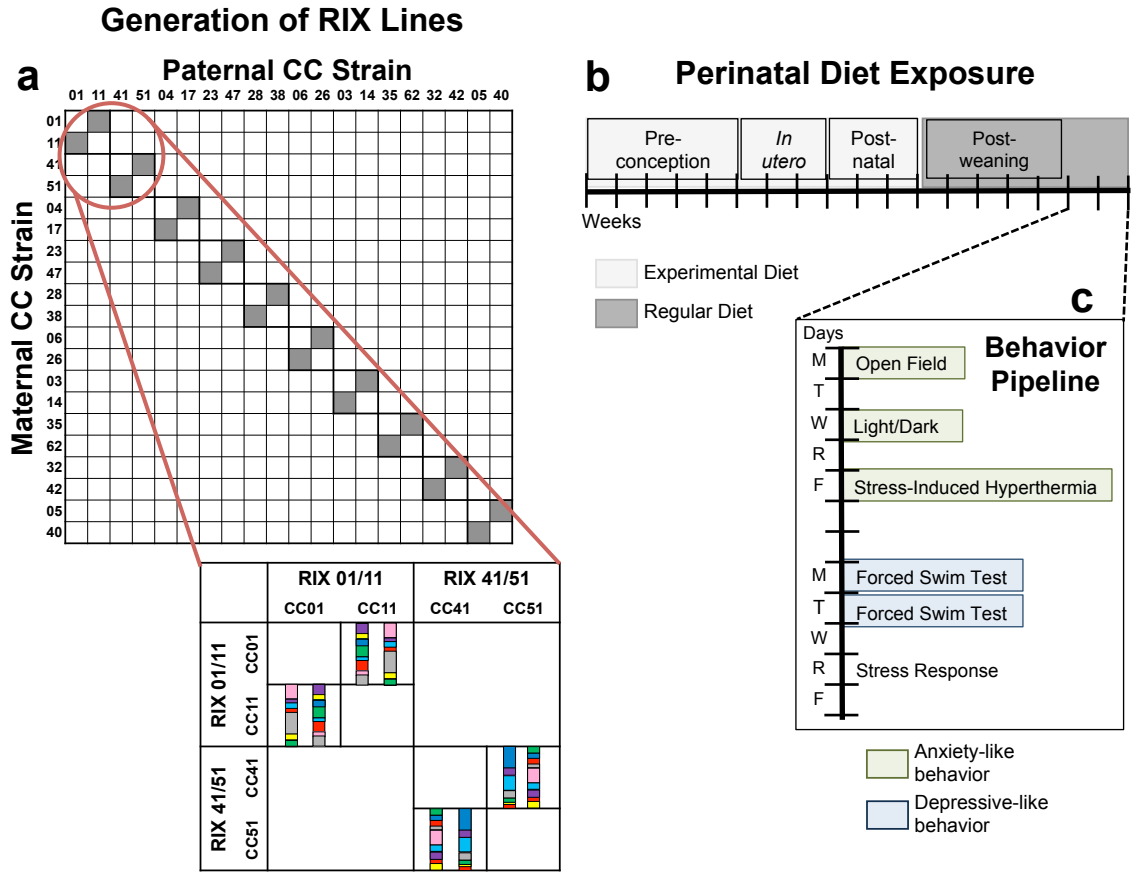
Significant values are shaded and **bolded**. CORT = corticosterone; deltaCORT = (Stress CORT – Basal CORT); deltaSIH = (T2-T1); Dist = distance; FST = forced swim test; Imb = immobile; LD = light/dark; OF= open field; PND = postnatal day; SIH = stress-induced hyperthermia; SIH-T1 = basal temperature; SIH-T2 = post-stress temperature; Vert = vertical

Table 2.3. Diet-by-PO effects on behavior

	RIX 01/11		RIX 41/51		RIX 04/17		RIX 23/47		RIX 06/26		RIX 03/14		RIX 35/62		RIX 32/42		RIX 05/40	
Phenotype	***p***	***q***	***p***	***q***	***p***	***q***	***p***	***q***	***p***	***q***	***p***	***q***	***p***	***q***	***p***	***q***	***p***	***q***
WeightPND21	0.51	0.76	**0.03**	0.19	**0.01**	0.19	0.47	0.85	**0.04**	0.15	**0.03**	0.57	0.94	0.99	**0.04**	0.68	**0.02**	0.47
WeightPND60	**0.03**	0.32	0.24	0.46	0.65	0.76	0.18	0.85	**0.04**	0.15	0.34	0.77	0.37	0.70	0.56	0.71	0.92	0.97
OF TotalDist	0.56	0.76	0.09	0.26	0.07	0.26	0.44	0.85	0.77	0.82	0.99	0.99	0.81	0.99	0.17	0.68	0.79	0.97
OF %Center	0.85	0.89	0.06	0.23	0.06	0.26	0.73	0.85	0.14	0.35	0.83	0.93	0.06	0.50	0.40	0.68	0.92	0.97
OF VertCount	0.71	0.78	0.72	0.85	**0.04**	0.26	0.61	0.85	0.58	0.68	0.13	0.57	0.35	0.70	0.21	0.68	0.37	0.93
OF Boli	0.19	0.54	0.81	0.85	0.41	0.64	**0.01**	0.17	0.35	0.68	0.18	0.57	0.06	0.50	0.64	0.73	0.92	0.97
LD TotalDist	0.09	0.48	0.46	0.71	0.48	0.64	0.74	0.85	0.46	0.68	0.78	0.93	0.18	0.70	0.43	0.68	0.99	0.99
LD DistDark	0.10	0.48	0.78	0.85	0.91	0.96	0.73	0.85	0.61	0.68	0.82	0.93	0.08	0.50	0.54	0.71	0.65	0.97
LD DistLight	0.12	0.49	0.28	0.46	0.10	0.28	0.49	0.85	0.18	0.40	0.87	0.93	0.22	0.70	0.51	0.71	0.67	0.97
LD Transition	0.17	0.54	0.07	0.23	0.36	0.64	0.60	0.85	0.45	0.68	0.85	0.93	0.90	0.99	0.31	0.68	0.73	0.97
LD %Light	0.61	0.76	0.62	0.85	0.26	0.64	0.23	0.85	0.51	0.68	0.18	0.57	0.14	0.70	0.69	0.73	0.42	0.93
LD Boli	**0.03**	0.32	0.94	0.94	0.08	0.26	**0.04**	0.36	0.58	0.68	0.11	0.57	1.00	1.00	0.12	0.68	0.47	0.93
SIH-T1	0.63	0.76	0.19	0.46	0.90	0.96	0.56	0.85	**0.02**	0.14	0.75	0.93	0.48	0.74	0.44	0.68	0.21	0.85
SIH-T2	0.47	0.76	**0.02**	0.19	0.59	0.73	0.82	0.87	0.54	0.68	0.88	0.93	0.38	0.70	0.67	0.73	0.72	0.97
deltaSIH	0.65	0.76	0.25	0.46	0.46	0.64	0.60	0.85	**0.01**	0.14	0.88	0.93	0.75	0.99	0.88	0.88	0.31	0.93
FST %Imb	0.35	0.71	0.74	0.85	**0.05**	0.26	0.91	0.91	0.53	0.68	0.25	0.63	0.88	0.99	0.15	0.68	0.07	0.70
FST Boli	0.51	0.76	0.24	0.46	0.96	0.96	0.20	0.85	0.07	0.23	0.82	0.93	0.31	0.70	0.44	0.68	0.12	0.78
BasalCORT	0.90	0.90	0.65	0.85	0.48	0.64	0.56	0.85	0.94	0.94	0.58	0.93	0.70	0.99	0.41	0.68	0.53	0.96
StressCORT	0.29	0.71	**0.02**	0.19	0.36	0.64	0.68	0.85	**0.02**	0.14	0.20	0.57	0.27	0.70	0.28	0.68	0.19	0.85
deltaCORT	0.35	0.71	0.06	0.23	0.39	0.64	0.77	0.85	0.10	0.27	0.18	0.57	0.45	0.74	0.25	0.68	0.43	0.93

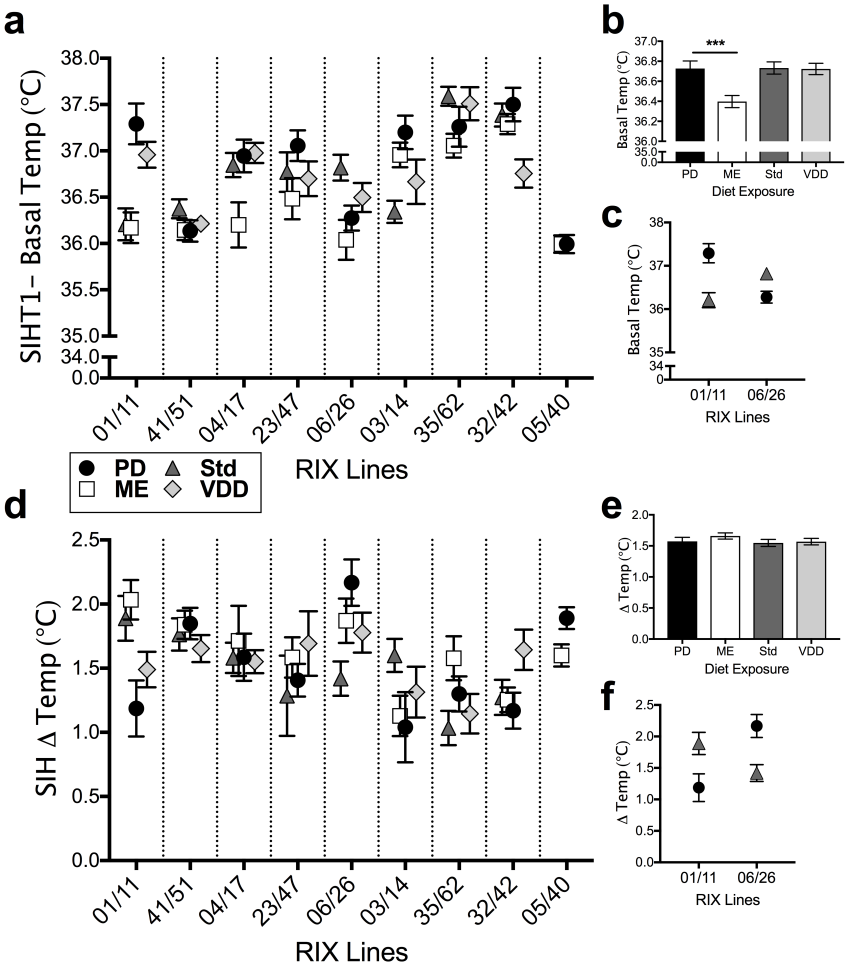
Significant values are shaded and **bolded**. CORT = corticosterone; deltaCORT = (Stress CORT – Basal CORT); deltaSIH = (T2-T1); Dist = distance; FST = forced swim test; Imb = immobile; LD = light/dark; OF= open field; PND = postnatal day; SIH = stress-induced hyperthermia; SIH-T1 = basal temperature; SIH-T2 = post-stress temperature; Vert = vertical

Figure 2.1. Experimental design



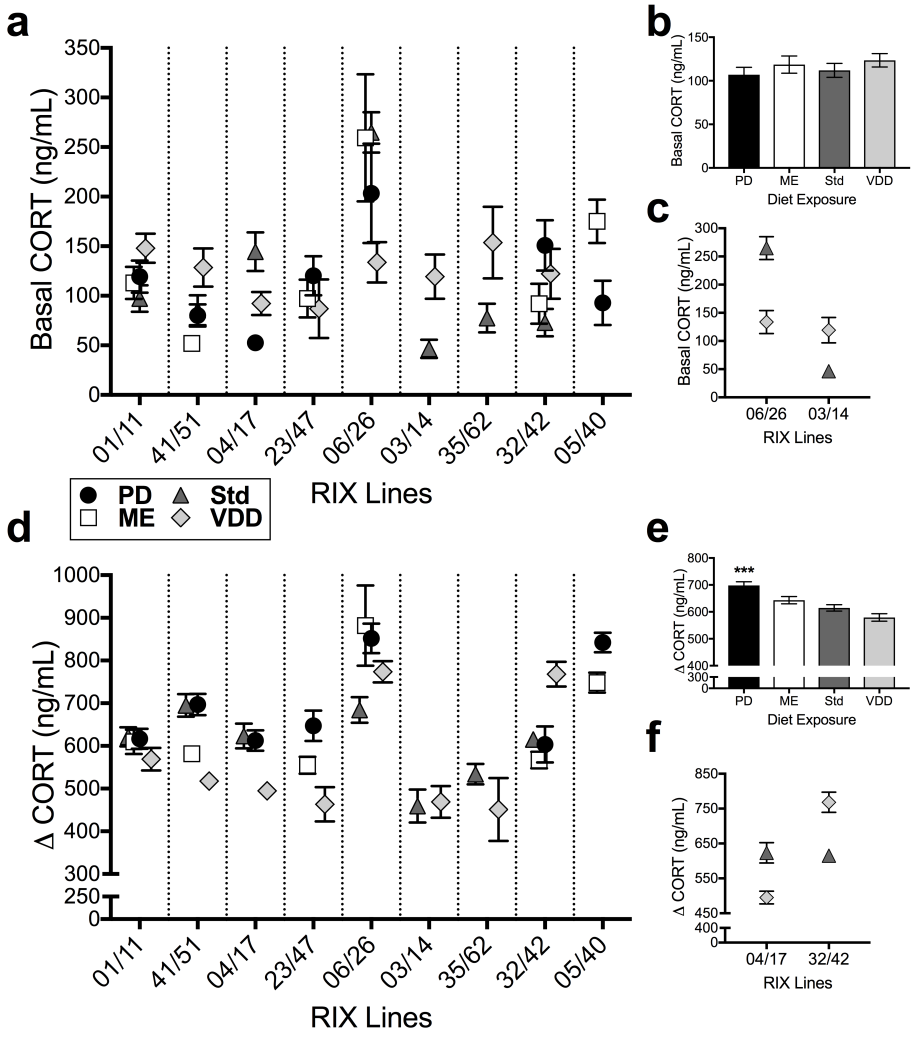
(a) Twenty different Collaborative Cross (CC) strains were used to generate ten recombinant inbred intercross (RIX) lines. Each RIX is a set of F1 reciprocal females that differ only in the parental origin of alleles. The CC alleles come from the 8 inbred strains (represented by 8 colors) that were used to generate the CC. Comparison of F1 reciprocal females within a RIX allows for detection of parent-of-origin effects. Comparison across RIX lines allows for detection of genetic background effects. **(b)** Multiple dams from each CC strain were put on one of four diets (PD, ME, VDD, Std) for 5 weeks prior to mating, throughout gestation and the postnatal period. F1 females were weaned onto a regular diet and remained on that diet until the completion of behavioral testing. **(c)** F1 females underwent a 2-week behavioral pipeline to assess anxiety- (OF, LD, SIH) and depressive-like (FST) behavior and stress response.

Figure 2.2. Basal and stress-induced temperature are affected by the interaction of perinatal diet exposure and genetic background.



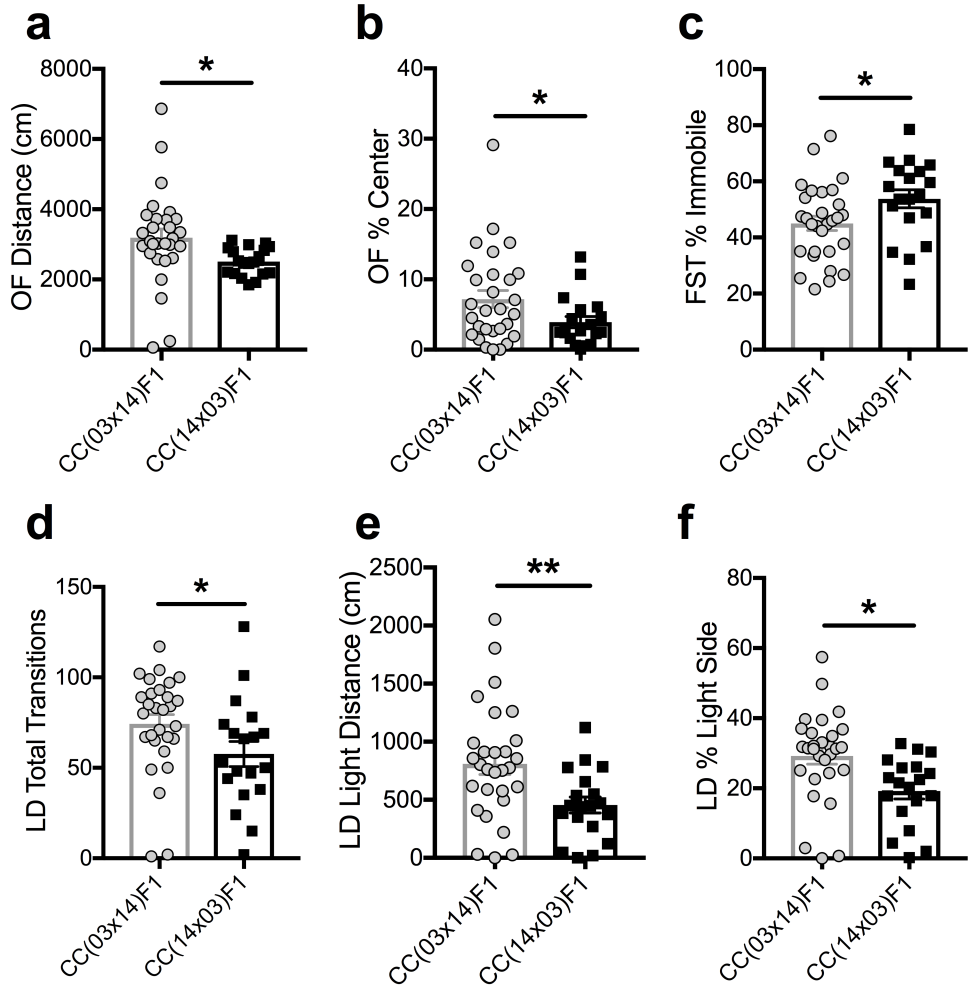
Data points are means of diet exposure groups. Error bars are SEM. There was a significant diet-by-RIX effect on (a) basal body temperature (p=3.6x10^{-7}, q=1.4x10^{-6}) and (d) change in temperature following a stressor (p=2.6x10^{-4}, q=0.001). There was an overall effect of perinatal diet on (b) basal temperature (p=0.0002, q=0.001) with ME exposed females showing decreased basal temperature compared to PD exposed females (posthoc ***p<0.001; N =157, 177, 170, 180 for PD, ME, Std & VDD, respectively). (e) No significant overall effect of diet was observed for change in temperature (p>0.05). An example of a diet-by-RIX interaction is shown for (c) basal and (f) change in temperature. (c) RIX 01/11 PD exposed females (N=31) show increased basal temperature (post hoc $p\leq$0.02) and (f) decreased change in temperature in response to stress (post hoc $p\leq$0.03) compared to Std exposed females. (N=28). (c) An opposing effect is seen in RIX 06/26 wherein PD exposed females (N=12) have decreased basal temperature (post hoc p<0.01) and (f) increased change in temperature in response to stress (post hoc $p\leq$0.009) compared to Std exposed females (N=21).

Figure 2.3. Perinatal diet interacts with genetic background to alter measures of stress response.



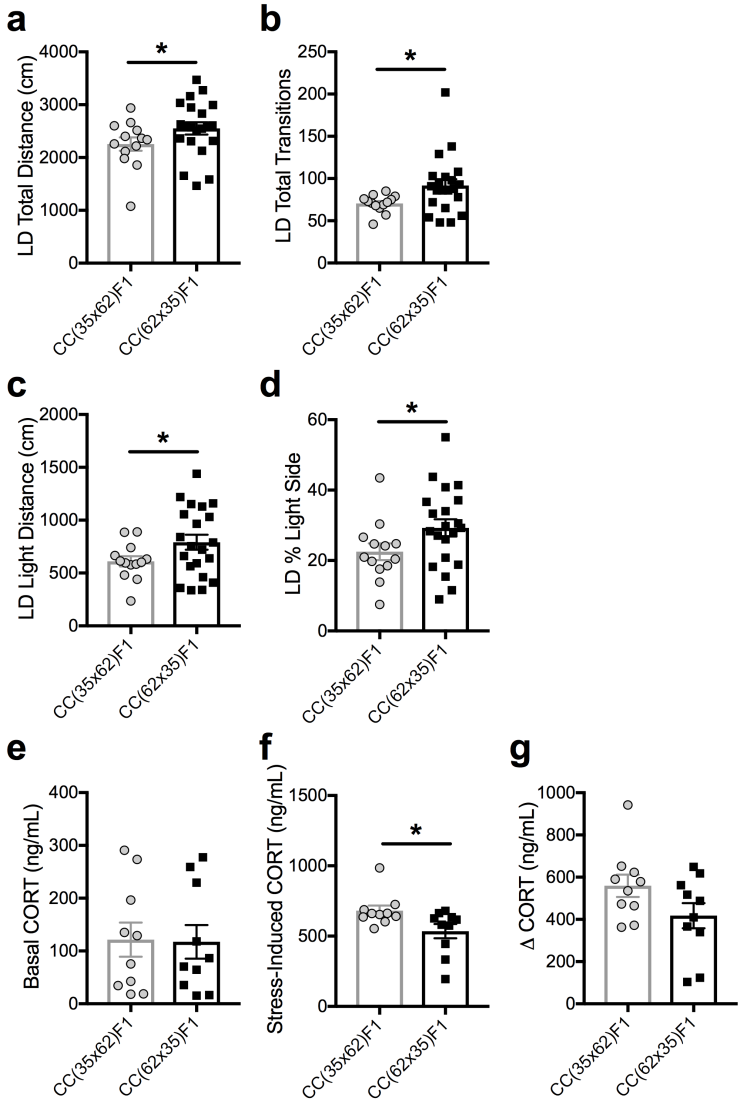
Data are means of diet exposure groups. Error bars are SEM. There was a significant diet-by-RIX effect on (a) basal CORT (p=1.5x10^{-10}, q=1.5x10^{-9}) and (d) change in CORT following a restraint stress (p=1.1x10^{-8}, q=7.6x10^{-8}). There was an overall effect of perinatal diet on (b) basal CORT (p=0.038, q=0.085) although post hoc tests revealed no significant effects. (e) There was a significant diet effect on change in CORT (p=2.1x10^{-5}, q=1.4x10^{-4}) with PD exposed females showing increased change in CORT (posthoc ***p<0.001; Ns=141, 150, 157, 171 for PD, ME, Std & VDD). Example of a diet-by-RIX interaction is shown for (c) basal CORT and (f) change in CORT. (c) In RIX 06/26, VDD exposure (N=23) resulted in decreased basal CORT compared to Std exposure (N=21) while in RIX 03/14, VDD exposed females (N=15) showed increased CORT compared to Std exposed females (N=21). (f) RIX 32/42 VDD exposed females (N=25) showed increased change in CORT compared to Std exposed females (N=30; post hoc p≤0.001) while RIX 04/17 VDD exposed females (N=27) showed decreased change in CORT compared to Std exposed females (N=27; post hoc p≤0.001).

Figure 2.4. Parent-of-origin affects anxiety- and depressive-like behaviors in RIX 03/14 females



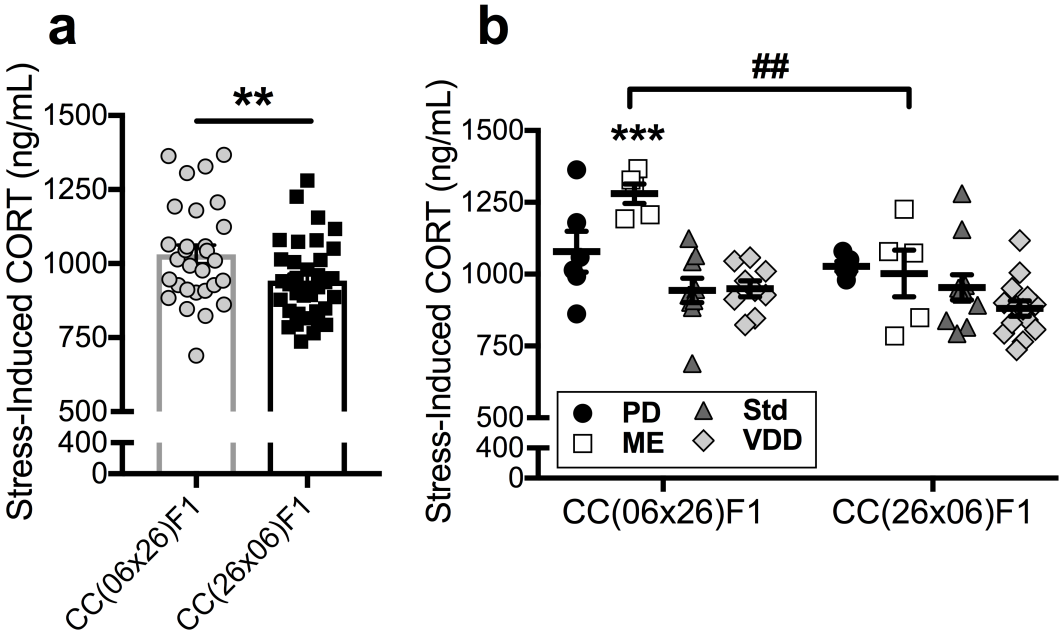
Data points are individual animals with bars indicating means. Error bars are SEM. CC(03x14)F1 females (N=29) showed (a) increased distance moved (*p=0.031, q=0.12), (b) increased time spent in the center of the OF (*p=0.045, q=0.15), (c) decreased % immobility in the FST (*p=0.02, q=0.12), (d) increased transitions (*p=0.026, q=0.12), (e) increased distance moved on the light side (**p=0.005, q=0.10), and (f) increased % time spent in the light side of the LD test (*p=0.012, q=0.12) in comparison to CC(14x03)F1 females (N=19).

Figure 2.5. Parent-of-origin affects locomotion, anxiety-like behavior and stress response in RIX 35/62 females



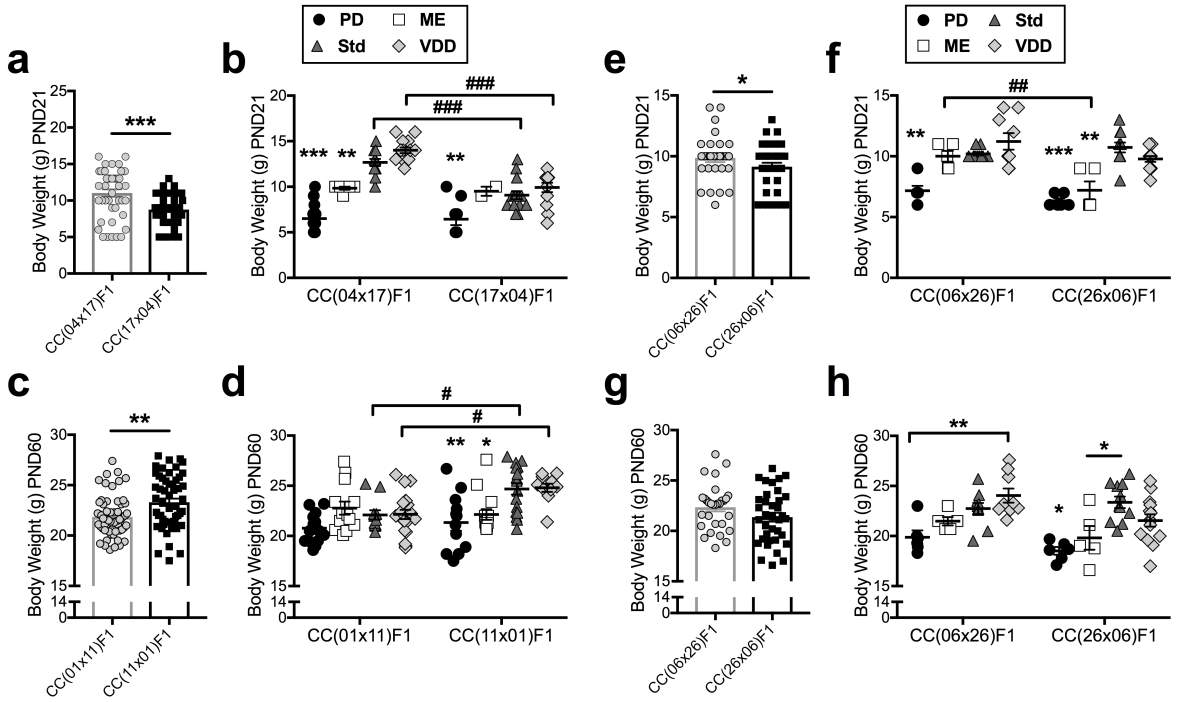
Data points are individual animals with bars indicating means. Error bars are SEM. CC(62x35)F1 females (N=21) showed (a) increased total distance (*p=0.041, q=0.16), (b) increased transitions (*p=0.03, q=0.15), (c) increased distance on the light side (*p=0.025, q=0.15) and (d) increased % time spent in the light side of the LD test (*p=0.028, q=0.15) compared to CC(35x62)F1 females (N=13). (e) RIX 35/62 reciprocal females did not differ in basal CORT levels (p>0.05). (f) CC(62x35)F1 females (N=10) showed decreased CORT levels following a restraint stress (*p=0.02, q=0.15) which resulted in (g) decreased change in CORT compared to CC(35x62)F1 females (N=10), although this effect did not reach statistical significance (p=0.07). Stress response is only reported for females exposed to VDD and Std due to the low N for ME and PD exposed groups.

Figure 2.6. Parent-of-origin and diet-by-PO effects on stress-induced CORT in RIX 06/26 females



(a) Data points are individual animals with bars indicating means. Error bars are SEM. There was a significant PO effect on stress-induced CORT levels in RIX 06/26 (**p=0.003, q=0.02) with CC(06x26)F1 females (N=29) showing increased CORT compared to CC(26x06)F1 females (N=35). (b) Data points shown are individual animals with mean (black bar). Error bars are SEM. There was a significant diet-by-PO effect on stress-induced CORT for RIX 06/26 (p=0.018, q=0.14). Posthoc analysis revealed that within CC(06x26)F1, ME exposed females (N=5; ***$p\leq$0.001) had increased CORT compared to PD (N=6), Std (N=9) and VDD (N=9) exposed females. No diet effects were observed within CC(26x06)F1 females (N=5,5,11,14). This resulted in a significant difference between ME exposed reciprocal females ($^{\#\#}$; $p\leq$0.01).

Figure 2.7. Perinatal diet interacts with parent-of-origin to affect body weight in multiple RIX lines



Data points are individual animals with bars indicating means. Error bars are SEM. There was a (a) PO effect (***p=2.0x10^{-5}, q=3.0x10^{-4}; N=44,39) and (b) diet-by-PO effect (p=0.01, q=0.19) on weight at weaning in RIX 04/17. Posthoc revealed that CC(04x17)F1 PD (***$p\leq$0.001) and ME (**$p\leq$0.01) exposed females weighed less than Std and VDD groups (N=12,6,12,14) and within CC(17x04)F1, only PD exposed females weighed less (**$p\leq$0.004; N=9, 2,15,13). Reciprocal females in the Std and VDD exposure groups also differed ($^{###}p\leq$0.001). For weight in adulthood in RIX 01/11, there was a (c) PO effect (**p=0.01, q=0.14; N=54,63) and (d) diet-by-PO effect (p=0.03, q=0.32). Posthoc showed that CC(11x01)F1 females exposed to PD (**$p\leq$0.002) and ME (*$p\leq$0.03) weighed less than Std and VDD groups (N=12,14,17,11) while within CC(01x11)F1 there was no effect of diet (N=19,13,11,20). Std ($^{#}p$=0.03) and VDD ($^{#}$=0.02) exposure also differed across reciprocal females. In RIX 06/26, there was a PO effect on (e) weight at weaning (*p=0.03, q=0.12; N=36,30) and a similar pattern in (g) adulthood (p>0.05) and a diet-by-PO effect on (f) weight at weaning (p=0.04, q=0.15) and (h) in adulthood (p=0.04, q=0.15). (f) Posthoc revealed that CC(26x06)F1 females exposed to PD (***$p\leq$0.001) and ME (**$p\leq$0.003) weighed less than Std and VDD groups (N=6,5,11,14) while in CC(06x26)F1, only PD exposed females weighed less (**$p\leq$0.006; N=6,5,10,9). Across reciprocal females, ME exposed groups differed ($^{##}p$=0.01). (h) Posthoc tests showed that CC(26x06)F1 females exposed to PD (*$p\leq$0.04) weighed less than Std and VDD groups but within CC(06x26)F1, PD exposed females only weigh less than VDD (**p=0.003). CC(26x06)F1 females exposed to ME and Std also differed (*p=0.02).

CHAPTER 3: CHARACTERIZATION OF TWO F1 HYBRID LINES OF COLLABORATIVE CROSS MICE AS MODELS FOR ADDICTION-RELATED BEHAVIOR [1,2]

INTRODUCTION

Substance use disorders (SUDs) are highly prevalent and result in significant health and financial burdens on affected individuals and society. Heritability estimates from twin studies range from 40 to 70% (Goldman *et al*, 2005) indicating that genetics play a significant role in the development of SUDs. Genetic differences are likely involved in multiple parts of the addiction cycle: the likelihood of initiating drug use (i.e. risk-taking personality or response to stressors), subjective effects caused by initial drug response or the transition to drug dependence after use is initiated (Kendler *et al*, 1999; Kendler *et al*, 2000). Identifying genetic differences in individuals that are susceptible to initiate drug use and those that are resilience to development of a SUD after drug use is initiated is crucial for the development of effective treatments.

Human studies are challenging due to the complex etiology of SUDs, the contribution of largely unknown environmental factors and previous or chronic drug exposures that make it difficult to parse the effects of the drug from potential predisposing factors. Moreover, genetic

[1]This chapter has been prepared for submission to the *Neuropsychopharmacology* journal. The authors will be: Sarah A Schoenrock, Padam Kumar, Alex Gomez-A, Joseph Farrington, Sofia Neira, Kyle Riker, Christiann H Gaines, Saad Khan, William Valdar, Fernando Pardo-Manuel de Villena, Donita L Robinson, and Lisa M Tarantino.

[2]Supplemental material for this chapter is provided in Appendix B

heterogeneity in human populations creates the need for extremely large sample sizes to identify genetic loci that increase risk.

Animal models have been developed and offer several advantages including control over the environment, baseline assessment before drug exposure and the ability to manipulate and access brain tissue for mechanistic studies. While animal models are limited in ability to recapitulate the full spectrum of diagnostic criteria observed in human SUDs, they do allow for evaluation of discrete phenotypes that model specific aspects of the addiction process (i.e. initial drug sensitivity, rewarding effects, etc). Commonly used models include selecting individual animals within an outbred population based on various predictive traits (i.e. novelty response, impulsivity) or initial drug response or screens of numerous inbred strains and selection of phenotypic outliers for a given phenotype (reviewed in Yamamoto *et al* (2013)). In these models, the individuals or inbred strain outliers are then used to investigate the relationship among addiction-related behaviors and probe for underlying mechanisms driving their divergent phenotypes.

In this study, we utilize a relatively new inbred mouse resource known as the Collaborative Cross (CC) (Churchill *et al*, 2004; Threadgill *et al*, 2012). The CC is a panel of recombinant inbred strains with genetic contributions from eight inbred strains; five classical strains (A/J, C57BL/6J, 129S1/SvImJ, NOD/ShiLtJ, NZO/HlLtJ) and three-wild derived (WSB/EiJ, CAST/EiJ, PWK/PhJ). The eight founder strains represent three subspecies of *Mus musculus*, resulting in novel combinations of alleles, and capture 90% of the genetic diversity present in the domesticated house mouse (Roberts *et al*, 2007; Srivastava *et al*, 2017). The increased genetic diversity of the CC results in a greater phenotypic range than observed in commonly used

models and could provide novel insight into the relationship among predictive and addiction-related behaviors and the underlying mechanisms.

In a previously published study, we assessed a panel of CC F1 hybrid female mice (referred to as Recombinant Inbred Intercrosses (RIX)) for locomotor behavior (Schoenrock *et al*, 2017). We identified two RIX lines that were outliers for novelty-induced locomotion in both a novel open field (OF) and light/dark (LD) arena (see **S Fig 5 in Appendix A**). Locomotor response to a novel environment is one commonly used animal model for studying addiction-related behaviors (Piazza *et al*, 1989), and is thought to reflect sensation-seeking in humans, a personality trait linked to increased drug initiation (Howard *et al*, 1997). Locomotor response to a novel arena has been shown to predict initial locomotor response (Hooks *et al*, 1991b), behavioral sensitization (Hooks *et al*, 1992; Hooks *et al*, 1991a) and intravenous self-administration (IVSA) behaviors (Marinelli and White, 2000; Piazza *et al*, 1989; Piazza *et al*, 1990; Pierre and Vezina, 1997) to psychostimulants in animal models.

We conducted a set of experiments to assess these two RIX lines for addiction-related behaviors including those modeling early (i.e. initial locomotor sensitivity) and subsequent (i.e. behavioral sensitization, reward and reinforcement) phases of the addiction process. We also explored mechanisms that might explain their divergent behavioral phenotypes including the hypothalamic-pituitary-adrenal (HPA) axis and dopaminergic system, both well-established in their role in addiction (Koob, 2008; Volkow *et al*, 2011). We believe these studies establish a framework for using the genetically diverse CC to identify phenotypic outliers that can aid in the dissection of complex behaviors including those that model drug addiction.

METHODS AND MATERIALS

Animals

CC mice were purchased from the UNC Systems Genetics Core Facility. CC mice were bred to generate F1 hybrids. In this paper, F1 mice from each cross are collectively referred to as a RIX line. CC004/TauUnc and CC017/Unc were crossed to create RIX 04/17; CC041/TauUnc and CC051/TauUnc were crossed to create RIX 41/51; CC005/TauUnc and CC040/TauUnc were crossed to create RIX 05/40. All crosses were performed reciprocally such that maternal and paternal contribution was balanced in the F1 offspring.

Mice were housed in a specific pathogen-free animal facility on a 12-hour light/dark cycle with lights on at 7:00 A.M. All procedures and animal care were approved by the UNC Institutional Animal Care and Use Committee and followed guidelines set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Food and water were provided *ad libitum* throughout the experiment. Breeder diet was AIN-93G (Dyets Inc, Bethlehem, PA) and offspring were weaned and maintained on Harlan Teklad 2920 (Envigo, Frederick, MD, USA).

Drugs

Cocaine HCl (COC; Sigma-Aldrich, St. Louis, MO) and methamphetamine (METH; Sigma-Aldrich, St. Louis, MO) were dissolved in 0.9% saline. COC was administered intraperitoneally (IP) at a dose of 20 mg/kg of body weight for all experiments except behavioral sensitization (10 mg/kg) and dose response (10, 20, 30, 40, 45, and 50 mg/kg). METH was administered IP at a dose of 2 mg/kg. Both drugs and saline controls were injected administered in a volume of 0.01mL/g of body weight.

Behavioral phenotyping

All behavioral testing was performed during the light portion of the light/dark cycle between 8:00 A.M. and 12:00 P.M. except for the 6-hr OF test and behavioral sensitization experiments which ran until 4:00 P.M. All experimental animals were naive to any previous testing at the start of each experiment. All animals were tested at a mean age of 65 ± 6.6 days.

Open field (OF) arena

The eight OF apparatuses (ENV-515-16, Med Associates, St. Albans, VT, USA) were 43.2x43.2x33 cm with white Plexiglas floors and clear Plexiglas walls. Infrared detection beams at 2.54 cm intervals on the x, y, and z-axes tracked the animals' position and activity automatically during testing. Each OF was enclosed in a sound-attenuating chamber (73.5x59x59 cm) illuminated by two overhead light fixtures containing 28-V lamps. Data were analyzed post-session using commercially available software (Activity Monitor 5.1, Med Associates). The OF was used to assess acute locomotor response to COC and METH, behavioral sensitization to COC and stress response in a novel environment. Eight identical OF arenas were used. For tests requiring repeated exposure to the OF, mice were tested in the same arena for all sessions.

Acute locomotor response to COC

For the initial test of COC-induced locomotion, we used a 6-hr OF test. Mice were weighed and placed into the OF arena for 1 hr, then removed and injected with saline and returned to the OF for 2 hrs. After 2 hrs, mice were injected with COC and returned to the OF for an additional 3 hrs. Distance moved was scored in 5-min bins. Distance moved in the first 10 mins of the test was used to assess novelty-induced locomotion and the sum of the distance moved post-COC

injection (180-360 min), referred to as area under the curve (AUC) was used to assess COC-induced locomotion.

COC dose response and methamphetamine response

Animals were tested on 3 consecutive days in the OF and all test sessions were 30-mins in length. On Day 1 (habituation) and Day 2 (baseline) animals were injected with saline and on Day 3, animals were injected with either saline, COC, or METH before being immediately placed into the OF arena. Distanced moved was recorded in 2-min bins. A difference score of total distance moved on Day 3 - Day 2 was used to assess COC- or METH-induced locomotion.

Behavioral sensitization to COC

Each day of the 19-day sensitization protocol consisted of a 90-min test session. On each test day, animals were placed into the OF for 30 min, then removed and injected with either saline or COC and returned to the arena for 60 min. Animals received saline injections on Days 1, 2, and 12 and COC injections on Days 3, 5, 7, 9, 11, and 19. Total distanced moved in the 60-min post injection was used to assess initiation of behavioral sensitization (Days 3,5,7,9,11), expression of sensitization (Day 19) and conditioned activation (comparison of Day 12 to Day 2).

Stress response to a novel environment

Corticosterone (CORT) was assessed at three time points immediately prior to and 30- and 120-min after a 10-min exposure to a novel OF arena. Blood was collected by retro-orbital bleed for the first two time points and trunk blood was collected for the third-time point. Whole blood was centrifuged at 11,700 rpm for 13 min to isolate serum, and CORT levels (measured in ng/mL) were analyzed by competitive radioimmunoassay following the manufacturer's protocol (MP Biomedicals, Santa Ana, CA, USA).

Conditioned Place Preference (CPP)

CPP was assessed using a 10-day protocol in a three-chambered arena (46.5×12.7×12.7 cm; MED-CPP-MSAT, Med Associates) that consisted of a grey middle chamber (7.2×12.7 cm; 21-24 lux lighting) with a smooth PVC floor flanked by white and black chambers (each 16.8×12.7 cm) on either side. The black chamber had a stainless-steel grid rod floor (3.2 mm rods, 7.9 mm centers; 25-27 lux lighting) and the white chamber had a stainless-steel mesh floor (6.35×6.35 mm; 13-15 lux lighting). The chambers were separated by guillotine doors that were open on habituation Day 1 and the preference test on Day 10 and closed on conditioning Days 2-9. Each CPP arena was enclosed in a sound-attenuating box (ENV-016MD, Med Associates). Two identical CPP arenas were used; each mouse was tested in the same arena throughout testing.

The 10-day CPP protocol is described in more detail elsewhere (Eisener-Dorman *et al*, 2011). On Day 1, mice were injected with saline before being placed in the middle compartment of the CPP apparatus with access to all 3 chambers for 30 min. On days 2, 4, 6, and 8, animals were given an injection of saline before being confined to the saline-paired compartment. On days 3, 5, 7, and 9, mice were given an injection of COC before being confined to the COC-paired chamber. The CPP apparatus was unbiased and mice were randomly assigned to receive COC in either the black or white chamber. Assignment to a training chamber was balanced within RIX line and sex. Day 10 served as the test day during which mice were given an injection of saline before being placed into the middle compartment with access to the entire apparatus for 30-minutes. Time spent in each chamber on Day 10 was recorded and compared to Day 1. A control group that received saline on all days was included to allow for comparison of drug effects within a RIX line.

Brain concentration of COC

Animals were injected with COC and returned to their home cage. At 10-min whole brain was collected, rinsed with ddH_2O, hemisected and snap-frozen in liquid nitrogen. This time point was chosen to correlate with the peak behavioral response. Brain concentrations of COC, and its metabolites (norcocaine (NOR); benzoylecgonine (BZE)) were quantified using liquid chromatography tandem mass spectrometry as previously described (Slawson *et al*, 2002).

Monoamine brain tissue content

Monoamine levels in the dorsal striatum (dST) and nucleus accumbens (NAc) were measured in both drug naïve and COC-exposed animals. For COC exposure, animals were given an injection of COC and returned to the home cage for 10 min prior to brain collection. Brain tissue was collected and immediately sectioned using a 1mm brain block that was kept cold on ice. One mm tissue punches were taken from dST and NAc, pooled across hemispheres for each animal and flash frozen in liquid nitrogen. Tissue content of norepinephrine (NE), dopamine (DA), serotonin (5-HT) and select metabolites (DOPAC, HVA, 3-MT, 5-HIAA) was measured by high performance liquid chromatography at the Vanderbilt Neurochemistry Core (medschool.vanderbilt.edu/vbi-core-labs/neurochemistry-core).

In vivo Fast Scan Cyclic Voltammetry (FSCV)[3]

Endogenous DA release was measured in anesthetized mice before and after COC administration according to published (Shnitko *et al*, 2016) and supplemental methods. Briefly,

[3]*In vivo* FSCV was performed through a Collaboration with the Robinson Lab at UNC (Fig 4.4, S Fig 1 & 2 in Appendix B): A. Gomez, S. Neira, K. Riker, D. Robinson

mice were anesthetized with urethane (50% w/w in saline, 1.8g/kg IP) and placed in a stereotaxic frame. A bipolar stimulating electrode was lowered -4.0mm from the skull surface into the ventral tegmental area (VTA; AP -3.8, ML -0.2). A carbon-fiber microelectrode was lowered into the NAc -4.0mm from dura (AP +1.2, ML -1.2). An Ag/AgCl reference electrode was inserted into contralateral cortex. A triangle waveform potential ramping from -0.4V to +1.3V to 0.4V at 400 V/s was applied to the carbon-fiber electrode at 10Hz and DA signals were obtained upon stimulation of the VTA. Evoked DA signals were collected five minutes apart. First, saline (vehicle) was administered IP and three evoked DA recordings were collected; this constituted the baseline measurement. Next, mice received either 20mg/kg COC or saline IP and evoked DA recordings were collected for 60 min. Evoked DA was background subtracted and confirmed via the cyclic voltammogram as previously described (Shnitko *et al*, 2016).

Statistical analysis

All statistical analyses were performed using SPSS v24 (IBM, Armonk NY, USA) and GraphPad Prism (GraphPad, La Jolla CA, USA). Within an experiment, analysis of variance (ANOVA) tests were performed to analyze the effects of RIX (referred to as strain), sex, time points and dose (as relevant) on the dependent variable. Dependent variables included distance moved, AUC, locomotor difference score, CORT levels, time in CPP chambers, monoamine tissue content, concentrations of COC and metabolites and electrically-evoked DA release. Significant main effects were followed up with Tukey's or Bonferroni's *posthoc* analyses. Mean differences were significant at $p < 0.05$. Pearson 2-tailed correlation was used for novelty- and COC-induced locomotion.

RESULTS

RIX 04/17 and 41/51 show phenotypically divergent initial locomotor sensitivity to COC

We tested the relationship between novelty-induced locomotion and COC-induced locomotion using a 6-hr OF test (**Fig 3.1a**). RIX 05/40 was included for comparison as it displayed normal novelty-induced locomotion among a panel of 9 RIX lines (Schoenrock *et al*, 2017). ANOVA of strain x sex revealed a significant effect of strain on novelty-induced locomotion as measured by the distance moved in the first 10 min ($F_{2,141}$=119.8, p=10.0x10^{-31}) and COC-induced locomotion as measured by the total distance moved post COC ($F_{2,141}$=72.3, p=4.2x10^{-22}). RIX 41/51 moved significantly less than RIX 04/17 and RIX 05/40 and RIX 04/17 moved significantly more than RIX 41/51 and 05/40 for both novelty- (p<0.001; **Fig 3.1b**) and COC-induced locomotion (p<0.001; **Fig 3.1c**).

Strain difference in initial COC locomotion is not dose-dependent

We tested whether the difference between RIX 04/17 and 41/51 for initial sensitivity to COC was dose-dependent. We assessed dose dependency of initial locomotor sensitivity between RIX 04/17 and 41/51 using the three-day OF test and six doses of COC (**Fig 3.1e**). An ANOVA of strain x sex x dose on locomotor response to COC revealed a significant effect of strain ($F_{1,258}$=484.3, p=1.3x10^{-58}) and dose ($F_{6,258}$=37.4, p=1.5x10^{-31}) and a strain x dose interaction ($F_{6,258}$=20.7, p=2.1x10^{-19}). RIX 41/51 mice were significantly more activated at 30, 40, 45, and 50 mg/kg compared to saline (p≤4.1x10^{-5}). RIX 04/17 mice were significantly more activated at all COC doses compared to saline (p≤1.6x10^{-5}). RIX 04/17 showed significantly higher locomotor response at every COC dose tested (p≤1.63x10^{-4}) compared to RIX 41/51. Peak

locomotor response for RIX 41/51 was at 40 mg/kg while RIX 04/17 showed peak activation at 20 mg/kg.

RIX 04/17 and 41/51 differ in initial locomotor response to METH

To assess whether the difference in initial locomotor sensitivity was specific to COC or extended to other psychostimulants, we measured locomotor response to METH using our 3-day protocol (**Fig 3.1f**). An ANOVA of strain x sex x dose on locomotor response revealed a significant effect of strain ($F_{1,75}$=30.0; p=6.7x10^{-7}), dose ($F_{1,75}$=39.3; p=2.9x10^{-8}) and a strain x dose interaction ($F_{1,75}$=28.3; p=1.0x10^{-6}), but no effect of sex (p>0.05). Follow-up analyses revealed that METH-treated animals of both strains moved significantly more than saline-treated animals (p≤0.007) but RIX 04/17 had greater METH-induced response compared to RIX 41/51 (p=2.2x10^{-7}).

Strain difference in initial locomotor response to COC is not due to brain concentrations of COC or metabolites

We tested for differences in brain concentrations of COC and its metabolites at 10-mins post drug exposure; the time point of peak behavioral activation (see **Fig 1a**). RIX 04/17 and 41/51 did not differ for COC, NOR or BZE concentrations in the brain. Nor were there any sex differences or sex by strain interaction effects (all p>0.05; **S Table 1).**

RIX 04/17 and 41/51 show behavioral sensitization to repeated exposures to COC

Behavioral sensitization was assessed by repeated measures ANOVA of distance moved post COC dosing for each exposure day (**Fig 3.2a**). There was a significant effect of day

($F_{2.5,64.2}$=13.1; p=4.0x10^{-6}) and a day x strain interaction effect ($F_{2.5,64.2}$=3.9; p=0.018). RIX 04/17 shows a significant increase in COC-induced locomotor activity from Day 3 to 5 (p=0.0002) and Day 7 to 9 (p=0.025) and appears to reach a plateau after the third exposure to COC. RIX 41/51 showed a significant increase in distance from Day 5 to 7 (p=0.01). These data indicate that both strains show behavioral sensitization, however, RIX 41/51 animals require more exposures to COC before showing a significant increase in locomotor behavior.

We also assessed conditioned activation by comparison of saline treatment on Day 2 (baseline) to Day 12 within each strain. Both RIX 41/51 ($F_{1,14}$=33.6; p=4.7x10^{-5}) and RIX 04/17 ($F_{1,14}$=12.5; p=0.003) showed a significant increase in locomotion on Day 12, indicating some level of conditioned activation. Although Day 12 locomotion is much lower than on the last exposure to COC on Day 11 in both RIX.

RIX 04/17 and 41/51 show conditioned place preference to COC

We measured CPP to a single dose of COC to determine if differences in initial locomotor sensitivity in these two RIX lines predicted differences in sensitivity to the rewarding effects of the drug (**Fig 3.2b)**. An ANOVA assessing the effects of strain, chamber, test day (pre- vs post-training), dose (COC vs saline) and sex on the time spent in the drug-paired chamber revealed a significant effect of chamber ($F_{2,436}$=12.2; p=7.0x10^{-6}), strain x chamber ($F_{2,436}$=31.8; p=1.6x10^{-13}), and dose x chamber ($F_{2,436}$=13.2; p=3.0x10^{-6}). RIX 41/51 spent significantly more time in the middle chamber during the habituation session on Day 1 ($p\leq$3.2x10^{-7}). However, both RIX 41/51 and 04/17 COC-trained mice spent significantly more time in the COC-paired chamber than the saline-paired chamber on Day 10, after eight conditioning days (all $p\leq$3.2x10^{-4}) and also spent significantly more time in the COC-paired chamber than saline control animals ($p\leq$0.015). We

observed no strain difference in time spent in the COC-paired chamber post conditioning (p>0.05) indicating that both strains show CPP for the COC-paired chamber.

RIX 41/51 males have alterations in stress reactivity and recovery to a novel environment

The HPA axis is involved in initiation of drug use, transition to drug dependence and the negative affective state that drives drug-seeking (Koob, 2008). We assessed the HPA axis at baseline and in response to a stressor in both RIX lines. A repeated measures ANOVA of serum CORT levels before (0 min) and after (30 and 120 min) exposure to novel OF stress yielded a significant effect of time ($F_{2,78}$=171.1; p=3.0x10^{-29}), time x strain ($F_{2,78}$=5.5; p=0.006), time x sex ($F_{2,78}$=6.3; p=0.03). Due to the significant sex difference, we analyzed females and males separately and observed a significant effect of time in both males (p=7.7x10^{-13}) and females (p=4.3x10^{-18}; **Fig 3.3b**) and a male-specific time x strain interaction (p=1.1x10^{-4}; **Fig 3.3a**). CORT was significantly increased at 30 min post stressor in males from both RIX lines (p≤0.001) but the level at 30 min was significantly lower in RIX 41/51 compared to RIX 04/17 (p=0.001). Additionally, only RIX 04/17 shows a significant decrease in CORT at 120 compared to 30 (p<0.001). Collectively, these data indicate that RIX 41/51 males have a blunted and prolonged elevation in CORT in comparison with RIX 04/17.

Specific strain differences in NE and 5-HT, but not DA striatal tissue content

Since DA has a known role in mediating both movement and addiction-related phenotypes, we assessed the dopaminergic system as a possible mechanism for the observed RIX differences. We assessed tissue content of monoamines and their metabolites basally and after exposure to COC in two key areas with DA neuronal projections, the dST and NAc, **(S Table 2)**. There were

no strain differences in DA tissue content in either brain region, but there were select differences in 5-HT and NE. RIX 41/51 has increased levels of 5-HT in the dST at basal conditions compared to RIX 04/17 ($F_{1,13}$=7.4, p=0.022) and in the NAc after acute exposure to COC ($F_{1,14}$=11.0, p=0.07). RIX 04/17 has increased basal levels of NE in the NAc compared to RIX 41/51 ($F_{1,11}$=16.8, p=0.003).

COC effects on DA dynamics is not different across strains

We used *in vivo* FSCV to assess potential strain differences in DA release and clearance at baseline and after COC exposure. We collapsed samples after the initial saline injection (i.e., baseline), plotting them as concentration versus time in **Fig 3.4a**. The peak DA concentration, or $[DA]_{max}$, at baseline was higher in RIX 41/51 than 04/17 mice, but this did not reach significance (**inset;** t=1.91, p=0.073). We compared the effect of saline versus COC on $[DA]_{max}$ by strain and found that COC increased evoked DA release similarly in each strain. Illustrating this, **Fig 3.4b** displays $[DA]_{max}$ as percent change from baseline (non-transformed data displayed in **S Fig 1**). A 3-way repeated measures ANOVA of strain x drug x time (calculated on non-transformed data), revealed a significant drug x time interaction ($F_{1,19}$=5.40, p<0.004) with significant main effects of time ($F_{1,19}$=4.12, p=0.014) and drug ($F_{1,19}$=10.43, p=0.004). Post-hoc analysis (collapsed across strain) indicated that DA release was three times higher after COC compared with saline (all t's>3.44, p<0.01), **Fig 3.4b, S Fig 1**). To further assess pharmacological effects of COC, we determined the sample containing the peak effect of COC on DA release for each mouse and calculated several parameters (**S Fig 2**). We found no strain differences on the peak $[DA]_{max}$ (t=1.82, p=0.093), the time of that peak (T_{max}, t=0.20, p=0.843), the latency from the end of the stimulation to the $[DA]_{max}$ (t=1.63, p=0.129), the full width of the DA signal at half height

(t=0.39, p=0.699), and the slope of the clearance curve (t=0.922, p=0.376). Together, these data indicate that the RIX lines show a similar pattern of DA dynamics after COC.

DISCUSSION

RIX 04/17 and 41/51 as models to study addiction-related behaviors

In this study, we selected two CC-RIX lines that were outliers for locomotor activity in a novel environment; a phenotype previously shown to predict addiction-related behaviors (Piazza *et al*, 1989). We characterized these lines for several addiction-related behaviors to establish their utility as mouse models for assessing the underlying genetic and mechanistic factors that drive these behaviors. We first assessed initial locomotor sensitivity to COC and found a significantly divergent response that was consistent across multiple doses. Comparison of their COC-induced locomotion at 20 mg/kg in the 3-day OF test (see **Fig 3.1e**) to a previously published survey of 45 traditional inbred mouse strains confirms that RIX 41/51 and 04/17 are at the phenotypic extremes. Only one strain (LG/J) showed a lower locomotor response to COC than RIX 41/51 and only one strain (C57BR/cdJ) was more responsive to COC than RIX 04/17, Furthermore, both RIX lines fell outside the range of the seven founders of the CC population that were tested in this study (Wiltshire *et al*, 2015).

Our ability to identify phenotypic outliers for locomotor sensitivity to COC highlights the genetic diversity present in the CC resulting from novel allele combinations of the three-major subspecies of *Mus musculus*. Expanded phenotypic range in the CC, relative to that observed in standard inbred strains, has also been observed for phenotypes related to immunology and toxicology (Gralinski *et al*, 2015; Mosedale *et al*, 2017; Venkatratnam *et al*, 2017; Vered *et al*, 2014). Ours is the first study to examine addiction-related behaviors in the CC.

Human studies have shown that individuals who experienced a positive or pleasurable first experience with COC have a higher lifetime use and increased risk for dependence (Davidson *et al*, 1993; Haertzen *et al*, 1983; Lambert *et al*, 2006). In animal models, however, locomotor sensitivity to COC does not always predict other addiction-related behaviors (Yamamoto *et al*, 2013). For this reason, we examined behaviors thought to reflect the rewarding and neuro-modulating properties of psychostimulants, CPP and behavioral sensitization, in the CC-RIX. We hypothesized that RIX 04/17 would show greater COC CPP compared to RIX 41/51. To the contrary, we found that both RIX lines showed a preference for COC after multiple training exposure days. These data would indicate that initial locomotor response to COC does not predict the rewarding effects in these lines. One consideration in the interpretation of CPP data is that our RIX 04/17 do exhibit much higher locomotor activity on COC training days compared to RIX 41/51, which may inhibit their ability to associate the compartment with the drug's effects (Cunningham *et al*, 2006). Another significant caveat was the use of only one dose of COC for CPP experiments. Additional experiments using lower, less activating doses of COC are needed to more fully explore the relationship between initial sensitivity and the rewarding effects of COC in these lines.

Behavioral sensitization is the phenomenon of increased locomotor response across repeated exposures to a drug and is thought to reflect underlying long-term neuroadaptations in response to the drug, although the mechanisms are a subject of debate in the literature (Rothenfluh and Cowan, 2013). We observed behavioral sensitization in both RIX lines but sensitization emerged earlier in RIX 04/17 compared to RIX 41/51. One caveat that must be considered when interpreting the behavioral sensitization data is that RIX 04/17 could have reached a ceiling in

locomotor activation as there appears to be a plateau after the third COC exposure. Additional sensitization studies with lower doses would be necessary to test this further.

The experiments described in this paper are in response to experimenter-administered drug. Additional studies are needed to assess the effects of drug in a contingent or self-administrated paradigm. Use of a paradigm such as IVSA will allow for the assessment of additional addiction-related behaviors such as acquisition of drug intake, maintenance, motivation to administer, extinction resistance and reinstatement behavior, all of which cannot be assessed in a non-contingent paradigm.

Investigation of possible underlying mechanisms for divergent behavioral phenotypes

Role of the HPA axis

The HPA axis thought to be involved in initiation of drug use, transition to drug dependence and the negative affective state that drives drug-seeking (Koob, 2008). We assessed the HPA axis at baseline and in response to a stressor and found differences in HPA axis reactivity and recovery in RIX 41/51 males compared with RIX 04/17. The sex-specific nature of this effect is difficult to interpret as we did not observe sex-specific differences in other behaviors. It is possible that if we had assessed behaviors that more directly model the negative reinforcement of withdrawal and reinstatement of drug use, sex-specific differences in RIX 41/51 might have been observed. One caveat in the interpretation of these data is that the initial blood sample collection is very stressful itself. Therefore, the increase in CORT seen following the novel OF exposure is likely due to a combination of the basal blood collection and the novel environment. A follow-up study using separate animals for the different time points would allow for assessment of HPA axis activity due solely to the novel environment.

Role of the dopaminergic system

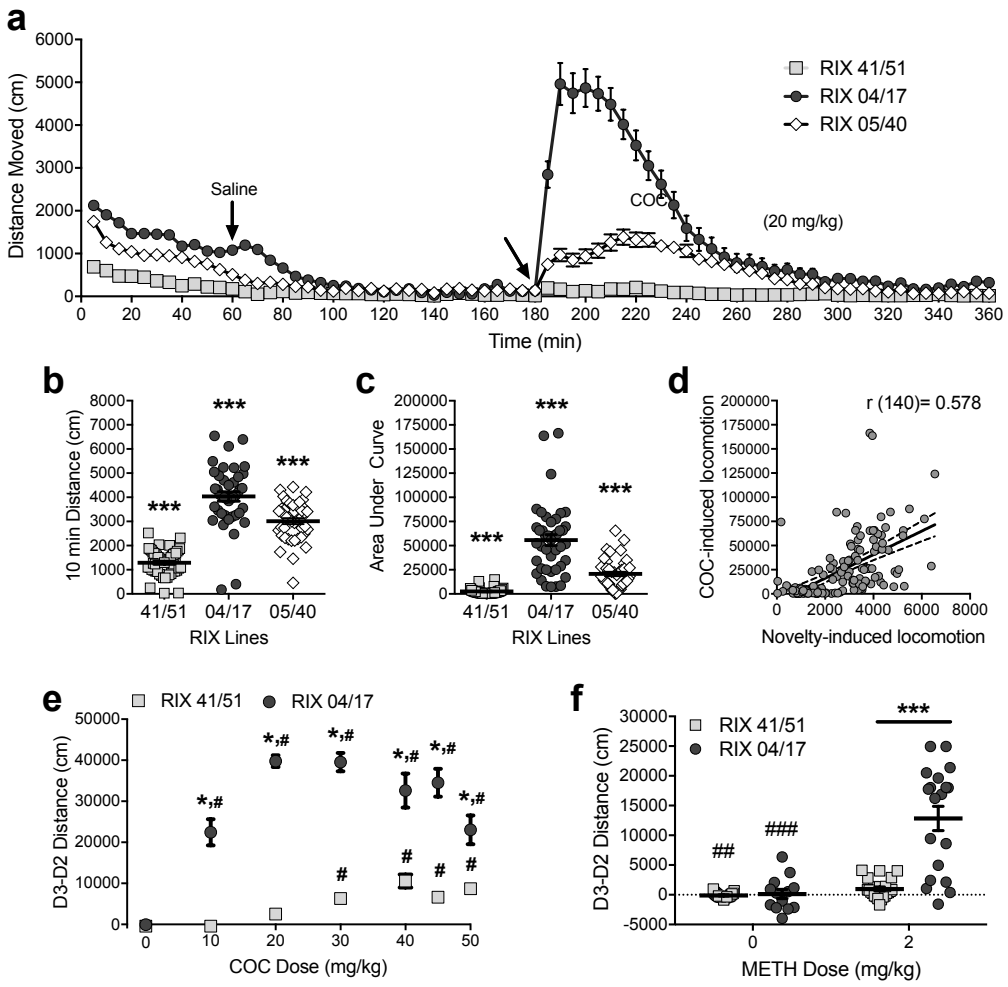
COC affects DA dynamics in subregions of the brain associated with motor behavior and rewarding properties of drugs, including NAc, dST and prefrontal cortex, (Hedou *et al*, 1999; Volkow *et al*, 2011). We found that COC-induced increases in evoked DA signals in the NAc were similar between the RIX lines, suggesting that DA release and clearance dynamics did not underlie RIX differences in COC-induced behavior. However, it should be noted that we chose to target the NAc core, whereas some published studies indicate that DA in the NAc shell more closely aligns with behavioral effects of stimulants (Hedou *et al*, 1999; Parkinson *et al*, 1999).

We did identify differences in other monoamines. Basal NE levels in the NAc were higher in RIX 04/17 than RIX 41/51. The NA shell receives NE projections mainly from the nucleus of the tractus solitarius and locus coeruleus (Delfs *et al*, 1998) and recent evidence has shown that noradrenergic neurotransmission might be implicated in COC sensitization. Jimenez-Rivera *et al* (2006) found that the alpha-2 receptor agonist clonidine attenuated the acute locomotor response to COC. We also observed a significant decrease in 5-HT in the dST at baseline and in the NAc after acute COC in RIX 04/17 mice. Low serotonergic activity has been observed in humans with a sensation-seeker personality (Netter *et al*, 1996), which would fit with the high novelty-induced phenotype observed in RIX 04/17, which is thought to model sensation-seeking behavior. It should be noted that we made all of our comparisons between RIX 41/51 and RIX04/17, and it is possible that divergent initial locomotor sensitivity to COC may not be due to the same mechanisms in these lines.

Conclusion and future directions

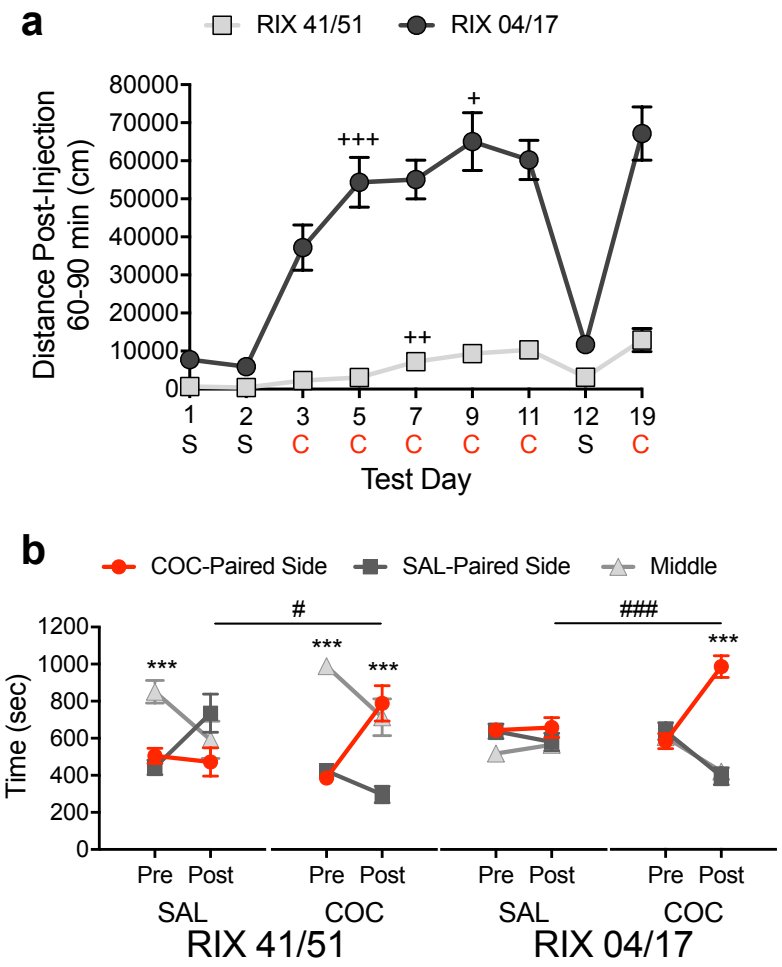
We have shown that RIX 41/51 and 04/17 are phenotypically divergent for initial locomotor response to COC and can be used as genetically diverse, yet stable models to study addiction-related behaviors and the underlying mechanisms. Their divergent behavior does not appear to be due to differences in the dopaminergic system, but could involve the noradrenergic or serotonergic systems which would be interesting as these circuits have a strong role in other phenotypes related to psychiatric disorders (i.e. mood, stress) but are less characterized as a mechanism in development of COC SUDs. Future studies utilizing these RIX lines to assess other addiction-related phenotypes such as impulsivity and IVSA should be performed to further establish the relationship among behaviors. Additionally, the genetic diversity present in the CC lines can be used to perform genetic analysis of initial locomotor sensitivity to COC using these RIX lines to identify genetic loci contributing to this phenotype.

Figure 3.1. RIX 04/17 and 41/51 show divergent initial locomotor sensitivity to cocaine



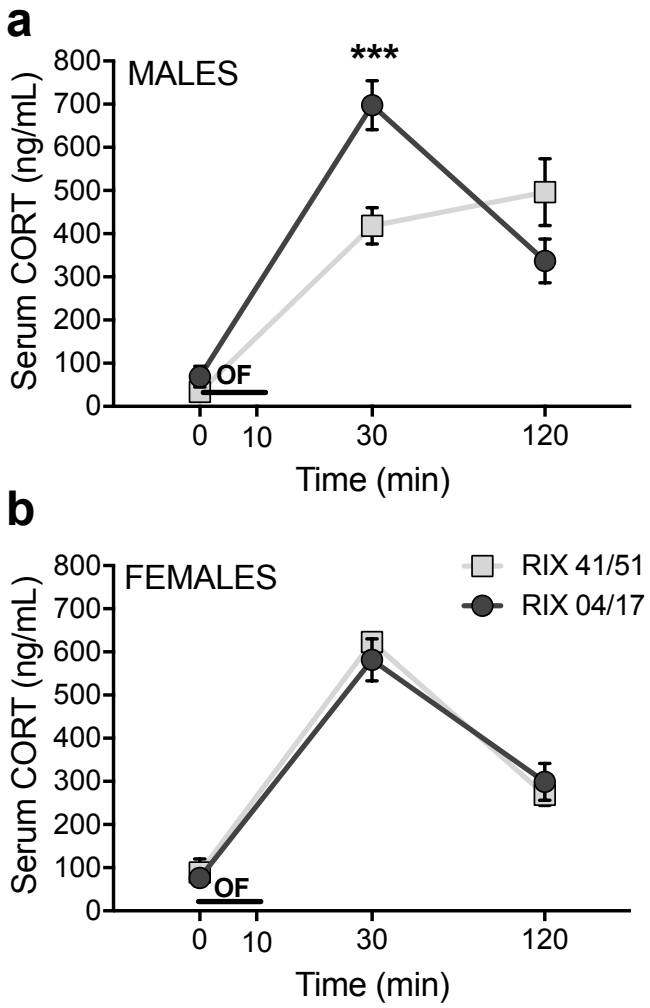
Data are either strain means or individual animals with think black bars representing strain mean. All error bars are SEM. **(a)** Distance moved in 5 min bins across a 6-hr OF test for RIX 41/51 (N=56), RIX 04/17 (N=42) and RIX 05/40 (N=44). From this test, there was a significant strain difference on **(b)** novelty-induced locomotion (distance moved in the first 10 mins) and **(c)** cocaine (COC) induced-locomotion (Area Under Curve (AUC) in the last 3-hrs post COC injection) (***strain effect, $p<0.001$). **(d)** Novelty- and COC-induced locomotion are positively correlated ($p<0.001$). **(e)** Day 3-Day 2 distance for RIX 41/51 (Ns=22,21,21,22,21,21,21) and RIX 04/17 (Ns=15,16,16,15,16,16,16) in a 3-day OF test. RIX 04/17 had COC-induced increase in locomotion at all doses while RIX 41/51 only had COC-induced increase at the four highest doses (#COC effect within strain compared to saline, $p<0.001$). There was a significant effect of strain at all doses of COC (*strain effect, $p<0.001$). **(f)** Day 3-Day 2 distance for RIX 41/51 (N=21, 23) and RIX 04/17 (N=13, 19). There was a significant effect of METH in both strains (##$p<0.01$, ###$p<0.001$) and a significant effect of strain on METH response (***$p<0.001$).

Figure 3.2. Strain differences in behavioral sensitization but not conditioned place preference to cocaine



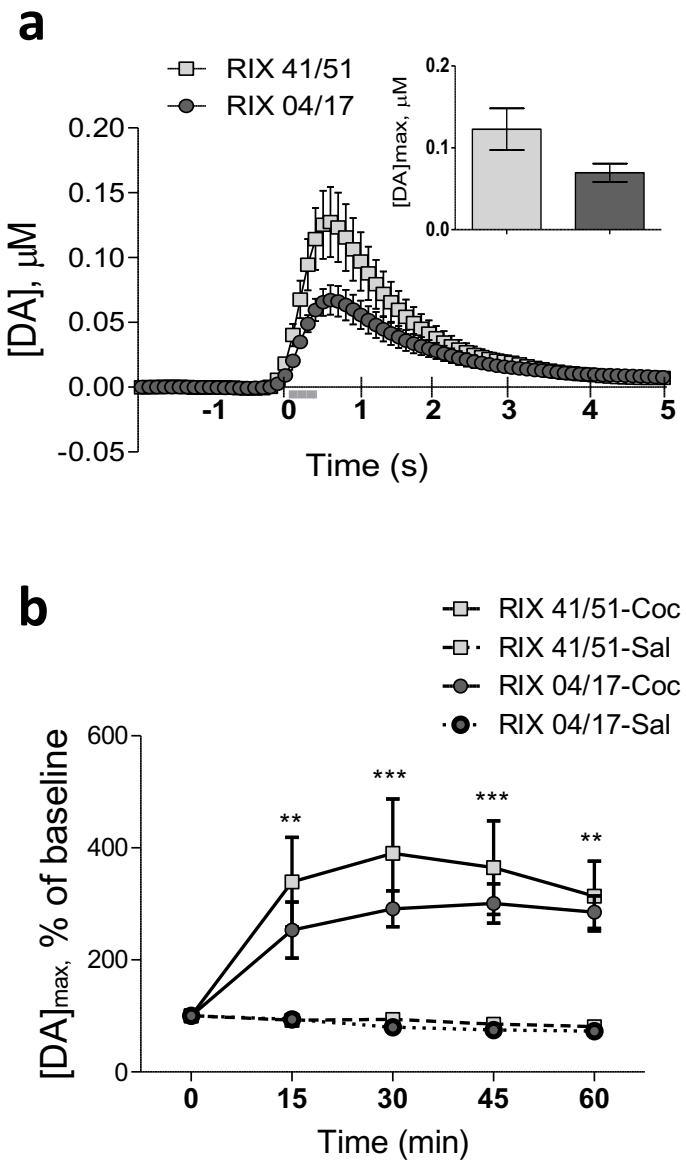
Data are either strain means with SEM error bars. **(a)** Distance post injection of saline (S) or 10 mg/kg cocaine (C) for RIX 41/51 (N=15) and 04/17 (N=16) animals. There was a significant effect of COC exposure day (p=4.0x10^{-6}) and COC exposure day x strain (p=0.018). Comparing consecutive exposures days, RIX 41/51 had a significant increase from Day 5-7 and RIX 04/17 from Day 3-5 and Day 7-9 (within RIX effect, +p<0.05, p<0.01, p<0.001). **(b)** Time spent for RIX 41/51 and 04/17 mice in each of the three chambers (cocaine (COC)-paired, saline (SAL)-paired and middle) before (Pre) or after (Post) training to COC (N=19, 20) or for saline-saline controls (N=17, 18). RIX 41/51 spent significantly more time in the middle chamber during the pre-test session on Day 1 (***p≤3.2x10^{-7}). Both RIX 41/51 and 04/17 mice that were trained with COC spent significantly more time in the COC-paired chamber than the SAL-paired chamber on the post-test session (***p≤3.2x10^{-4}). Within RIX, COC trained animals spent more time in the COC-paired chamber than saline-saline (SAL) control animals (#p<0.015, ###p<0.001).

Figure 3.3. Dysregulation of stress response in RIX 41/51 males



Data are strain means with SEM error bars for RIX 41/51 and RIX 04/17 **(a)** males (N=11,12) and **(b)** females (N=11,12) before and after a 10-min open field (OF) exposure. **(a)** There was a significant time x strain in males (p=1.1x10^{-4}). *Post hoc* analysis in males showed increased CORT at 30 min compared to 0 in both strains ($p \leq 0.001$) but increased CORT at 30 min was lower in RIX 41/51 (***p=0.001, strain effect). Only RIX 04/17 shows a significant decrease in CORT at 120 compared to 30 (p<0.001). **(b)** There was a significant effect of time (p=7.7x10^{-13}) in females but no strain differences.

Figure 3.4. Cocaine enhanced electrically evoked dopamine release in the NAc of RIX 41/51 and RIX 04/17 anesthetized mice



(a) Concentration-versus-time traces of evoked dopamine (DA) release in RIX 41/51 (n=14) and RIX 04/17 (n=12) mice during saline/pre-drug recordings; the stimulation is indicated by the gray line. Inset: $[DA]_{max}$ did not significantly differ between strains, p=0.073. **(b)** Cocaine (COC) increased evoked DA release compared to saline (Sal) administration in both strains. Data are expressed as percent of the baseline, mean ± SEM (n=6-8/group). Collapsed across strain, significantly different from Sal groups, ** p<0.01, *** p<0.001.

CHAPTER 4- GENETIC ANALYSIS OF INITIAL LOCOMOTOR RESPONSE TO COCAINE USING THE COLLABORATIVE CROSS

INTRODUCTION

Approximately two million individuals report current cocaine (COC) use in the United States despite the drug being illegal. Among addictive substances, COC has the highest risk of addiction (Goldstein and Kalant, 1990). In the most recent survey, one million individuals in the United States met the diagnosis criteria for a COC Substance Use Disorder (SUD) (CBHSQ, 2016). Despite the significant personal, societal and financial burden of COC use disorders, there are currently no FDA-approved treatments. Increased knowledge of the underlying mechanisms that lead to COC use disorders are needed to develop effective treatments.

Twin studies indicate that the heritability (h^2) of COC addiction ranges from 0.4 - 0.7 indicating that genetics plays a significant role (Ducci *et al*, 2012; Goldman *et al*, 2005). Approaches to identify genes involved in SUDs include candidate gene and genome-wide association studies (GWAS). Candidate genes studies are hypothesis-driven, based on what is known about a drug (i.e. target system, kinetics, etc) while GWAS are hypothesis-free. A GWAS study for COC dependence found a genome-wide significant association with a single nucleotide polymorphism (SNP) in *FAM53B* (family with sequence similarity 53, member B) on Chr 10 (Gelernter *et al*, 2014). The Chr 10 region was previously identified by linkage analysis in a COC dependence study (Gelernter *et al*, 2005). Despite these few successful genetic associations with SUD, GWAS studies have been limited by insufficient sample sizes that reduce power to

detect the likely numerous genetic variants as that drive COC use and abuse (Goldman *et al*, 2005).

Genome-wide mapping approaches have been employed in rodents and overcome some of the obstacles present in humans. Such studies include mapping of quantitative trait loci (QTL) – genetic loci that contribute to phenotypic variation. In mouse QTL studies, acute psychomotor response to COC is commonly used as a measure of initial sensitivity to a drug. Human studies have shown that initial response to a drug predicts future use and abuse (de Wit and Phillips, 2012). Numerous QTL studies have been conducted using standard recombinant inbred (RI) strains (Boyle and Gill, 2001; Boyle and Gill, 2009; Gill and Boyle, 2003, 2008; Jones *et al*, 1999; Miner and Marley, 1995; Phillips *et al*, 1998; Tolliver *et al*, 1994) or C57BL/6 substrains (Kumar *et al*, 2013) and have identified genomic regions that are associated with initial locomotor response to COC. While these studies identified some overlapping QTL regions, only one genetic variant in the *Cyfip2* gene has been identified and validated for this phenotype (Kumar *et al*, 2013). Identification of genes that influence initial response to COC in QTL studies will further our understanding of its mechanisms and provide candidate genes for analysis in human studies.

In the present study, we utilized a new resource, the Collaborative Cross (CC). The CC was created by intercrossing eight inbred mouse strains including five classical – A/J, C57BL/6J (B6J), 129S1/SvImJ (129), NOD/ShiLtJ (NOD), NZO/HlLt (NZO) and three wild-derived – WSB/EiJ (WSB), CAST/EiJ (CAST), PWK/PhJ (PWK). This set of inbred strains represents the three subspecies of *Mus musculus* (Churchill *et al*, 2004; Srivastava *et al*, 2017) and the diversity allows for novel combinations of alleles and the observation of expanded phenotypic diversity

across the population in comparison to traditional inbred and RI strains (Graham *et al*, 2015; Gralinski *et al*, 2015; Mosedale *et al*, 2017; Rasmussen *et al*, 2014).

In a previous study, we identified two CC F1 hybrid lines (referred to as Recombinant Inbred Intercrosses or RIX), RIX 41/51 and 04/17 that showed very low or high initial locomotor response to COC, respectively (see **Fig 3.1**). Therefore, we wanted to use these two RIX lines to conduct QTL mapping to identify candidate genes that contribute to either low or high initial locomotor response to COC. Since each RIX is an F1 hybrid of two CC strains, we investigated whether the phenotype was due to one or both of the CC parental strains in order to develop an effective mapping strategy. For both RIX 41/51 and 04/17, we identified a single CC parent that displayed a similar COC locomotor phenotype, CC041/TauUnc and CC004/TauUnc respectively. We crossed each of these CC strains to C57BL/6NJ (B6N) to generate two separate F2 populations. QTL mapping using the CC041 X B6N F2 and identified genome-wide significant QTLs on Chrs 7, 11 and 14 for low COC locomotor response. This study is the first to use the CC to perform QTL mapping for an addiction-related phenotype, validates previously identified QTLs and proposes several candidate genes for follow-up analysis.

METHODS AND MATERIALS

Animals

CC004/TauUnc (CC004), CC017/Unc (CC017), CC041/TauUnc (CC041) and CC051/TauUnc (CC051) were purchased from the Systems Genetics Core Facility at the University of North Carolina (UNC; http://csbio.unc.edu/CCstatus/index.py). C57BL/6NJ (B6N) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in a specific pathogen-free facility on a 12-hour light/dark cycle with lights on at 7:00 A.M. All

procedures were approved by the UNC Institutional Animal Care and Use Committee and followed guidelines set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Food and water were available *ad libitum* throughout the experiment. Breeder diet was Harlan Teklad 2919 and post-weaning diet was Harlan Teklad 2920 (Envigo, Frederick, MD, USA).

Drugs

Cocaine HCl (COC; Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% saline and a dose of 20 mg/kg of body weight was used to test initial locomotor response in the 6-hr and 3-day open field (OF) test.

Phenotyping RIX parental strains for initial locomotor sensitivity to COC

RIX 41/51 and 04/17 show divergent initial locomotor response to COC. Each RIX is an F1 hybrid of two CC strains: CC041 and CC051 were crossed to make RIX 41/51 (**Fig 4.1a**) and CC004 and CC017 were crossed to make RIX 04/17 (**Fig 4.2a**). We phenotyped these four CC strains for initial locomotor sensitivity using a 6-hr OF test to determine if one, or both CC parental strains contribute to the phenotype of the RIX. Mice were weighed and placed into a 43.2x43.2x33 cm OF arena (ENV-515-16, Med Associates, St. Albans, VT, USA) and tracked by infrared detectors that surrounded the arena at 2.54 cm intervals on the x, y, and z axes. After 1hr, mice were removed, injected with saline and returned to the OF for 2 hrs and then injected with 20 mg/kg COC and returned to the OF for the remaining 3 hrs. Distance moved (in centimeters) was analyzed in 5-min bins and the sum of the distance moved post-COC injection (180-360 min), referred to as area under the curve (AUC), was used to assess COC-induced

locomotion. Data from the CC strains was compared to data previously collected in RIX 04/17 (N=42) and 41/51 (N=56) (see **Fig 3.1a**).

Statistical analysis

An ANOVA of the effects of strain and sex on AUC was performed using SPSS v24 for Mac OS X 10.6+. Tukey's *post hoc* was used to follow-up on any significant main effects of strain. Graphs were generated using Graphpad Prism 7.0c for Mac OS X.

F2 mapping population

Two separate F2 populations were created. Low responding CC041 animals were reciprocally bred to B6N to generate CC041xB6N and B6NxCC041 F1s. These F1s were crossed in all combinations (CC041xB6NXCC041xB6N, CC041xB6NXB6NxCC041, B6NxCC041X CC041xB6N, B6NxCC041XB6NxCC041) to generate an F2 population (**Fig 4.1c**). High COC responding CC004 animals were bred to B6N animals in the same fashion to generate a CC004xB6N F2 population (**Fig 4.2c**).

Phenotyping F2 populations for initial locomotor sensitivity to COC

Table 4.1 provides the number of mice tested in each mapping population. F2 animals were tested using a high-throughput 3-day test in the OF arena. On Day 1 (habituation) and Day 2 (baseline) animals were injected with saline before being placed into the OF arena for 30 mins. On Day 3, animals were injected with 20 mg/kg COC before being placed into the OF for 30 mins. Distanced moved was recorded in 2-min bins for the entire test. Total distance moved on

Day 3-Day 2 was used to assess COC-induced locomotion. All data was transformed using a two-step inverse rank transformation to normality in SPSS.

Genotyping

Genotyping was performed for the CC041 X B6N F2 population. Mice were euthanized immediately following testing on Day 3 and DNA was extracted from tail tissue from CC041, B6N, F1 breeders and the F2 population using the DNeasy Blood & Tissue kit (Qiagen). Genotypes were determined using the Mouse Universal Genotyping Array (MUGA) that consists of 7851 SNP markers on an Illumina Infinium platform that are distributed throughout the genome (average spacing of 325 kb) and were chosen to be maximally informative and for the eight founder strains of the CC (Morgan *et al*, 2015). Nucleotide genotypes were processed and converted to haplotype calls (i.e. B6N, CC, or HET) for use in QTL mapping using the *argyle* package (version 0.2.0) in R Studio (Morgan, 2015). A series of genotype checks were performed and markers were eliminated for any of the following reasons: they were not informative between B6N and CC041, did not meet the Chi-square distribution of expected genotypes for an intercross population (showed segregation distortion) or had a large number of missing calls (>40). This left 2701 markers with an average spacing of 0.5 cM and maximum gap of 8.8 cM, ample coverage for an F2 population.

QTL mapping

QTL mapping was performed using the *qtl* package (version 1.40-8; (Broman, 2014; Broman *et al*, 2003) in R Studio (version 1.0.136). Single scan QTL analyses using the *scanone* function were performed for transformed total distance data for Day 1, Day 2 and Day 3 and D3-D2

distance. Only the autosomes were assessed as there was no difference in response between males with either B6N or CC041 mothers. Sex and F2 breeding cross direction were included as covariates. A Haley-knot regression approximation model of interval mapping was used based on the density of the genotyping array and the amount of recombination present in the F2 intercross. Genome-wide significant thresholds for logarithm of the odds (LOD) scores (measure of genotype to phenotype association) were determined using 1000 permutations. For each QTL peak identified, the 1.5 LOD support interval was used and it thought to reflect the 95% confidence interval, or that the true QTL lies within this region 95% of the time (Dupuis and Siegmund, 1999). The MUGA markers closest to the outer limits of the 1.5 LOD interval was identified and used as the megabase (Mb) locations flanking the region (mm10, GRCm38). Genotype data provided by Srivastava *et al* (2017) was used to determine the CC parental strain haplotype in the 1.5 LOD intervals for each QTL. Using the *fitqtl* function and an equation assuming an additive model, $y = QTL\ 1 + QTL\ 2 + QTL\ 3$, the amount of variance explained for the QTL peaks of a given phenotype was estimated.

Two scan QTL analysis using the *scantwo* function in *R/qtl* was performed for the significant QTLs identified for each of the measures with sex and cross direction as covariates. Both full (LOD_{fv1}) and additive models (LOD_{av1}), allowing for the possibility of epistasis or not, respectively, were fit by comparing pairs of loci on the two chromosomes to the single-QTL model. LOD_i indicates evidence for an interaction of the two loci by comparing the fit of the two models (LOD_{fv1} - LOD_{av1}). LOD thresholds of 6, 4, 3 for LOD_{fv1}, LOD_i, LOD_{av1} were used to determine a significant pair of QTLs (Gary Churchill, personal communication). Effect plots at each QTL peak and for significantly interacting markers were generated using the *effectplot* function.

Prioritizing candidate genes in QTL regions

To prioritize genes in the QTL intervals as potential candidates we used the process outlined in **Fig 4.6**. Using the BioMart tool in Ensemble (http://www.ensembl.org/biomart/) we identified protein-coding, micro RNAs (miRNA) and long non-coding RNAs (lncRNA). We then used the Mouse Phylogeny Viewer tool (http://msub.csbio.unc.edu/, Wang *et al* (2012)) to identify regions that were identical-by-descent (IBD) between B6J and the haplotype of CC041 and eliminated any genes in those regions. Using multiple resources (https://tissues.jensenlab.org/Search; http://www.informatics.jax.org/; http://biogps.org/#goto=welcome; http://amp.pharm.mssm.edu/Enrichr/#find; https://www.ebi.ac.uk/gxa/home) we eliminated any genes not expressed in the brain. From this list, we prioritized any genes that contained non-synonymous SNPs or insertions/deletions (indels) between B6J and the CC parent haplotype in the QTL region (i.e. NOD). We used the Sanger Institute SNP Viewer tool (http://www.sanger.ac.uk/sanger/Mouse_SnpViewer/rel-1505) and limited the types of SNPs to those that were introduced or inserted a stop or were present in coding regions, regulatory regions, splice regions or induced missense and frameshift variants. From this list, we prioritized genes that met all three of the following criteria: 1) genes with previously-identified phenotypes that affect brain morphology or function (i.e. axon development, brain size, dendrite morphology, etc.), were identified as a GWAS hit for a psychiatric disorder or were linked with relevant behavioral phenotypes (i.e. abnormal locomotion, psychostimulant response, etc.); 2) genes that overlap with previously-identified QTL regions for initial COC locomotor response; 3) genes that show differential gene expression

in response to acute or chronic COC (using www.GeneWeaver.org and the search term 'cocaine').

RESULTS

Initial locomotor sensitivity to COC is driven by one CC parental strain for both RIX lines

We assessed initial locomotor sensitivity to COC using the 6-hr OF test in all four CC strains that were used to produce the RIX lines. For RIX41/51, the low COC response line, there was a significant effect of strain ($F_{(2,96)}$=24.8; p=2.5x10^{-9}) with *post hoc* tests showing CC041 < RIX41/51 < CC051 ($p \leq 0.016$; **Fig 4.1b**). For RIX04/17, the high COC response line, there was a significant effect of strain ($F_{(2,78)}$=8.0; p=0.001) with *post hoc* tests showing RIX 04/17 = CC004 > CC017 ($p \leq 0.002$; **Fig 4.2b**). There were no effects of sex (p>0.05). The data for RIX41/51 and RIX04/17 were previously shown in **Fig 3.1a**. These data indicate that only one of the CC parental strains showed a similar phenotype for initial locomotor sensitivity to COC for both RIX 41/51 and 04/17.

Low locomotor response to COC in CC041 is dominant

CC041, CC004, B6N, F1s and F2s were phenotyped in a 3-day OF test. For the low COC response population, the F1s showed similar response to CC041, indicating that this phenotype is dominant. The F2 population showed a wider range of responses, although there was a large proportion at the lower end of the phenotypic distribution (**Fig 4.1d**). For the high COC response population, there was a range of response in the F2 population, although very few mice showed the extreme response seen in CC004 (**Fig 4.2d**). Due to the dominant nature of the low COC

response, we chose to conduct QTL analysis in this population as we believed we would have greater power to detect causative loci than in the high responding population.

QTL on Chr 7, 11 and 14 for low initial locomotor response to COC

QTLs that passed the p<0.1 genome-wide LOD threshold based on 1000 permutations are shown in **Table 4.2**. We identified 3 significant QTL for locomotor response to COC (Day3-Day2 distance) on Chr 7, 11 and 14 (**Fig 4.3a**). The QTL on Chr 7 had a peak at 17.2cM or 29.74Mb (LOD=4.21, *p*=0.023) that accounted for 4.3% of the variance. The effect plot at this peak showed that both the CC041/CC041 and HET genotypes were driving the low COC response (**Fig 4.3b**), indicating a dominant phenotypic effect at this locus similar to that observed in the parental and F1 populations (see **Fig 4.1d**). The LOD interval (27.5-40.3 Mb) of the Chr 7 peak is 12.8 Mb and contains 203 protein-coding genes. CC041 has a NOD haplotype that transitions to NZO at approximately 36.05Mb in this interval. The QTL on Chr 11 had a peak at 14.3cM or 20.98Mb (LOD=6.54, *p*=0.001) and accounted for 6.1% of the variance. The effect plot at this peak showed that the B6N/B6N genotype was driving the low COC response (**Fig 4.3c**) indicating that this is a transgressive QTL (see **Fig 4.1d**). The LOD interval (12.6-37.7Mb) of the Chr 11 peak is 25.1 Mb and contains 106 protein-coding genes. CC041 has a NOD haplotype that transitions to a WSB haplotype at approximately 36.8 Mb. The Chr 14 QTL had a peak at 42.7cM or 79.67Mb (LOD=3.88, *p*=0.051) that accounted for 4% of the variance. The effect plot at this peak showed that the CC041/CC041 genotype was driving the low COC response (**Fig 4.3d**). The LOD interval (9.1-97.4) on Chr 14 was very large spanning most of the chromosome (88.3 Mb) due to the appearance of multiple peaks towards the proximal end (see

Fig 4.3a), therefore we did not perform follow-up analysis of potential candidate genes in this region.

Single QTL analysis of Day 1, Day 2 and Day 3 distance revealed several overlapping regions (**Table 4.1, Fig 4.4**). Interestingly, a peak on Chr 11 was identified for all days in similar locations to the peak identified for COC locomotor response (peaks at 30.88 or 35.7 Mb compared to 20.9 Mb) and in every case, the B6N/B6N genotype was associated with the low response. A QTL peak on Chr 7 was identified for Day 2 and Day 3 distance with the Day 3 peak being the same as the one identified for Day 3-Day 2 and in every case, the CC041/CC041 and CC041/B6N genotypes was associated with low response. A QTL peak on Chr 14 was identified for Day 1 and Day 3 with the Day 3 peak being the same as the one identified for Day 3-Day 2 and in all cases only the CC041/CC041 genotype showed the low response. Two separate QTLs on Chr 6 were specific to non-COC exposures on Day 1 (peak at 97.0 Mb) and Day 2 (peak at 37.0 Mb). For both Chr 6 QTL, the CC041/CC041 genotype was associated with low locomotor response. A QTL on Chr 2 (peak at 152.7 Mb) was unique for the Day 3 distance phenotype with the CC041/CC041 genotype driving the low response.

Collectively, the single scan QTL analysis in the low responding population indicates that QTL identified are associated with locomotor phenotypes on multiple days in the 3-day OF test, a finding that could be due to the significant correlation among the phenotypes (**Table 4.3**). Pearson correlation showed that Day 1, Day 2, Day 3 and Day 3-Day 2 distance were all significantly correlated ($r(6)=0.31\text{-}0.99$; $p \leq 1 \times 10^{-15}$). The strongest correlation was observed between Day 3 distance and D3-D2 distance ($r(6)=0.99$; $p \leq 1 \times 10^{-15}$).

Two-locus analysis shows additive effect of QTLs for low COC response

Most complex traits, such as initial drug sensitivity, are thought to be due to the action of multiple genetic loci, some of which may act in concert to affect the phenotype. Therefore, we performed a two-locus analysis to determine if the QTLs identified in the single scan are linked and if so, in what manner (i.e. additive or epistasis). For each phenotype, only the chromosomes with significant QTLs in the single scan were analyzed. **Table 4.4** shows all loci that met the threshold in either a full model (allows for possibility of epistasis; $LOD_{fv1} \geq 6.0$) or additive model (assumed no epistasis; $LOD_{av1} \geq 3.0$).

For Day3-Day2, we found strong evidence for an interaction among a pair of QTL at the same location identified in the single scan analyses on Chrs 7 and 11 at 29.74 and 20.98 Mb, respectively. The presence of a homozygous B6N genotype on Chr 11 results in low response to COC independent of their genotype on Chr 7 (**Fig 4.5a**). There was also evidence for an interaction between QTL on Chrs 7 and 14 at 29.74 and 79.67 Mb, respectively (**Fig 4.5b**). There was also evidence for interaction between a pair of QTL on Chrs 11 and 14 at 20.98 and 79.67 Mb, respectively (**Fig 4.5c**). Collectively, the findings presented here and in Table 4 support the idea that the QTLs identified in the single scan analysis are linked and act in a predominantly additive manner to contribute to the low locomotor response to COC seen in CC041.

Prioritization of interesting candidate genes at Chr 7 and 11 QTL regions

The strategy outlined in **Fig 4.6** was used to identify potential candidate genes for COC locomotor response at the QTL regions on Chr 7 and 11. The 12.8 Mb QTL region on Chr 7 contained 203 protein-coding genes. 63 of these genes were in regions of IBD between B6N and the CC parental strain (NOD or NZO) and were eliminated from our priority list of candidates.

124 genes in the interval are expressed in the brain. From those 124, we prioritized the 54 that had a deleterious SNP or indel present. From this list, we then identified genes that met all three of the criteria described above. Five genes met all criteria: *Chst8, Pepd, Slc7a10, Ankrd27,* and *Dpy19l3* are discussed below as potential candidates.

The Chr 11 QTL interval contained 106 protein-coding genes, all of which were expressed in the brain. 45 genes contained either a nonsynonymous SNP or indel. Using the same criteria described above, we identified six genes that met our three criteria for inclusion as candidates: *Xpo1, Ccdc88a, Rtn4, Hba-a1, Slit3, and Rars*.

DISCUSSION

In this study, we used an F2 intercross of a traditional inbred mouse strain (B6N) and a CC strain (CC041) to map three QTLs on Chr 7, 11 and 14 that are involved in locomotor response to COC. The association between genotype and phenotype on Chr 7 best matches the mode of inheritance observed in the analyses of the parental lines, F1 and F2 populations. Interestingly, at the Chr 11 region, we made the observation that the B6N genotype was responsible for low COC response. All three QTL are acting in an additive manner, although there is also evidence for an epistatic interaction of allelic status at Chr11 with both the Chr 7 and 14 loci. Using a bioinformatics approach, we identified potential candidate genes in the Chr 7 and Chr 11 regions that could be mediating low COC locomotor response. Below we compare our QTL regions to those previously identified and discuss potential candidate genes.

Previous QTL studies for initial COC locomotor response

Several previous studies have performed QTL mapping for initial locomotor response to COC, mostly using a panel of RI strains (Boyle *et al*, 2001; Boyle *et al*, 2009; Gill *et al*, 2003, 2008; Jones *et al*, 1999; Miner *et al*, 1995; Phillips *et al*, 1998; Tolliver *et al*, 1994). Three studies assessing initial COC locomotor response identified regions that overlap with our QTL regions on Chr 7 and 11. A study by Gill *et al* (2003) used males and females from 36 strains of AcB/BcA which are congenic strains with small regions of B6J (B) introgressed on an A/J (A) background or small regions of A/J introgressed on a B6J background. This study identified several QTLs associated with initial COC locomotor response including one on Chr 7 (peak at 26.5 cM or 51.7 Mb) for which increased COC response was driven by the B6J donor allele. This study did not include a QTL interval since associations of genotype and phenotype were done by correlation rather than interval mapping. However, using a 25 Mb region around the peak at 51.7 Mb gives us a QTL region of 33.2-76.9 Mb which overlaps with the proximal end of our Chr 7 region. Tolliver *et al* (1994) used males from 16 BXD strains and identified multiple significant loci including one on Chr 11 (peak at 5 cM or 12.3 Mb) where the B6J genotype was associated with higher COC response than DBA genotype. QTL intervals were not used at this time either, but using the 25 Mb region around the peak gives us a QTL region of 0-33.4 Mb which overlaps with our Chr 11 region. Another study by Jones *et al* (1999) used males and females from 26 BXD strains to assess initial COC locomotor response, COC-related behaviors (center time, nosepokes, repeated movements) and D1R, D2R and DAT densities in the brain. They identified several QTL for COC-related behaviors where the B6J genotype was associated with increased COC response on Chr 11 (peaks 2-11 cM or 12.3-19.7 Mb), which overlaps with our Chr 11 region and the region observed by Tolliver *et al* (1994).

Two additional studies identified significant QTL in close proximity to ours. A study by Kumar *et al* (2013) used an F2 intercross of two B6 substrains, B6N and B6J, and identified a QTL peak on Chr 11 (1.5 LOD interval 35-57 Mb). Within this region, they identified a variant in the *Cyfip2* gene as the causative SNP for the low locomotor response to COC in B6N mice. While *Cyfip2* does not fall within the 1.5 or 2.0 LOD interval of our Chr 11 QTL, we cannot rule out the possibility that the *Cyfip2* gene is causing the B6N-driven low COC response we observed. The B6N variant of *Cyfip2* has also been identified as a causative gene in a QTL mapping study of binge-eating behavior in mice (Kirkpatrick *et al*, 2017) indicating that *Cyfip2* may be modulating a phenotype associated with multiple psychiatric disorders (i.e., behavioral disinhibition).

A study by Dickson *et al* (2016) performed QTL mapping for intravenous self-administration (IVSA) of COC using males and females from 44 BXD strains. While IVSA behaviors are not the same as initial locomotor response, it was interesting that the two QTL regions they identified for IVSA dose response curves were on Chr 7 (30.4-30.6 Mb) which is within our region and Chr 11 (46.18-50.57 Mb) which is slightly outside our region but does contain *Cyfip2* as candidate gene. For both QTL, the B6J genotype showing higher and more consistent COC infusions at lower doses of COC compared to animals with the DBA genotype. They also performed a eQTL using gene expression from midbrain tissue and found that *Fam53b* (located on Chr 7 at 139.9 Mb) had a trans-eQTL that overlapped with the Chr 11 QTL region. *FAM53B* was previously identified as a candidate in a human GWAS for COC dependence (Gelernter *et al*, 2014). Furthermore, they showed a correlation between the expression of *Fam53b* and *Cyfip2*, indicating a relationship between these two genes on Chr 7 and 11 for COC-related behavior.

Potential candidate genes controlling locomotor response to COC

We wanted to prioritize potential candidate genes within each of the QTL regions. Previous studies have used a variety of techniques to prioritize candidate genes including pre-existing knowledge about the function of genes in relation to the drug's effects (i.e. genes involved in the dopaminergic system), genes with SNPs between the two parental strains of the mapping cross, and correlation with gene expression changes. We employed a variety of these and additional tools to propose five potential candidate genes in the Chr 7 region (*Chst8, Pepd, Slc7a10, Ankrd27, Dpy19l3*) and six potential candidate genes in the Chr 11 region (*Xpo1, Ccdc88a, Rtn4, Hba-a1, Slit3, and Rars*). We briefly discuss why we believe each gene is a potential candidate and might warrant further investigation.

Chst8 (carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 8) has been shown to be differentially expressed in the hippocampus (Hipp) and prefrontal cortex (PFC) of mice exposed to chronic COC and in postmortem tissue from COC addicts (Farris *et al*, 2015; Krasnova *et al*, 2008). A SNP in *Chst8* was also identified in a human GWAS for antipsychotic regulation of working memory in schizophrenia patients (McClay *et al*, 2011). Additionally, *Chst8* knockout (KO) mice show overall hypoactivity, possibly indicating an alteration in the underlying circuity for activity which includes dopamine (MGI:1916197). Collectively, *Chst8* is a good potential candidate due to its differential expression after COC exposure in brain tissue from mice and humans, its association with the mechanisms of antipsychotic drugs and its effect on overall activity in mice.

Pepd (peptidase D) plays a key role in recycling of the amino acid proline. *Pepd* is a potential candidate gene as chronic COC exposure can alter its gene expression in the brain (Krasnova *et al*, 2008) and mice lacking *Pepd* show abnormal brain morphology (MGI:2683289).

Slc7a10 (solute carrier family 7, cationic amino acid transporter, y+ system member 10) is a transmembrane transporter for small neutral amino acids. *Slc7a10* has been shown to be differentially expressed in the brain after chronic exposure to COC (Krasnova *et al*, 2008) and *Slc7a10* KO mice show multiple neuro-related abnormalities including overall hypoactivity, seizures, ataxia, spontaneous tremors, and abnormal synaptic transmission (Xie *et al*, 2005). Collectively, this indicates that *Slc7a10* has an effect on multiple movement-related phenotypes and therefore is a potential candidate for follow-up analysis.

Ankrd27 (ankyrin repeat domain 27) serves as a guanine-exchange factor (GEF) that acts on the GTPase, Rab21, to influence neurite outgrowth (Burgo *et al*, 2009). *Ankrd27* has been shown to be epigenetically dysregulated in Hipp tissue from COC addicts (Farris *et al*, 2015). *Ankrd27* KO mice display abnormal motor capabilities, coordination, and movements (MGI:211773). Due to the widespread action of GEFs within the brain, previous connections to movement-related phenotypes and evidence from human studies of COC addicts, *Ankrd27* is a potential candidate gene for follow-up studies.

Dpy1913 (dpy-19-like 3) was selected as a potential candidate gene due to previous identification in a human GWAS for a psychiatric disorder, Bipolar Disorder, and as a gene that is epigenetically dysregulated in the NAc after exposure to chronic COC in mice (Renthal *et al*, 2007; Smith *et al*, 2009). Additionally, there is evidence that *Dpy1913* is involved in development of glutamatergic neurons and glutamate has been shown to play a role in addiction (Koob *et al*, 2010; Watanabe *et al*, 2011).

Xpo1 (exportin 1) is a nuclear export protein that is a popular target for cancer treatments as it has wide-spread actions within the body. In humans, it is known to be involved in the 2p15p16.1 microdeletion syndrome that presents with many autistic features. The XPO1 protein was identified by meta-analysis to be associated with psychiatric disorders (Bagheri *et al*, 2016; Zhao *et al*, 2016). *Xpo1* has also been identified as differentially regulated by chronic exposure to COC in the PFC, Hipp and NAc of mice (Krasnova *et al*, 2008; Renthal *et al*, 2009).

Ccdc88a (coiled coil domain containing 88A) was identified as a potential candidate gene for multiple reasons. KO mice display multiple neuro-related phenotypes including hypoactivity, impaired learning and memory, impaired sensorimotor gating and abnormal dendritic spine morphology and synaptic transmission (Nakai *et al*, 2014). In humans, *Ccdc88a* is involved in PEHO syndrome in which patients present with severe mental retardation and epileptic seizures (Nahorski *et al*, 2016). *Ccdc88a* has also been shown to be downregulated by CREB, a key transcription factor in the rewarding effects of repeated drug exposure (McClung and Nestler, 2003).

Rtn4 (reticulon 4) was selected as a potential candidate gene due to its known involvement in axonal outgrowth, cell migration and synaptic plasticity (Su *et al*, 2008; Sui *et al*, 2015). Additionally, *Rtn4* was shown to be differentially expressed in mice exposed to chronic COC (Krasnova *et al*, 2008; Renthal *et al*, 2007) and epigenetically dysregulated in the Hipp of COC addicts (Farris *et al*, 2015).

Hba-a1 (hemoglobin alpha, adult chain 1) is a potential candidate gene due to studies that shown that its gene expression can be regulated by exposure to stress (Stankiewicz *et al*, 2015; Stankiewicz *et al*, 2014), which is thought to play a key role in the development and maintenance

of SUDs (Koob, 2008). Additionally, *Hba-a1* expression is dysregulated in the NAc of mice exposed to chronic COC (Feng *et al*, 2014).

Slit3 (slit homolog 3) was selected as a candidate gene due to its known role in axon guidance during brain development. A SNP in *Slit3* was also identified in GWA studies for psychiatric disorders, including MDD and Schizophrenia, further supporting its role in the brain (Glessner *et al*, 2010; Shi *et al*, 2004). *Slit3* was also identified as epigenetically dysregulated in the NAc of mice exposed to chronic COC (Renthal *et al*, 2007).

Rars (arginyl-tRNA synthetase) is a key player in myelination and is known to be involved in human disorders of hypomyelination (Wolf *et al*, 2014). *Rars* has been shown to be differentially regulated in the Hipp and PFC of mice exposed to chronic COC and in the Hipp of COC addicts and alcoholics (Farris *et al*, 2015; Krasnova *et al*, 2008). Due to the critical role of myelination in synaptic transmission and it differential expression by COC, we prioritized *Rars* as a candidate gene for follow-up studies.

Reflections on the generation of the QTL mapping population

One unexpected finding was the large QTL on Chr 11 in which the low response to COC was driven by the B6N allele and not the CC041 allele. This is surprising given the data the CC041 mice show lower COC response than B6N (see **Fig 4.1d**). We chose to cross CC041 to B6N as it is closely related genetically to B6J, which is one of the eight founder strains of the CC and therefore, would not introduce an entirely new genetic background in with the already genetically diverse background of the CC strain. However, investigation of the founder contribution in CC041 revealed that there was only 0.05% B6J present. Consequently, we did introduce an entirely new genetic background including allelic combinations that would not have

been present in this particular CC strain, making the transgressive nature of the Chr 11 QTL less surprising. Identification of causative variants in the Chr 11 region are clearly contributing to low response to COC, however this variant is likely not responsible for the phenotypic effect observed in CC041. Future studies are needed to investigate the Chr 11 locus to determine if it is *Cyfip2* or something regulating (i.e. gene expression) or physically linked to *Cyfip2*, and how that gene is interacting with the loci on Chr 7 and 14. Additionally, studies are needed to narrow the identified QTL regions to reduce the number of potential candidate genes and/or validate the potential candidate genes that we prioritized.

Table 4.1: Number of mice phenotyped in COC mapping populations

Population	Generation	Cross	# F	# M	Total
Cocaine low response	F0	CC041/TauUnc (CC041)	12	12	24
		C57BL/6NJ (B6N)*	11	13	24
	F1	B6NxCC041	13	13	26
		CC041xB6N	11	12	23
					49
	F2	B6NxCC041 X B6NxCC041	58	62	120
		CC041xB6N X CC041xB6N	59	53	112
		CC041xB6N X B6NxCC041	66	63	129
		B6NxCC041 X CC041xB6N	37	50	87
					448
Cocaine high response	F0	CC004/TauUnc (CC004)	11	14	25
		C57BL/6NJ (B6N)*	11	13	24
	F1	B6NxCC004	9	8	17
		CC004xB6N	9	10	19
					36
	F2	B6NxCC004 X B6NxCC004	38	39	77
		CC004xB6N X B6NxCC004	38	39	77
		CC004xB6N X CC004xB6N	38	40	78
		B6NxCC004 X CC004xB6N	39	38	77
					309

*Same animals

Table 4.2. QTL regions identified in the 3-day open field test

	Chr	Position (cM)	Position (Mb)	Marker Name	LOD	*p* value	1.5 LOD Interval (Mb)	Interval Size (Mb)	Decreasing Allele	CC Parent Haplotype
Day 1 Distance	6	44.9	97.01	backupUNC060149691	6.39	0.001	51.5-111.7	60.2	CC041	CC
	11	21.2	35.74	UNC111423133	4.03	0.033	18.0-70.8	52.8	B6N	DD/HH
	14	43.5	84.63	JAX00385742	6.66	0.001	73.3-94.6	21.3	CC041	FF
Day 2 Distance	6	15.9	37.09	backupUNC060073738	5.45	0.001	21.9-111.7	89.8	CC041	CC
	7	16.9	28.90	UNC070790469	3.79	0.061	4.0-41.5	37.5	CC041	DD/EE/DD
	11	21.2	35.74	UNC111423133	5.12	0.002	21.7-51.5	29.8	B6N	DD/HH
Day 3 Distance	2	75.3	152.70	UNC020230950	3.71	0.075	141.5-180.8	39.3	CC041	HH/CC/EE
	7	17.2	29.74	UNC070628845	6.28	0.001	27.5-37.9	10.4	CC041	DD/EE
	11	18.2	30.88	backupJAX00025840	9.31	0.000	18.0-38.5	20.5	B6N	DD/HH
	14	42.7	79.67	backupUNC140618860	4.34	0.015	12.6-97.1	84.5	CC041	DD/CC/FF
D3-D2 Distance	7	17.2	29.74	UNC070628845	4.21	0.023	27.5-40.3	12.8	CC041	DD/EE
	11	14.3	20.98	UNC111412965	6.54	0.001	12.6-37.7	25.1	B6N	DDHH
	14	42.7	79.67	backupUNC140618860	3.88	0.051	9.1-97.4	88.3	CC041	DD/CC/FF

All QTL regions above the suggestive genome-wide threshold (p=0.01) using 1000 permutation for phenotypes in the 3-day open field test in the low-cocaine responding F2 population. Decreasing allele indicates the genotype associated with the lowest phenotype (CC041 = CC041/TauUnc; B6N = C57BL/6NJ). Collaborative Cross (CC) parental strain haplotype in QTL region is indicated by CC (129S1/SvlmJ), DD (NOD/ShiLtJ), EE (NZO/HlLtJ), FF (CAST/EiJ), HH (WSB/EiJ). Chr = chromosome; cM = centimorgan; Mb = megabase position in mm10 mouse genome

Table 4.3: Correlation of locomotor behavior in the 3-day open field test for the low COC response F2 population

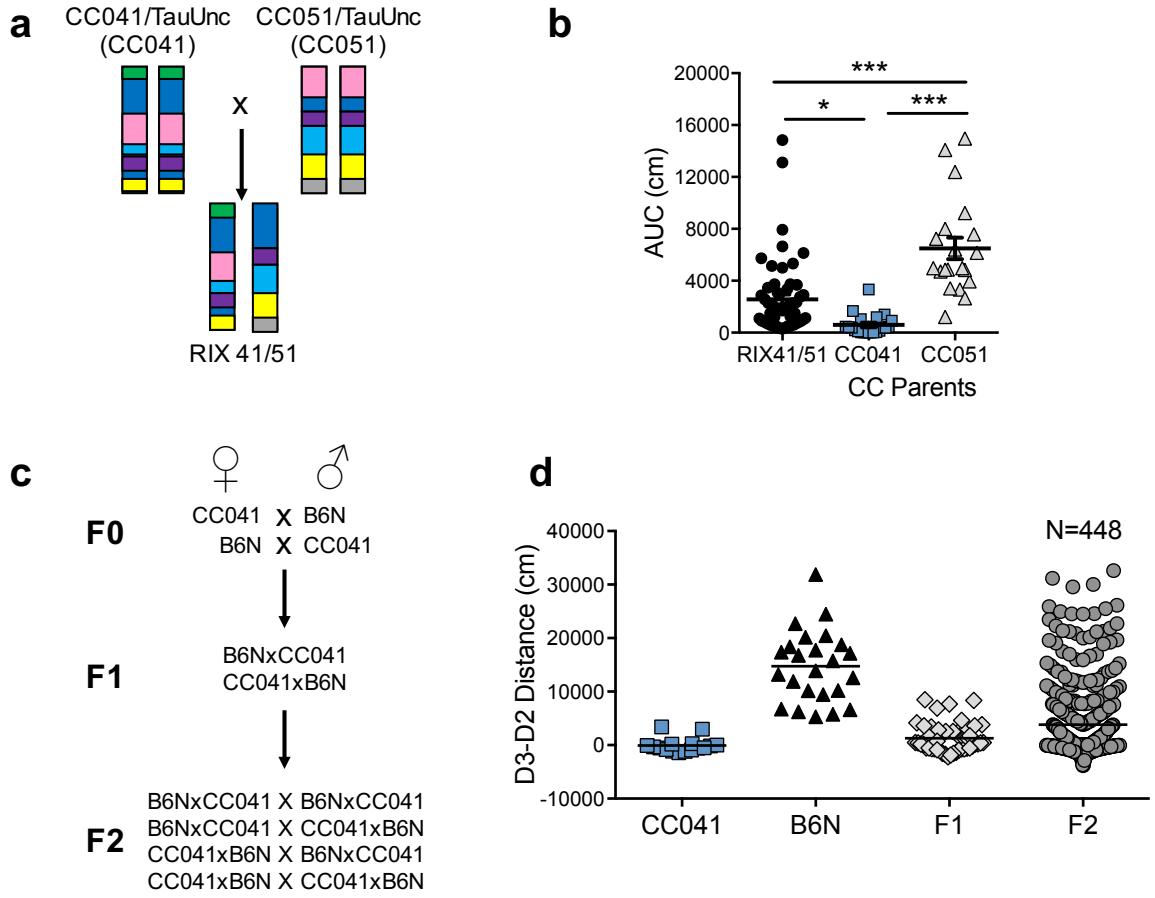
Phenotypes		**r**	***p***
Day 1	Day 2	0.764	$7.2x10^{-87}$
Day 1	Day 3	0.424	$5.1x10^{-21}$
Day 2	Day 3	0.518	$4.0x10^{-32}$
Day 1	Day 3-Day 2	0.311	$1.7x10^{-11}$
Day 2	Day 3-Day 2	0.366	$1.2x10^{-15}$
Day 3	Day 3-Day 2	0.986	0.00E+00

Table 4.4. Two scan QTL results for the low COC response population

		Full Model (allows for interaction)				**Additive Model** (no interaction allowed)		
	Chr(Pos1:Pos2)	Position 1 (Mb)	Position 2 (Mb)	LOD_{fv1}	LOD_i	Position 1 (Mb)	Position 2 (Mb)	LOD_{av1}
	Threshold			6.0	4.0			3.0
Day 1 Distance	Chr6:Chr11	71.06	31.50	6.4	2.8	97.01	35.74	3.6
	Chr6:Chr14	71.06	84.63$^+$	8.0	1.6	85.82	84.63$^+$	6.4
	Chr11:Chr14	36.50$^+$	84.63$^+$	4.8	0.8	36.50$^+$	84.63$^+$	4.0
Day 2 Distance	Chr6:Chr7	105.06	39.67	4.3	0.6	37.09	28.90	3.7
	Chr6:Chr11	37.09$^+$	35.74$^+$	6.4	1.6	37.09$^+$	35.74$^+$	4.8
	Chr7:Chr11	28.90$^+$	35.74$^+$	4.7	0.4	28.90$^+$	35.74$^+$	4.2
Day 3 Distance	Chr2:Chr7	164.19	29.74$^+$	5.5	1.2	152.70	29.74$^+$	4.2
	Chr2:Chr11	171.55	29.64	6.0	1.7	152.70	30.88	4.3
	Chr2:Chr14	152.70	78.22	4.7	0.3	163.21	79.67	4.3
	Chr7:Chr11	29.74$^+$	20.98	7.9	1.0	29.74$^+$	35.73	6.9
	Chr7:Chr14	29.74$^+$	79.67$^+$	5.5	0.4	29.74$^+$	79.67$^+$	5.1
	Chr11:Chr14	29.64	81.00	6.6	2.0	20.98	78.22	4.6
Day 3-Day 2 Distance	Chr7:Chr11	29.74$^+$	20.98$^+$	6.1	1.8	29.74$^+$	20.98$^+$	4.3
	Chr7:Chr14	36.76	26.48	4.7	0.3	29.74	79.67	4.4
	Chr11:Chr14	20.98$^+$	72.60	5.8	1.7	20.98$^+$	79.67	4.0

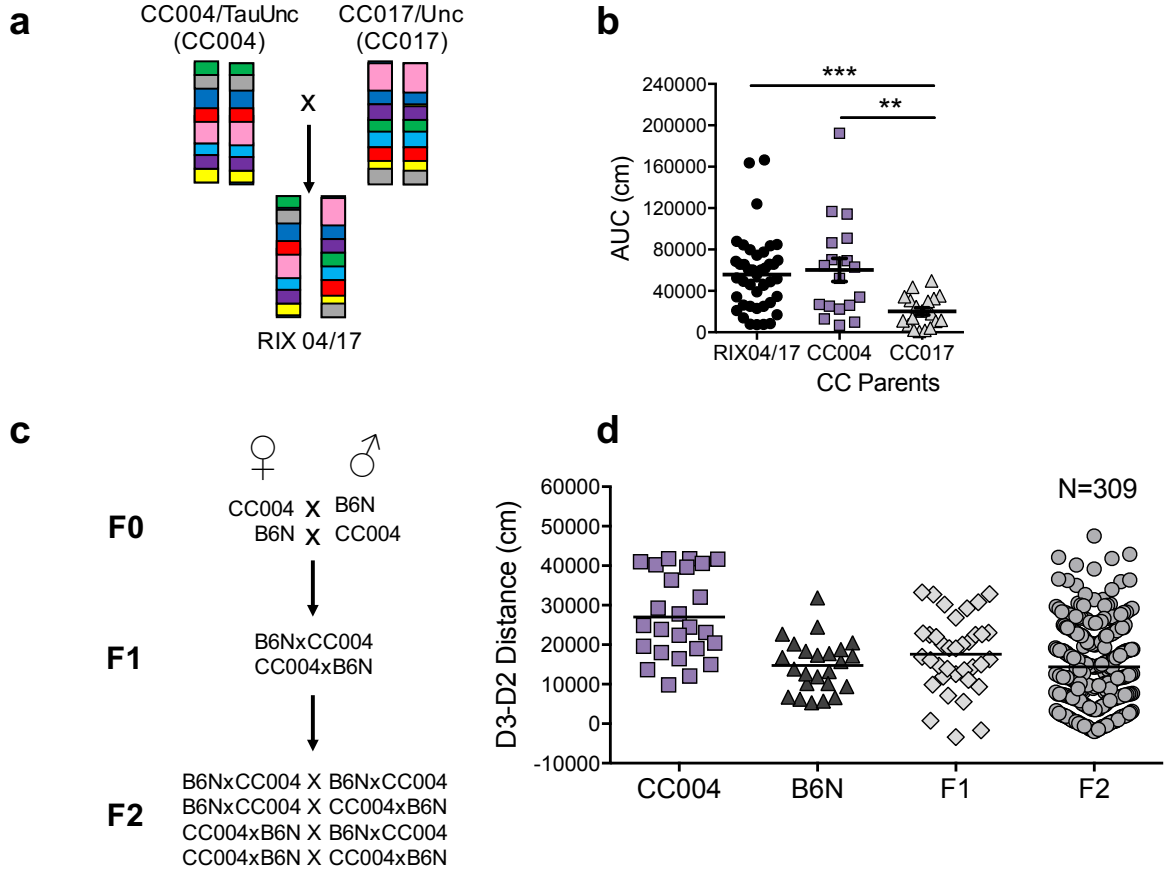
Gray shading indicates LOD scores that passed a lenient significance threshold. LOD_{fv1} = comparing full model with QTL on the two chromosomes (ChrPos1:Pos2) to the single-QTL model, indicates evidence for a second QTL, allowing for the possibility of epistasis; LOD_{av1} = comparing additive model with QTL on the two chromosomes (ChrPos1:Pos2) to the single-QTL model, indicates evidence for a second QTL, assuming no epistasis. LOD_i = improvement in fit of full model over additive model (LOD_{fv1} - LOD_{av1}), indicates evidence for interaction; $^+$indicates same Chr position for full and additive model

Figure 4.1. Low initial locomotor response to cocaine in CC041



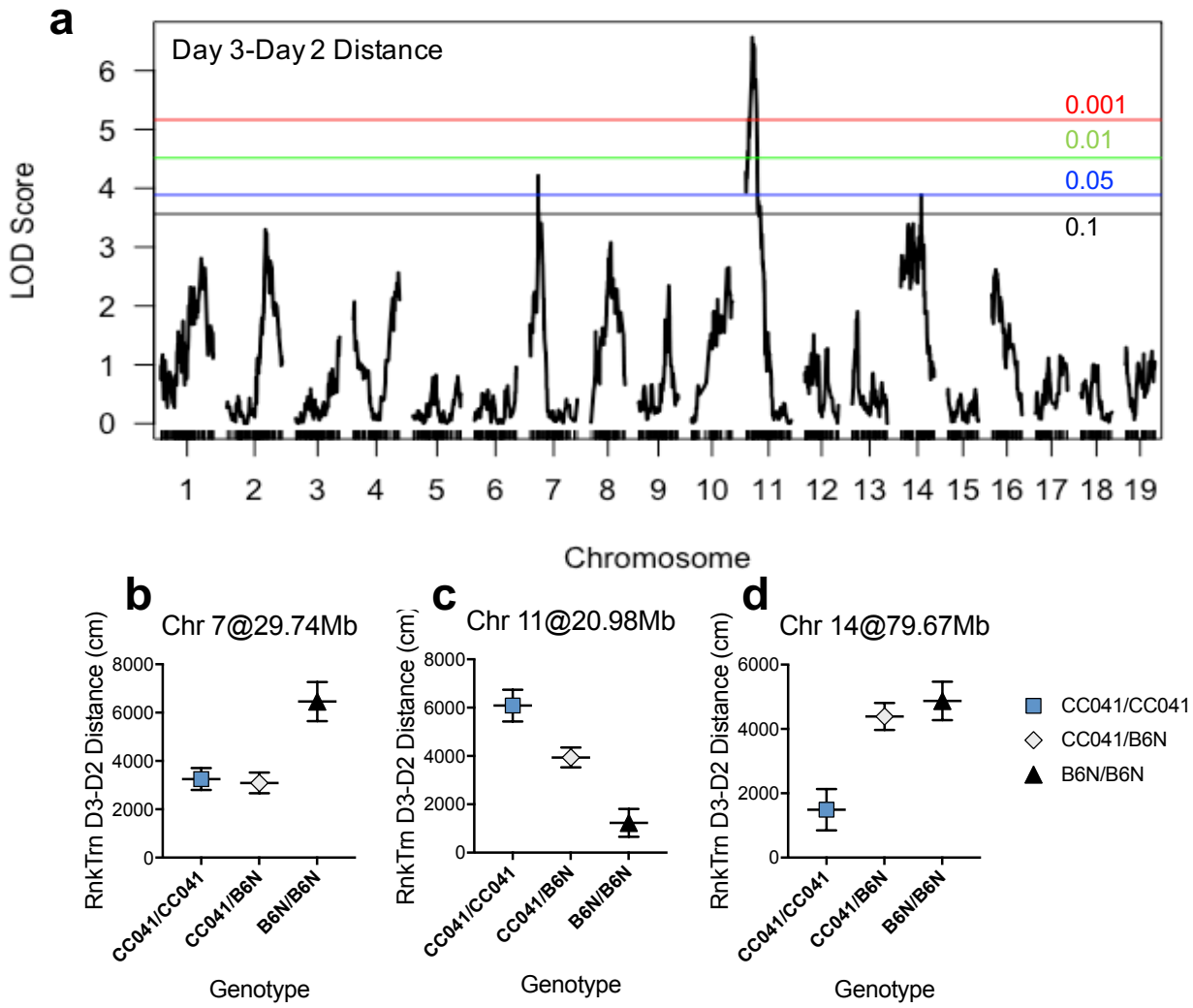
(**a**) RIX 41/51 is a F1 hybrid of CC041 and CC051, which are RI strains containing the genome of six inbred strains (color blocks). (**b**) Both CC parental strains and RIX 41/51 were phenotyped for initial locomotor response to cocaine (20 mg/kg), measured at the area under the curve (AUC) or total distance moved in the 3 hrs post-injection in a 6-hr open field test. There was a significant effect of strain (p=2.5x10^{-9}) with *post hoc* showing CC041 < RIX41/51 < CC051 (*p<0.05, **p<0.01, ***p<0.001). (**c**) To map low COC initial locomotor response, a reciprocal cross of CC041 to C57BL/6NJ (B6N) was made. F1s were then crossed in all combinations to generate an F2 population. (**d**) CC041, B6N, F1s and F2s (Ns=24, 24, 49, 448) were phenotyped in a 3-day open field test to assess initial cocaine response, measured as Day 3 (20 mg/kg cocaine) minus Day 2 (saline) distance.

Figure 4.2. High initial locomotor response to cocaine in CC004



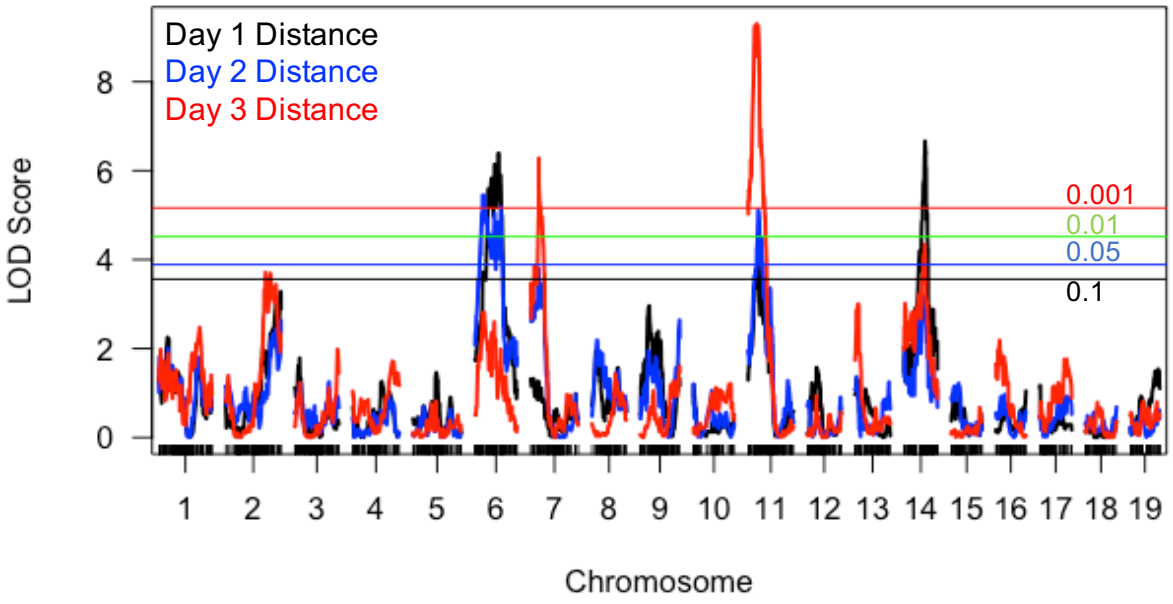
(**a**) RIX 04/17 is a F1 hybrid of CC004 and CC017, which are RI strains containing the genome of eight inbred strains (color blocks). (**b**) Both CC parental strains and RIX 04/17 were phenotyped for initial locomotor response to cocaine (20 mg/kg), measured at the area under the curve (AUC) or total distance moved in the 3 hrs post-injection in a 6-hr open field test. There was a significant effect of strain (p=0.001) with *post hoc* showing RIX 04/17 = CC004 > CC017 (**p<0.01, ***p<0.001). (**c**) To map low COC initial locomotor response, a reciprocal cross of CC004 to C57BL/6NJ (B6N) was made. F1s were then crossed in all combinations to generate an F2 population. (**d**) CC004, B6N, F1s and F2s (Ns=25, 24, 36, 309) were phenotyped in a 3-day open field test to assess initial cocaine response, measured as Day 3 (20 mg/kg cocaine) minus Day 2 (saline) distance.

Figure 4.3. Significant QTLs on Chr 7, 11 and 14 for for Day 3- Day 2 distance moved in the low COC responding F2 population



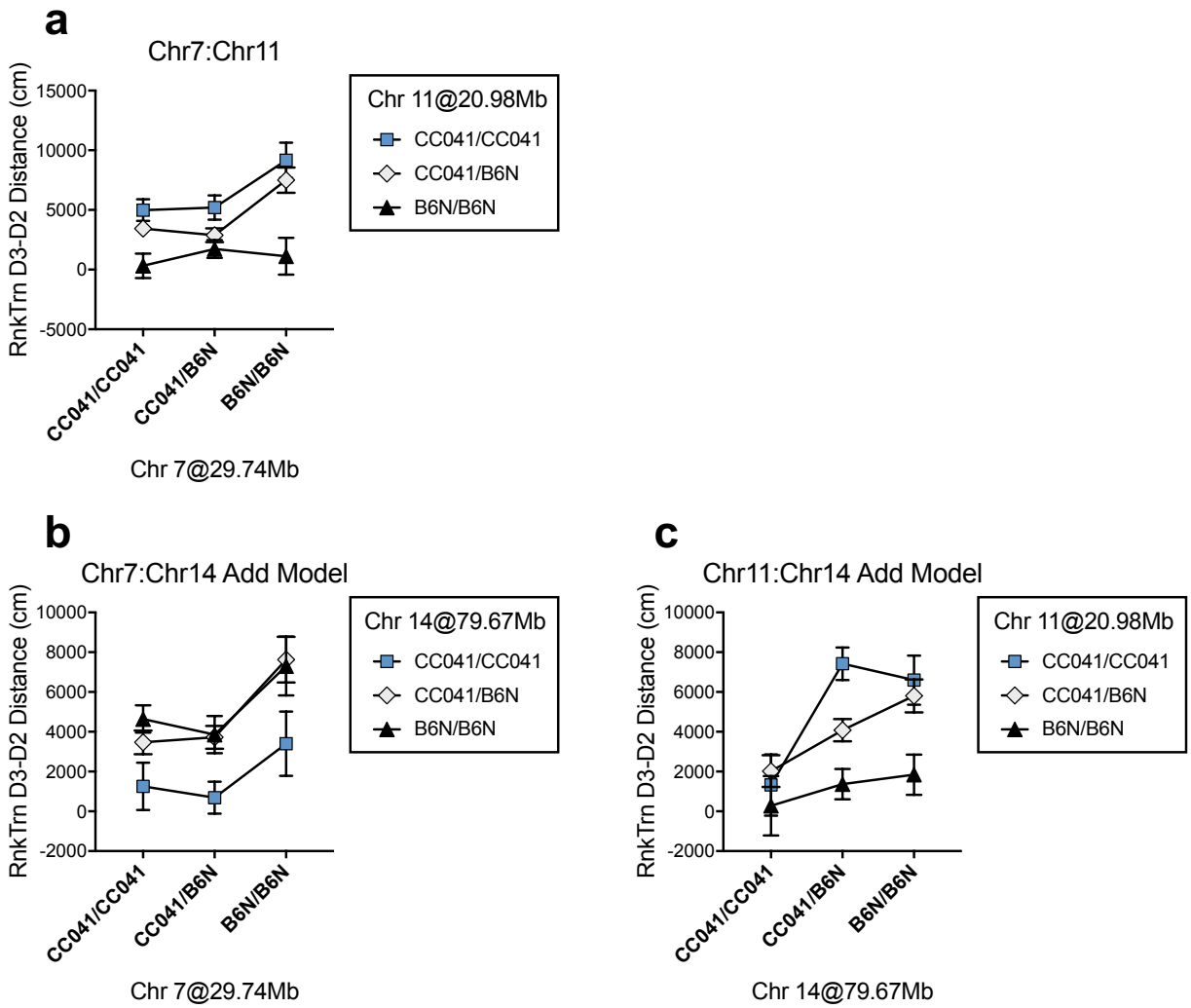
(a) Single scan QTL for initial COC response measured as Day 3 (20 mg/kg cocaine) minus Day 2 (saline). Genome-wide significant LOD thresholds based on 1000 permutations for p=0.001 (red line), p=0.01 (green line), p=0.05 (blue line), and suggestive at p=0.1 (black line). (**b**) Chr 7 had a peak at 29.74Mb (LOD=4.21; p=0.023; LOD interval=27.5-40.3 Mb) with both the CC041/CC041 and HET genotypes driving low COC response. (**c**) Chr 11 had a peak at 20.98Mb (LOD=6.54; p=0.001; LOD interval=12.6-37.7Mb) with B6N/B6N genotype driving low COC response. (**d**) Chr 14 had a peak at 79.67Mb (LOD=3.88; p=0.051; LOD interval=9.1-97.4) with CC041/CC041 genotype driving low COC response.

Figure 4.4. Significant QTLs for Day 1, 2 and 3 distance moved in the low COC responding F2 population



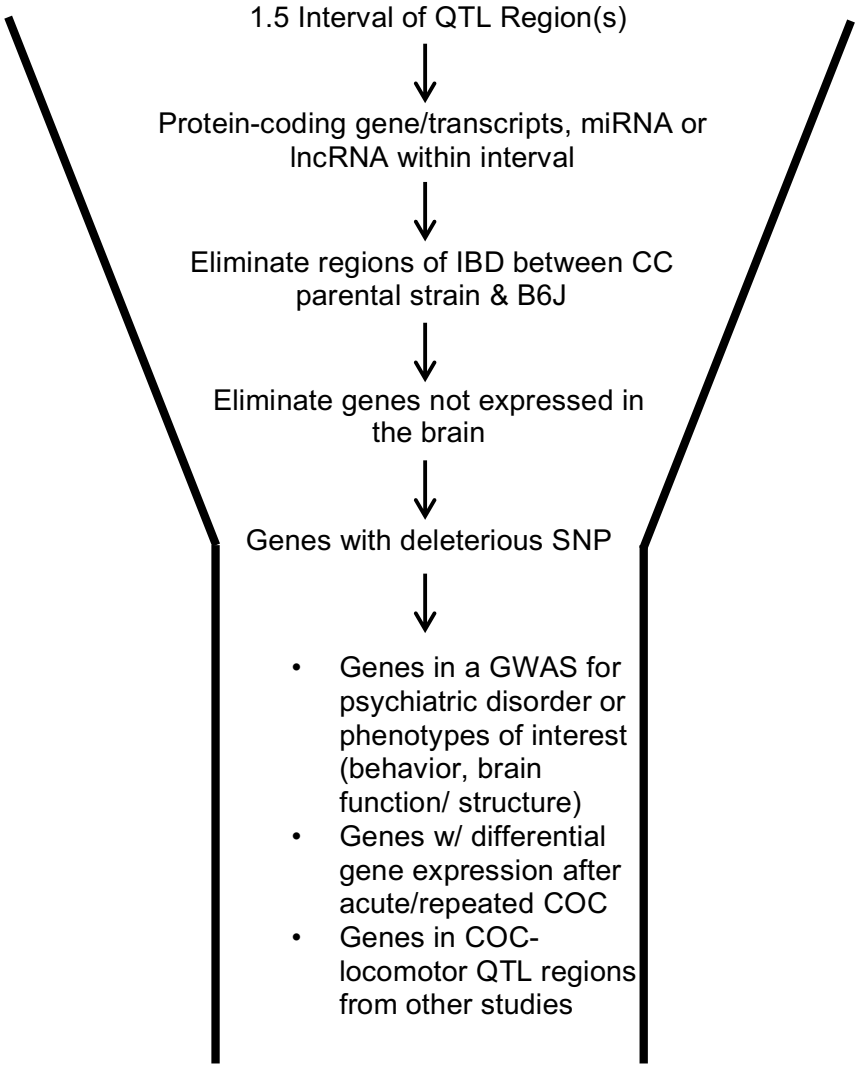
Single scan QTL for Day 1 (saline; black line), Day 2 (saline; blue line) and Day 3 (20 mg/kg cocaine; red line) distance moved. Genome-wide significant LOD thresholds based on 1000 permutations for p=0.001 (red line), p=0.01 (green line), p=0.05 (blue line), and suggestive at p=0.1 (black line). A peak on Chr 11 was present for all three days. A QTL peak on Chr 7 was identified for Day 2 and Day 3 and a peak on Chr 14 was present for Day 1 and Day 3. QTLs on Chr 6 were specific to non-COC exposures days (Day 1 and 2) and a QTL on Chr 2 was unique to cocaine exposure (Day 3).

Figure 4.5. Paired loci at QTLs on Chr 7, 11, and 14 for Day 3– Day 2 locomotion



(**a**) Evidence for a pair of QTL on Chr 7 at 29.74 Mb and Chr 11 at 20.98 Mb in both full or additive (Add) model (LOD_{fv1}= 6.1, LOD_{av1}=4.3); B6N/B6N genotype on Chr 11 shows low response independent of genotype on Chr 7. (**b**) Evidence for a pair of QTL on Chr 7 at 29.74 Mb and Chr 14 at 79.67 Mb only in the additive model (LOD_{av1}=4.4). (**c**) Evidence for a pair of QTL on Chr 11 at 20.98 Mb and Chr 14 at 79.67 Mb only in the additive model (LOD_{av1}=4.0); B6N/B6N on Chr 11 shows low response independent of Chr 14 genotype.

Figure 4.6. Strategy for identifying potential candidate genes within QTL regions



For Chr 11 and Chr 7 QTL regions we used the following strategy to identity potential candidate genes. Using the BioMart tool, we identified a list of protein-coding genes, lncRNA, and miRNAs. From this list, we eliminated any that were in a region of IBD between B6J and the CC parental strain using the Mouse Phylogeny Viewer. We then eliminated any genes not expressed in the brain. We then prioritized genes that had a nonsynonymous SNP between B6J and the CC parental strain, specifically targeting SNPs in coding sequence, splice regions, stop regions, regulatory regions, frameshift and missense using the Sanger Institute SNP Viewer tool. From this list, we then prioritized genes that met all three of the following criteria: previously identified with a phenotype of interest (i.e. brain structure/function or behavioral); expression differentially regulated by COC (using www.GeneWeaver.org); region that overlapped with previous QTL studies for initial COC-induced locomotion.

CHAPTER 5: GENERAL DISCUSSION

Conclusions of my Thesis Research

The work presented in this thesis utilized a relatively new recombinant mouse population, the Collaborative Cross (CC), to study complex behaviors. In Chapter 2, I studied the effects of genetic background, parent-of-origin (PO) and perinatal nutrition, as well as interactions among the three on stress response and anxiety- and depressive-like behaviors in adult F1 hybrids of CC strains (Recombinant Inbred Intercross, RIX). We found that perinatal diet exposure interacted with genetic background to affect anxiety-like behavior and stress response. We also found PO effects on multiple phenotypes that were dependent on genetic background. In Chapter 3, I characterized addiction-related behaviors to cocaine (COC) in two of these RIX lines (RIX 04/17 and 41/51) that I identified as phenotypic outliers for novelty-induced locomotion. RIX 04/17 and 41/51 exhibited divergent initial COC locomotor response and this phenotypic difference was not due to COC metabolism, hypothalamic-pituitary-adrenal (HPA) axis dysregulation or dopamine (DA) dynamics in the nucleus accumbens (NAc). In Chapter 4, I performed genetic mapping of initial COC response and determined that low initial sensitivity to COC present in RIX 41/51 was driven by the CC041/TauUnc parent strain and has a dominant mode of inheritance. I identified three significant quantitative trait loci (QTL) on Chr 7, 11 and 14 and used a bioinformatics approach to identify potential candidate genes for follow-up studies.

Below, I discuss my views on the following topics in the field of behavioral genetics: the utility of the CC population to study complex behaviors; the necessity to consider genetic

background when selecting rat or mouse models for behavior or physiological experiments; the challenges and need for improvement in identification and validation of genetic variants controlling complex behaviors; and a systems-level approach to the way we study addiction-related behaviors based on the notion that there are multiple pathways to development of a SUD.

Utility of the Collaborative Cross Population for Studying Complex Behaviors

The CC was designed to enable a system genetics approach to study complex traits as a panel of recombinant inbred (RI) strains with increased allelic diversity and low levels of linkage disequilibrium (Churchill *et al*, 2004; Collaborative Cross, 2012; Threadgill *et al*, 2012). The ability to perform system genetics is directly related to the number of CC strains available and the range of observed phenotypes. The original design of the CC population described production of 1000 strains, yet only 75 strains are currently available for distribution at an inbred status (csbio.unc.edu/CCstatus/index.py). During the construction of the CC, breeding and logistical issues arose resulting in high levels of extinction in pre-CC lines (Chesler *et al*, 2008; Iraqi *et al*, 2008; Welsh *et al*, 2012) A recent study of ~350 extinct CC strains showed that male infertility played a major role in extinction and is thought to be due to incompatibilities of crossing inbred strains from the different *Mus musculus* subspecies (Shorter *et al*, 2017). Despite these breeding difficulties, I believe the 75 available strains will still provide significant genetic and phenotypic expansion to existing inbred mouse strains based on the results from the studies described below.

Studies using the eight CC founder strains, pre-CC lines, CC strains, and various F1s have proven to provide increased phenotypic range for a variety of complex traits including: symptoms and susceptibility for viral and bacterial infections (Ferris *et al*, 2013; Gralinski *et al*,

2015; Rasmussen *et al*, 2014; Vered *et al*, 2014), bone microarchitecture (Levy *et al*, 2015), chemical metabolism (Mosedale *et al*, 2017; Venkatratnam *et al*, 2017); immune system (Phillippi *et al*, 2014), and susceptibility to melanoma (Ferguson *et al*, 2015). Additionally, the increase in diversity has provided new insights among previously established correlations of phenotypes. Furthermore, they have provided novel mouse models for disease states seen in humans that are not present in traditional mouse lines such as Ebola hemorrhagic fever symptoms and spontaneous colitis (Rasmussen *et al*, 2014; Rogala *et al*, 2014).

QTL analyses were performed in several of the studies mentioned above and relatively small regions containing a feasible numbers of candidate genes were identified (Ferris *et al*, 2013; Gralinski *et al*, 2015; Levy *et al*, 2015; Philip *et al*, 2011; Vered *et al*, 2014). Of note, most of these studies were conducted on large panels of pre-CC lines or F1s of these lines and the ability to successful map small QTL intervals exclusively in the 75 currently available strains is less documented as all of these strains reaching inbred status is relatively new.

The CC population offer several other advantages including publicly available dense genotyping for mapping, eliminating the need to perform genotyping in a mapping study using CC strains. DNA sequences and tools for genetic analysis including SNPs analysis and haplotype reconstructions are also available (Morgan *et al*, 2015; Srivastava *et al*, 2017). Additional data being generated, such as gene expression levels in brain tissue will also add in the ability to map eQTLs or investigate candidate genes in mapping studies using CC strains. Therefore, I believe the CC are a valuable resource to further our knowledge of complex traits such as addiction-related behaviors allowing for elucidation of the genetic relationship among phenotypes and identification of candidate genes. The data presented in Chapter 3 and 4 are the first to characterize CC strains and RIX of CC strains for addiction-related behaviors. To date,

behavioral analysis of CC strains has been published for rotarod performance (Mao *et al*, 2015), locomotor activity in the OF, and sleep behavior (Philip *et al*, 2011). More studies are needed to further characterize the currently available CC strains for neurobehavioral phenotypes.

Consideration of Genetic Background in Studies of Complex Behaviors

Studies comparing multiple inbred strains of mice have clearly shown a role of genetics to influence anxiety- and depressive-like behavior (O'Leary et al, 2013; Schoenrock et al, 2016; Can et al 2011; Liu et al 2001; Bothe et al 2005; Miller et al 2010), stress response (Trullas et al 1993), and addiction-related behaviors (Wiltshire et al, 2015; Thomsen et al, 2011; Eisener-Dorman et al 2011). Additionally, studies have shown that the relationship between behaviors differs across genetic background (Thomsen et al, 2011). These studies in combination with the data presented Chapter 2 and 3 further support the consideration of genetic background when selecting a mouse line for behavioral or physiological experiments. This is not to say that some underlying mechanisms of these behaviors are not shared among all genetic backgrounds. However, if we limit ourselves to behavioral research in only one genetic background, we might come to the wrong conclusions about relationships between behaviors (i.e. initial COC locomotor response and COC CPP; see **Fig 3.3b**) or the mechanisms underlying a specific phenotype (i.e. DA release in the NAc and COC locomotion; see **Fig 3.4**).

Phenotyping numerous strains is a costly endeavor both in time and money. However, due to the stable genetic background of inbred mouse strains, a specific strain only needs to be phenotyped once (although, validation across multiple laboratories is ideal). The Mouse Phenome Database (MPD; https://phenome.jax.org), curated by The Jackson Laboratory, is a resource that contains thousands of datasets for a wide range of phenotypes in various

populations (i.e., RIs, classical inbred strains, CC, F1 of strains, etc). Resources like the MPD allow for comparison across studies to obtain a more complete picture of the phenotypes associated for a given strain, so that researchers can select the appropriate strain(s) for addressing their specific research question.

One current limitation in the field is the small number of genetic backgrounds used for generation of transgenic mice (i.e., reporter lines, cre-lines, etc) that have proven valuable for furthering our knowledge of complex behaviors. Most of the commonly used transgenic lines in behavioral neuroscience are in substrains of C57BL/6, 129, and FVB (see www.jax.org/jax-mice-and-services). Depending on the phenotype of interest, this may not be problematic, however, there is an overall need for more genetic diversity in commonly used transgenic lines for mechanistic studies (i.e. circuitry and electrophysiology *in vivo* and slice preparations). I believe that generating common transgenic lines (i.e., targeting 5-HT, GABA, Glutamate, DA neuronal types) in a variety of genetically divergent inbred strains, including the inbred strains used to generate the CC (A/J, NOD, NZO, PWK, CAST, WSB) would greatly enhance our ability to consider genetic background in experimental designs and would increase the attractiveness and feasibility of using the CC and its companion population, the Diversity Outbred (DO), to study neuroscience-related phenotypes.

Future Directions for the Field of Behavioral Genetics for Gene Identification

The field of behavioral genetics has used various approaches to identifying genes that are associated with complex behaviors. In humans and rodents, GWAS and QTL experiments are commonly used approaches designed to allow for unbiased identification of causative genes. While reviewing the QTL literature for COC-related behaviors, it was striking to find that while

there are have been numerous QTL regions identified over the past 20 years, very few studies have led to the identification of causative variants and even fewer have validated candidate variants. Instead, like the QTL data I presented in Chapter 4, a list of possible candidate genes is proposed based on pre-existing knowledge of the systems involved for the mapped phenotype. This approach eliminates the possibility of identifying novel genes or pathways. The question then becomes how to we overcome these barriers to identifying causative genetic variants?

The first issue to address is identification of smaller QTL regions, which has been mainly hindered due to the limited recombination in common mapping populations (i.e. F2 intercross or backcross). One approach is to increase the number of generations of intercrossing thereby increasing recombination events. A study by Parker *et al* (2012) compared an F2 and F8 intercross population of B6J and DBA and showed a 65% decrease of the QTL region in the F8 line compared to the F2 intercross for initial METH locomotor response. Although this approach substantially reduced the QTL region, it also drastically increased the time and cost associated with breeding multiple generations.

An alternative approach is to increase the number of unique genetic backgrounds– either individual animals in an F2 or backcross or number of strains in an RI panel. The most commonly used RI strains, the BXD, have proven even more valuable as the availability of strains has increased, especially with the inclusion of lines derived from advanced intercross F9 and F14 generations. A study by Dickson *et al* (2016) using 44 BXD strains identified three QTL regions <1-4 Mb in size that control COC IVSA behavior and identified candidate genes within these regions, one of which, *Fam53b,* was previously identified in a human GWAS for COC dependence (Gelernter *et al*, 2014). The BXD will continue to be a useful mapping population as new strains are being produced and there is an extensive resource of phenotypic, genetic and

molecular data publically available (www.genenetwork.org). However, the usefulness of this population is limited to identification of genetic variants that differ between the two parental strains.

A third approach is the use of a relatively new mouse resource, the Diversity Outbred (DO) population, which was generated from the same initial intercrossing of the eight founders in the CC and has high levels of recombination that are increasing with each generation (Churchill *et al*, 2012). QTL analysis using the DO has identified significantly smaller QTL regions for several complex traits and behaviors (Gatti *et al*, 2014; Logan *et al*, 2013; Recla *et al*, 2014; Smallwood *et al*, 2014; Svenson *et al*, 2012). Allelic effects of the eight founders have been used to further narrow QTL regions and candidate genes have been identified for acute thermal pain sensitivity (Recla *et al*, 2014) and atherosclerosis (Smallwood *et al*, 2014). Due to the outbred nature and high level of recombination of the DO, dense genotyping is required for every individual which can be expensive and QTL analysis is more complicated, although a genotyping platform (GigaMUGA) and statistical programs have been developed to help overcome these obstacles (Gatti *et al*, 2014; Morgan, 2015; Morgan *et al*, 2015).

The second issue to address is identification and validation of candidate genes within smaller QTL regions. One common fine-mapping approach is to use gene expression data to perform eQTL analysis and identify regions that overlap with behavioral QTL (Dickson *et al*, 2016; Palmer *et al*, 2005; Parker *et al*, 2012; Parker *et al*, 2016). This method can be expensive, requiring genome-wide gene expression via RNA sequencing or microarrays for either whole brain or targeted regions implicated in the behavior (i.e. striatum). For mapping populations using inbred strains such as RIs, gene expression data only needs to be generated once. However,

for populations in which each individual is unique (F2 intercross, backcross, DO), gene expression would have to be generated for each animal.

Another approach is to generate congenic lines containing subsections of the QTL region. Yazdani *et al* (2015) employed this technique to narrow a QTL region for acute METH locomotor response on Chr 11 to two genes and used various approaches including sequencing, gene expression and targeted-gene editing to validate a novel gene, *Hnrnph1,* as the causative gene. Disadvantages of this approach include the time and costs necessary to generate the lines and the possibility of losing the phenotype due to multiple QTL and interactions with loci on other chromosomes.

Once a candidate genetic variant is identified, it has to be validated and the mechanism of action determined. Successful examples include a variant in *Cyfip2* that influences initial COC locomotor response (Kumar *et al*, 2013) and *Rgs2* that influences anxiety-like behavior (Yalcin *et al*, 2004). Few other genetic variants have been reported thus far. The inability to move from QTL to QT gene to specific variant is due in part to difficulties in identifying causative variant as many are thought to be in intronic or regulatory regions. Gene knockouts are among the most frequently used tools for validation, but are limited to genetic background on which the KO was made. Additionally, knocking out a gene is not always useful if your genetic variant results in something other than complete loss of protein or protein function. One technology that may aide in validation of causative genetic variants in the CRISPR/Cas9 system which is becoming more feasible to induce specific changes on a variety of genetic backgrounds. Additional approaches that may aid in the process of linking genes with phenotypes are the integration of more system-level analyses (Chesler, 2014). Above I mentioned the use of gene expression; other emerging

methods are the inclusion of metabolite analysis, epigenetic regulation (i.e., DNA and histone methylation), and ribosomal profiles (reviewed in Moreno-Moral and Petretto (2016)).

Multiple Paths to the Development of Addiction

Development of a SUD is a multi-step process with three key transitions: initiation of first use; first use to subsequent use; and repeated use to development of a SUD. Numerous studies in humans and animal models have identified factors that increase likelihood at each of these transitions. However, what is becoming clearer is that there are individual differences in the progression to development of an SUD (Egervari *et al*, 2017). How should this knowledge shape the way we study SUDs?

I believe we need to integrate multiple levels including genes, molecules, cells, circuits and physiology for specific behaviors associated in each phase of the multi-step process; similar to the Research Domain Criteria (RDoC) Initiative set forth by NIMH (Insel *et al*, 2010). One way to do this is establishing animal models that represent different pathways to the development of an addiction. Comparing these models across a thorough screen for the five levels mentioned above will help uncover what leads to the divergent paths. This is a similar goal behind the work being performed across four institutions as part of The Center for Systems Neurogenetics of Addiction (CSNA). Preliminary data shows a similar novelty-induced locomotion, initial COC locomotor response and behavioral sensitization behavior in CC004 and CC041 relative to RIX 04/17 and RIX 41/51, confirming our findings in Chapter 4 that these parents are driving the response seen in the RIX lines. Additionally, preliminarily IVSA shows that CC004 rapidly acquires IVSA at 1 mg/kg while CC041 mice do not acquire at this dose even after 24 daily sessions. This data in combination with the characterization of RIX 04/17 and 41/51 in Chapter 2

and 3 could establish these two strains as examples of diverging paths beginning with differences in predictive traits (response to novelty), initial drug response, and acquisition of self-administration, but similarities in the rewarding effects of a drug upon non-contingent administration. These strains are of particular interest to me for follow-up analyses as our work shows that locomotor differences at baseline and after acute COC are not due to underlying differences in the DA system, but may involve the NE or 5-HT systems.

In conclusion, I believe my PhD research adds to the existing knowledge in the field of behavioral genetics in the following ways: providing additional support for the key role that genetic background plays in mediating complex behaviors; offering new insights into the role of PO effects on complex behaviors that had not been previously reported; establishing initial data for the study of environmental and genetic factors mediating complex behavior in the emerging mouse population, the CC; identification of two novel CC mouse lines that can be used to study addiction-related behaviors and the underlying mechanisms including the identification of novel candidate genes that may mediate a protective initial response to COC.

APPENDIX A

Supplemental Material for Chapter 2

Supplemental Table 1. CC strains used to generate RIX lines and Ns of F1 females phenotyped

RIX Name	CC Strain	Abbreviated CC Name	Reciprocal Name	# F1 Females Phenotyped				Total RIX Ns
				PD	ME	Std	VDD	
01/11	CC001/Unc	01	CC(01x11)F1	19	13	11	20	117
	CC011/Unc	11	CC(11x01)F1	12	14	17	11	
41/51	CC041/TauUnc	41	CC(41x51)F1	12	24	11	20	122
	CC051/TauUnc	51	CC(51x41)F1	13	12	16	14	
04/17	CC004/TauUnc	04	CC(04x17)F1	12	6	12	14	83
	CC017/Unc	17	CC(17x04)F1	9	2	15	13	
23/47	CC023/GeniUnc	23	CC(23x47)F1	10	9	4	8	50
	CC047/Unc	47	CC(47x23)F1	6	4	3	6	
28/38	CC028/GeniUnc	28	CC(28x38)F1	Eliminated from study due to no viable CC(38x28)F1 offspring				
	CC038/GeniUnc	38	CC(38x28)F1					
06/26	CC006/TauUnc	06	CC(06x26)F1	6	5	10	9	66
	CC026/GeniUnc	26	CC(26x06)F1	6	5	11	14	
03/14	CC003/Unc	03	CC(03x14)F1	4	3	13	9	48
	CC014/Unc*	14	CC(14x03)F1	1	4	8	6	
35/62	CC035/Unc	35	CC(35x62)F1	1	2	5	5	34
	CC062/Unc	62	CC(62x35)F1	4	7	4	6	
32/42	CC032/GeniUnc	32	CC(32x42)F1	7	19	21	19	96
	CC042/GeniUnc	42	CC(42x32)F1	6	9	9	6	
05/40	CC005/TauUnc	05	CC(05x40)F1	14	24	NA	NA	69
	CC040/TauUnc	40	CC(40x05)F1	16	15	NA	NA	
			Total Diet Ns	158	177	170	180	

*CC014/Unc is now extinct; CC = Collaborative Cross; Geni = Geniad, Australia; ME = methyl enriched; NA = Not-attempted; PD = protein deficient; RIX = Recombinant Inbred Intercross or F1 hybrid females; Std = Standard; Tau = Tel Aviv University, Israel; Unc = University of North Carolina, US; VDD = Vitamin D Deficient

Supplemental Table 2. Nutritional content of experimental diets

	AIN-93G Standard (Std;110700)		Protein Deficient (PD;102787)		Vitamin D Deficient (VDD;119266)		Methyl Enriched (ME;518893)	
Ingredient	**g/kg**	**kcal/kg**	**g/kg**	**kcal/kg**	**g/kg**	**kcal/kg**	**g/kg**	**kcal/kg**
Casein	200	716	75	269	200*	716	-	-
L-Cystine	3	12	0.9	3.6	3	12	-	-
Sucrose	100	400	100	400	100	400	382.19	1528.76
Cornstarch	397.486	1430.9496	481.196	1732.3056	397.486	1430.9496	100	360
Dyetrose	132	501.6	160	608	132	501.6	100+	363
Soybean Oil	70	630	70	630	70	630	50++	450
t-Butyl hydroquinone	0.014	0	0.014	0	0.014	0	-	-
Cellulose	50	0	50	0	50	0	50	0
Choline Bitartrate	2.5	0	2.5	0	2.5	0	14.48	0
Mineral Mix #210025	35	30.8	35[@] (#213266)	30.8	35	30.8	-	-
Vitamin Mix # 310025	10	38.7	10	38.7	10** (#319255)	38.7	10	38.7[@@] (#300050)
Calcium Phosphate Dibasic	-	-	10.97	0	-	-	-	-
Calcium Carbonate	-	-	4.42	0	-	-	-	-
Primex	-	-	-	-	-	-	100	900
Salt Mix #215001 (no Fe Added)	-	-	-	-	-	-	35	16.45
Sodium Bicarbonate	-	-	-	-	-	-	4.3	0
Ferric Citrate, U.S.P.	-	-	-	-	-	-	0.33	0
Succinyl Sulfathiazole	-	-	-	-	-	-	10	0
L-AA	-	-	-	-	-	-	143.7	574.8

The PD and VDD diets were nutritionally matched to the Std diet. ME was matched to a methyl donor deficient diet that was used in pilot studies and eliminated from these experiments due to the inability of dams to produce viable offspring when exposed to the diet. Red indicates the main nutritional component that was changed in each diet (pelleted). The product number associated with each diet, vitamin mix, and mineral mix are provided (Dyets, Inc; Bethlehem, PA). [@]Ca and P free, [@@]Vitamin K1/Dextrose mix free w/ addition of menadione sodium bisulfite; *vitamin free, ** no vitamin D, +dextrin instead of dyetrose, ++corn oil instead of soybean oil

Supplemental Table 3. Breeding statistics

RIX Line	CC Strain	Diet	# Batches	# Dams Mated	% Productive Matings	# Pups Born	Avg Litter Size	% Survival Weaning	Sex Ratio
01/11	CC001/Unc	PD	9	19	89	92	5.41	61	0.53
		ME	7	17	71	66	5.50	44	0.83
		Std	6	11	82	63	7.00	82	0.62
		VDD	4	8	100	49	6.13	88	0.49
	CC011/Unc	PD	9	20	65	64	4.92	82	0.43
		ME	7	19	47	65	7.22	95	0.54
		Std	6	12	67	60	7.50	95	0.50
		VDD	4	8	63	32	6.40	100	0.43
41/51	CC041/TauUnc	PD	4	7	100	52	7.43	75	0.50
		ME	7	17	71	98	8.17	92	0.46
		Std	6	12	67	55	6.88	98	0.51
		VDD	4	8	88	46	6.57	100	0.50
	CC051/TauUnc	PD	4	8	75	41	6.83	86	0.54
		ME	7	18	61	54	4.91	70	0.58
		Std	6	11	73	40	5.00	75	0.59
		VDD	4	8	75	33	5.50	100	0.53
04/17	CC004/TauUnc	PD	6	14	64	48	5.33	88	0.53
		ME	8	15	80	79	6.58	94	0.57
		Std	3	6	100	41	6.83	94	0.55
		VDD	6	10	100	61	6.10	97	0.54
	CC017/Unc	PD	6	16	75	51	4.25	88	0.57
		ME	8	19	53	36	3.60	41	0.45
		Std	3	6	100	34	5.67	88	0.66
		VDD	6	13	54	33	4.71	95	0.60
23/47	CC023/GeniUnc	PD	4	12	83	35	3.50	92	0.56
		ME	4	8	75	21	3.50	100	0.55
		Std	3	7	100	26	3.71	97	0.42
		VDD	4	9	89	26	3.25	88	0.69
	CC047/Unc	PD	4	8	88	33	4.71	57	0.55
		ME	4	10	100	31	3.10	43	0.43
		Std	3	6	83	23	4.60	48	0.56
		VDD	4	7	100	31	4.43	40	0.62

Supplemental Table 3 continued

RIX Line	CC Strain	Diet	# Batches	# Dams Mated	% Productive Matings	# Pups Born	Avg Litter Size	% Survival Weaning	Sex Ratio
28/38	CC028/GeniUnc	PD	3	4	100	22	5.50	42	0.35
		ME	2	4	50	10	5.00	100	0.42
		Std	2	2	100	7	3.50	50	0.50
		VDD	3	4	100	18	4.50	25	0.50
	CC038/GeniUnc	PD	3	4	25	1	1.00	0	NA
		ME	2	4	50	3	1.50	0	NA
		Std	2	2	50	1	1.00	0	NA
		VDD	3	4	25	2	2.00	0	NA
06/26	CC006/TauUnc	PD	6	12	50	23	3.8	92	0.82
		ME	6	15	47	35	5.0	57	0.44
		Std	3	6	67	20	5.0	75	0.78
		VDD	5	11	73	26	3.3	69	0.52
	CC026/GeniUnc	PD	6	13	69	46	5.1	61	0.48
		ME	6	13	85	53	4.8	76	0.48
		Std	3	6	100	29	4.8	97	0.52
		VDD	5	9	89	43	5.4	78	0.56
03/14	CC003/Unc	PD	4	7	57	22	5.5	75	0.49
		ME	3	6	67	20	5.0	96	0.65
		Std	5	10	90	43	4.8	92	0.44
		VDD	3	6	83	34	6.8	97	0.49
	CC014/Unc	PD	4	8	63	13	2.6	73	0.33
		ME	3	6	67	11	2.8	75	0.59
		Std	5	10	60	23	3.8	83	0.64
		VDD	3	6	83	17	3.4	76	0.52
35/62	CC035/Unc	PD	5	8	25	11	5.5	50	0.25
		ME	7	11	36	21	5.3	63	0.37
		Std	3	3	67	17	8.5	90	0.41
		VDD	3	5	80	24	6.0	100	0.59
	CC062/Unc	PD	5	7	86	27	4.5	90	0.66
		ME	7	14	100	80	5.7	76	0.51
		Std	3	4	100	23	5.8	100	0.51
		VDD	3	6	50	20	6.7	100	0.71

Supplemental Table 3 continued

RIX Line	CC Strain	Diet	# Batches	# Dams Mated	% Productive Matings	# Pups Born	Avg Litter Size	% Survival Weaning	Sex Ratio
32/42	CC032/GeniUnc	PD	6	11	100	73	6.6	76	0.49
		ME	5	9	78	52	7.4	100	0.47
		Std	4	8	100	78	9.8	75	0.48
		VDD	7	12	100	96	8.0	83	0.56
	CC042/GeniUnc	PD	6	21	62	34	2.6	23	0.62
		ME	5	16	56	43	4.8	85	0.31
		Std	4	12	92	31	2.8	82	0.43
		VDD	7	18	56	34	3.4	71	0.74
05/40	CC005/TauUnc	PD	8	29	79	102	4.4	62	0.46
		ME	5	16	94	79	5.3	87	0.55
		Std				Not Attempted			
		VDD				Not Attempted			
	CC040/TauUnc	PD	8	27	59	50	3.1	97	0.55
		ME	5	16	75	43	3.5	83	0.64
		Std				Not Attempted			
		VDD				Not Attempted			
	Overall Diet Means	PD	27	255	71	840	3.3±2.8	72±38	.52±.28
		ME	27	253	68	900	3.6±3.1	75±39	.53±.27
		Std	18	134	82	614	4.8±3.2	84±32	.53±.24
		VDD	23	152	78	625	4.1±3.0	82±36	.57±.22

Grey shading indicates RIX 28/38 was removed from study due to inability to produce viable CC(38x28)F1 females at weaning on all diets. Twenty-eight separate breeding batches were set up over a two-year period to produce all the female offspring needed. Within each batch at least two diets and RIX were included, the number of times a RIX/diet combination was attempted is indicated as # Batches (of 28). % productive matings = ((# litters born/# dams mated) x 100); % survival weaning = ((# pups at weaning/# pups born) x 100); sex ratio = (# F pups weaning/total pups at weaning); ME = methyl enriched; PD = protein deficient; Std = standard; VDD = vitamin D deficient

Supplemental Table 4. Statistical analysis on breeding of RIX lines

RIX Line	Effect	% Productive Matings	Avg Litter Size	% Survival Weaning	Sex Ratio
01/11	PO	**0.005**	0.242	**0.002**	**0.015**
	Diet	0.229	**0.003**	0.750	**0.029**
	Diet-by-PO	0.895	0.215	0.231	0.503
41/51	PO	0.316	**0.002**	0.202	0.283
	Diet	0.387	0.404	0.168	0.973
	Diet-by-PO	0.770	0.238	0.179	0.941
04/17	PO	0.096	**0.001**	**0.024**	0.773
	Diet	0.122	0.221	**0.006**	0.853
	Diet-by-PO	0.088	0.372	**0.004**	0.751
23/47	PO	0.452	0.063	**7.0E-06**	0.853
	Diet	0.836	0.359	0.849	0.644
	Diet-by-PO	0.339	0.368	0.847	0.826
28/38	PO	**0.010**	**0.034**	0.070	NA
	Diet	0.827	0.940	0.734	NA
	Diet-by-PO	0.318	0.940	0.734	NA
06/26	PO	**0.012**	0.179	0.660	0.100
	Diet	0.294	0.811	0.690	0.220
	Diet-by-PO	0.815	0.354	0.295	0.147
03/14	PO	0.624	**0.004**	0.196	0.988
	Diet	0.592	0.692	0.756	0.496
	Diet-by-PO	0.668	0.611	0.884	0.499
35/62	PO	**0.009**	0.400	0.183	0.093
	Diet	0.488	0.279	0.118	0.392
	Diet-by-PO	**0.014**	0.406	0.694	0.817
32/42	PO	**0.001**	**1.9E-17**	**0.036**	0.702
	Diet	0.143	**0.016**	**0.005**	**0.014**
	Diet-by-PO	0.411	**0.007**	0.081	0.178
05/40	PO	**0.043**	**0.001**	0.054	0.219
	Diet	0.113	0.143	0.446	0.236
	Diet-by-PO	0.945	0.669	**0.016**	0.976
Overall	**RIX**	**8.8E-7**	**3.7E-39**	**3.0E-12**	0.542
	Diet	**0.010, *ME<Std**	0.139	0.069	0.504
	Diet-by-RIX	0.498	0.103	0.110	0.601

Data are *p* values for effect of PO, diet and diet-by-PO within each RIX line and an overall effect of RIX, diet and diet-by-RIX across all 20 CC strains used. Significant values are shaded grey and **bolded**. % productive matings = ((# litters born/# dams mated) x 100); % survival weaning = ((# pups at weaning/# pups born) x 100); sex ratio = (# F pups weaning/total pups at weaning); ME = methyl enriched; Std = standard

Supplemental Table 5. Tukey's post-hoc analyses of overall diet effects

Phenotype	*p*-value	*q*-value	Tukey's Post-hoc
WeightPND21	**2.2E-16**	**2.2E-15**	ME & PD < all ($p<1.0x10^{-7}$)
WeightPND60	**2.2E-16**	**2.2E-15**	ME & PD < VDD & Std ($p<1.0x10^{-7}$)
OF TotalDist	0.772	0.881	
OF % Center	0.807	0.881	
OF VertCount	0.998	0.998	
OF Boli	0.192	0.350	
LD TotalDist	0.228	0.381	
LD DistDark	**0.023**	0.058	none
LD DistLight	0.805	0.881	
LD Transition	0.837	0.881	
LD % Light	**0.048**	0.096	ME < VDD (p=0.024)
LD Boli	0.352	0.502	
SIH-T1	**2.0E-04**	**0.001**	ME < PD ($p=1.3x10^{-4}$)
SIH-T2	**1.9E-04**	**0.001**	PD > ME ($p=6.1x10^{-6}$); PD > VDD (p=0.047); PD > Std (p=0.018)
deltaSIH	0.342	0.502	
FST % Imb	0.377	0.502	
FST Boli	**0.023**	0.058	Std > VDD (p=0.018)
BasalCORT	**0.038**	0.085	none
StressCORT	**0.003**	**0.010**	PD > ME (p=0.001); PD > VDD (p=0.003)
deltaCORT	**2.1E-05**	**1.4E-04**	PD > ME ($p=7.2x10^{-5}$); PD > VDD ($p=1.2x10^{-5}$)

For each phenotype that had a significant effect of diet (shaded grey and **bold**) at the *p* value level, a Tukey's post-hoc was performed. CORT = corticosterone; deltaCORT = (Stress CORT – Basal CORT); Dist = distance; Imb = Immobile; LD = light/dark; OF = open field; ME = methyl enriched; PND = postnatal day; PD = protein deficient; SIH-T1 = basal temperature; SIH-T2 = post-stress temperature; deltaSIH = (T2-T1); Std = Standard; VDD = vitamin D deficient; Vert = vertical

Supplemental Table 6. Perinatal diet effects on behavior within each RIX line

	RIX 01/11			RIX 41/51			RIX 04/17		
Phenotype	***p***	***q***	**Tukey's posthoc**	***p***	***q***	**Tukey's posthoc**	***p***	***q***	**Tukey's posthoc**
WeightPND21	**2E-04**	**4E-03**	PD<all (p≤0.001)	**1E-12**	**2E-11**	PD&ME<all (p≤0.001)	**8E-09**	**2E-07**	PD<all (p≤0.001)
WeightPND60	**0.03**	0.10	PD<Std & VDD (p≤0.001)	**5E-06**	**5E-05**	PD&ME<all (p≤0.02)	**4E-06**	**3E-05**	PD<Std (p≤0.001); VDD>all (p≤0.04)
OF TotalDist	0.47	0.74		0.08	0.13		0.29	0.64	
OF %Center	0.74	0.82		**0.02**	0.09	PD>VDD (p<0.02)	0.90	0.96	
OF VertCount	0.65	0.82		0.07	0.13		0.49	0.87	
OF Boli	**0.03**	0.10	none	0.80	0.80		0.61	0.87	
LD TotalDist	0.68	0.82		0.09	0.13		0.59	0.87	
LD DistDark	0.48	0.74		0.09	0.13		0.36	0.72	
LD DistLight	0.74	0.82		0.14	0.18		0.72	0.87	
LD Transition	0.97	0.97		0.08	0.13		0.95	0.96	
LD %Light	0.33	0.60		0.06	0.13		0.72	0.87	
LD Boli	**4E-03**	**0.03**	Std<ME (p<0.008)	**0.03**	0.11	none	0.74	0.87	
SIH-T1	**2E-03**	**0.02**	PD&VDD>ME&Std (p≤0.02)	0.27	0.32		0.17	0.48	
SIH-T2	**0.05**	0.15		**0.05**	0.13	Std>VDD (p<0.02)	**0.01**	**0.04**	ME<all (p≤0.007)
deltaSIH	**0.01**	0.07	PD<ME&Std (p≤0.03)	0.09	0.13		0.20	0.50	
FST %Imb	0.56	0.80		0.36	0.40		0.53	0.87	
FST Boli	0.28	0.56		0.76	0.80		0.96	0.96	
BasalCORT	0.13	0.32		0.25	0.31		**2E-04**	**8E-04**	Std>all (p≤0.04)
StressCORT	0.95	0.97		**5E-04**	**3E-03**	Std&PD>ME&VDD (p≤0.001)	**5E-06**	**3E-05**	Std>all (p≤0.05)
deltaCORT	0.28	0.56		**2E-03**	**9E-03**	Std&PD>ME&VDD (p≤0.002)	**1E-04**	**6E-04**	VDD<all (p≤0.001)

Supplemental Table 6 continued

Phenotype	RIX 23/47			RIX 06/26			RIX 03/14		
	p	***q***	**Tukey's posthoc**	***p***	***q***	**Tukey's posthoc**	***p***	***q***	**Tukey's posthoc**
WeightPND21	**8E-05**	**2E-03**	PD<all (p≤0.01)	**7E-07**	**7E-06**	PD&ME<all (p≤0.001)	**0.05**	0.13	PD<VDD (p≤0.01)
WeightPND60	**6E-04**	**6E-03**	VDD>all (p≤0.005)	**4E-07**	**7E-06**	PD&ME<Std&VDD (p≤0.04)	**0.01**	0.10	ME<Std (p≤0.02)
OF TotalDist	0.13	0.21		0.25	0.50		0.65	0.72	
OF %Center	0.16	0.22		0.64	0.80		0.26	0.33	
OF VertCount	**0.04**	0.10	VDD>PD (p<0.04)	0.46	0.61		**0.01**	0.10	PD<VDD (p<0.02)
OF Boli	0.08	0.16		0.13	0.38		**0.03**	0.13	ME&PD<VDD&Std (p≤0.04)
LD TotalDist	0.30	0.34		0.45	0.61		0.17	0.27	
LD DistDark	0.43	0.45		0.33	0.51		**0.04**	0.13	ME>VDD (p<0.04)
LD DistLight	**3E-03**	**0.01**	Std>all (p≤0.04)	0.83	0.94		0.32	0.37	
LD Transition	0.18	0.22		0.31	0.51		0.93	0.98	
LD %Light	**0.03**	0.07	Std>VDD (p<0.04)	0.90	0.95		0.16	0.27	
LD Boli	**0.01**	**0.04**	Std<ME&VDD (p≤0.04)	0.18	0.39		**0.04**	0.13	none
SIH-T1	0.23	0.27		**3E-03**	**0.01**	ME<Std (p<0.01)	**0.02**	0.10	none
SIH-T2	0.11	0.19		0.15	0.39		0.24	0.32	
deltaSIH	0.16	0.22		**7E-03**	**0.02**	Std<PD (p<0.009)	0.14	0.27	
FST %Imb	**0.01**	**0.04**	ME<PD&VDD (p≤0.02)	0.99	0.99		0.15	0.27	
FST Boli	0.09	0.16		**2E-03**	**0.01**	Std>ME&PD (p≤0.05)	0.17	0.27	
BasalCORT	0.56	0.56		0.29	0.51		0.13	0.27	
StressCORT	**2E-03**	**0.01**	PD>ME>VDD (p≤0.01)	**4E-04**	**3E-03**	ME&PD>Std&VDD (p≤0.03)	0.21	0.29	
deltaCORT	**0.02**	**0.05**	PD>VDD (p<0.002)	0.85	0.94		1.00	1.00	

Supplemental Table 6 continued

Phenotype	RIX 35/62			RIX 32/42			RIX 05/40		
	p	***q***	**Tukey's posthoc**	***p***	***q***	**Tukey's posthoc**	***p***	***q***	**Tukey's posthoc**
WeightPND21	0.80	0.89		**1E-04**	**6E-04**	PD&ME<Std (p≤0.03)	0.16	0.28	
WeightPND60	**5E-03**	**0.03**	none	0.22	0.40		0.31	0.39	
OF TotalDist	0.27	0.44		0.99	1.00		0.10	0.19	
OF %Center	**2E-05**	**4E-04**	VDD<all (p≤0.02)	1.00	1.00		0.09	0.19	
OF VertCount	0.22	0.43		0.94	1.00		0.29	0.39	
OF Boli	0.17	0.38		0.53	0.66		0.25	0.37	
LD TotalDist	**8E-03**	**0.04**	VDD<ME (p<0.04)	0.28	0.47		**8E-03**	**0.05**	ME>PD
LD DistDark	**0.01**	**0.04**	ME>VDD (p<0.01)	0.14	0.31		**0.04**	0.11	ME>PD
LD DistLight	**9E-03**	**0.04**	PD>VDD (p<0.04)	0.51	0.66		**0.04**	0.11	ME>PD
LD Transition	0.24	0.43		0.47	0.66		**0.01**	**0.05**	ME>PD
LD %Light	**0.03**	0.08	none	0.90	1.00		0.17	0.28	
LD Boli	0.73	0.89		0.16	0.31		0.49	0.54	
SIH-T1	0.06	0.16		**3E-04**	**1E-03**	VDD<all (p≤0.03)	0.83	0.83	
SIH-T2	0.96	0.96		**0.05**	0.15		**0.02**	**0.05**	ME<PD
deltaSIH	0.32	0.46		0.13	0.31		**0.02**	**0.05**	ME<PD
FST %Imb	0.78	0.89		0.36	0.56		0.26	0.37	
FST Boli	**4E-03**	**0.03**	ME>Std&VDD (p≤0.05)	**0.04**	0.13	none	0.40	0.47	
BasalCORT	0.29	0.44		**0.04**	0.13	none	**5E-03**	**0.05**	ME>PD
StressCORT	0.89	0.93		**1E-07**	**3E-06**	VDD>all (p≤0.005)	0.81	0.83	
deltaCORT	0.36	0.48		**4E-05**	**4E-04**	VDD>all (p≤0.001)	**9E-03**	**0.05**	ME<PD

Grey shading indicates phenotypes that had an overall significant diet-by-RIX effect and gold shading are phenotypes that did not have an overall effect but do have an effect of diet exposure within that particular RIX line. Tukey's posthoc analysis was performed to determine which diet group was causing the effect. CORT = corticosterone; deltaCORT = (Stress CORT – Basal CORT); deltaSIH = (T2-T1); FST = forced swim test; Imb = immobile; LD = light/dark; OF = open field; ME = methyl enriched; PND = postnatal day; PD = protein deficient; SIH = stress-induced hyperthermia; SIH-T1 = basal temperature; SIH-T2 = post-stress temperature; Std = Standard; VDD = vitamin D deficient; Vert = vertical

Supplemental Table 7. Percent variance explained by PO, diet, and diet-by-PO

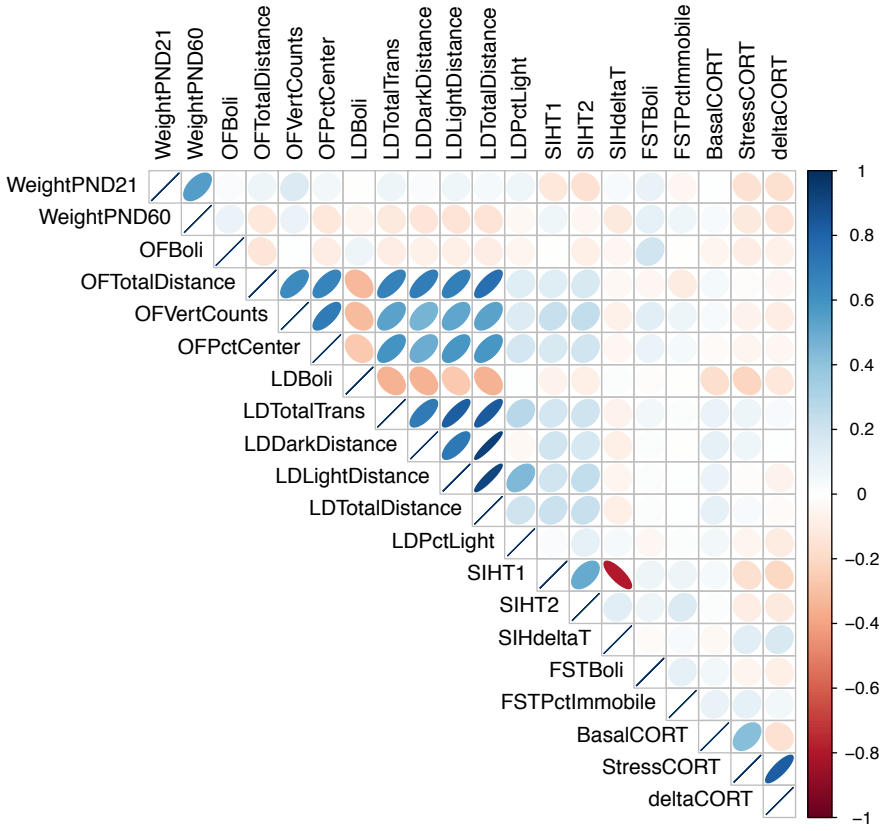
	RIX 01/11				RIX 41/51				RIX 04/17				RIX 23/47				RIX 06/26			
Phenotype	**PO**	**Diet**	**DxP**	**R**	**PO**	**Diet**	**DxP**	**R**	**PO**	**Diet**	**DxP**	**R**	**PO**	**Diet**	**DxP**	**R**	**PO**	**Diet**	**DxP**	**R**
WeightPND21	0	**76**	9	15	**6**	**81**	**7**	6	**22**	**64**	**10**	4	**59**	**37**	2	2	**7**	**71**	**14**	8
WeightPND60	**16**	**26**	**12**	45	**5**	**54**	3	37	**13**	**44**	2	41	**49**	**33**	6	12	4	**41**	**10**	45
OF TotalDist	1	7	3	89	0	8	6	86	2	13	10	75	0	11	5	84	**14**	5	1	80
OF %Center	0	1	1	97	0	**7**	6	87	2	1	9	89	3	11	3	84	4	2	7	86
OF VertCount	**13**	4	3	81	**6**	13	1	79	**10**	6	**10**	74	**12**	**24**	4	59	**19**	4	2	75
OF Boli	0	**7**	4	88	1	2	1	95	**7**	4	4	86	0	14	**19**	67	1	8	4	87
LD TotalDist	3	3	6	89	1	7	2	90	0	6	3	90	0	11	3	86	2	4	4	90
LD DistDark	2	5	6	88	0	7	1	92	1	12	1	86	2	13	3	82	1	5	3	91
LD DistLight	3	1	5	90	4	8	3	85	0	3	9	88	0	**24**	4	72	3	1	7	88
LD Transition	1	1	5	94	0	7	7	86	2	1	4	93	1	9	3	87	1	5	4	91
LD %Light	0	3	2	96	1	8	2	89	2	3	6	88	2	**15**	7	76	3	1	4	92
LD Boli	0	**10**	**7**	83	0	**7**	0	93	0	2	8	89	1	**19**	**14**	67	1	7	3	90
SIH-T1	0	**23**	2	75	2	6	5	87	**21**	24	1	54	0	31	4	65	0	**17**	**12**	70
SIH-T2	0	**14**	4	82	4	**9**	**12**	74	**5**	**23**	2	70	6	16	2	76	0	16	4	80
deltaSIH	0	**13**	1	85	0	11	3	85	**13**	15	3	69	1	26	3	69	0	**14**	**13**	73
FST %Imb	**5**	2	3	90	0	3	1	96	2	5	**9**	84	**17**	**22**	1	61	1	0	3	95
FST Boli	4	5	2	89	1	2	5	92	0	1	0	99	2	17	8	72	**5**	**17**	8	70
BasalCORT	0	5	1	94	0	9	2	89	**4**	**53**	1	42	0	3	3	94	**13**	20	1	67
StressCORT	3	0	3	94	0	**29**	**7**	63	**16**	**24**	2	58	3	**64**	1	32	**8**	**28**	**9**	54
deltaCORT	3	4	3	90	0	**33**	5	61	**12**	**19**	2	67	2	**30**	1	66	0	12	10	77

Supplemental Table 7 continued

	RIX 03/14				RIX 35/62				RIX 32/42				RIX 05/40			
Phenotype	**PO**	**Diet**	**DxP**	**R**	**PO**	**Diet**	**DxP**	**R**	**PO**	**Diet**	**DxP**	**R**	**PO**	**Diet**	**DxP**	**R**
WeightPND21	1	**33**	**38**	27	22	35	12	30	4	**67**	**15**	14	**47**	9	**24**	20
WeightPND60	0	**32**	7	61	1	**29**	6	64	2	14	4	80	**58**	2	0	40
OF TotalDist	**9**	3	0	88	7	27	2	64	0	0	5	95	**7**	4	0	89
OF %Center	**8**	7	2	83	3	**52**	10	35	1	0	3	96	5	9	0	87
OF VertCount	3	**18**	9	69	1	12	9	77	0	0	5	95	2	2	1	95
OF Boli	1	**47**	6	46	1	12	18	69	0	3	2	95	4	2	0	94
LD TotalDist	6	11	2	81	**8**	**24**	9	59	0	5	3	92	**18**	**10**	0	71
LD DistDark	0	**16**	2	82	1	**33**	15	50	2	6	2	90	**19**	**5**	0	76
LD DistLight	**15**	7	1	77	**9**	**26**	9	56	0	2	2	95	**5**	**6**	0	89
LD Transition	**11**	1	2	87	**12**	10	1	76	1	3	4	92	**10**	**8**	0	82
LD %Light	**18**	9	8	66	**10**	**20**	11	59	0	1	1	98	0	3	1	96
LD Boli	2	**15**	11	73	0	43	0	56	0	7	6	87	7	1	1	91
SIH-T1	0	**20**	2	77	3	17	6	74	2	**29**	2	67	1	0	5	94
SIH-T2	0	9	2	89	4	1	10	85	0	**10**	2	88	0	**11**	0	88
deltaSIH	0	11	1	88	4	20	3	74	1	14	1	84	3	**10**	1	86
FST %Imb	**9**	9	7	76	5	4	2	89	1	7	8	84	10	3	6	81
FST Boli	2	14	2	82	6	**28**	6	60	3	**10**	3	85	2	1	4	93
BasalCORT	0	48	1	51	0	28	1	71	0	**15**	3	82	3	**18**	1	78
StressCORT	2	7	5	87	**23**	0	5	72	1	**41**	2	55	0	0	4	96
deltaCORT	2	0	10	88	15	3	2	79	1	**36**	4	59	0	**19**	2	79

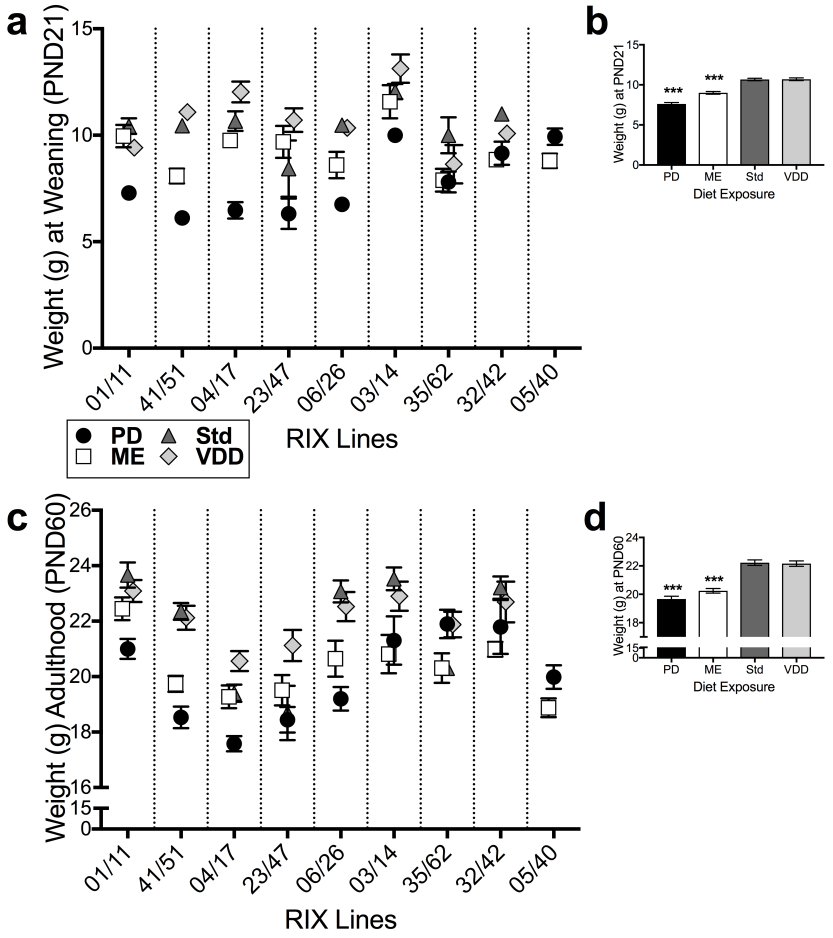
Percent variance explained by either parent-of-origin (PO), diet, diet-by-PO, or the residuals of the lmer models (R; i.e. random effects, noise of the phenotype) are shown. Phenotypes that had a significant effect at the *p* value level are shaded grey and **bolded.** CORT = corticosterone; deltaCORT = (Stress CORT – Basal CORT); Dist = distance; Imb = Immobile; LD = light/dark; OF = open field; PND = postnatal day; SIH-T1 = basal temperature; SIH-T2 = post-stress temperature; deltaSIH = (T2-T1); Vert = vertical

Supplemental Figure 1. Correlation of behaviors



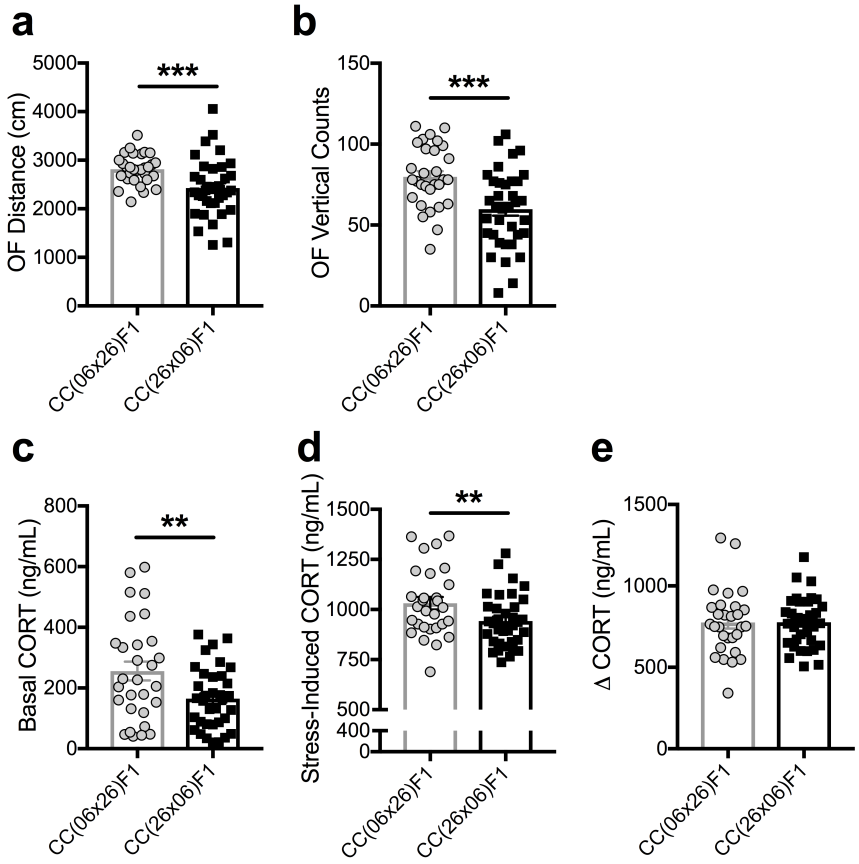
Spearman correlation of 20 phenotypes. Color and direction of the ellipse indicate direction of the correlation. Intensity of color and size of ellipse indicate strength of correlation. The *p* value threshold for significance was adjusted to $p<0.00026$ to correct for multiple tests.

Supplemental Figure 2. Body weight is influenced by diet-by-RIX interactions



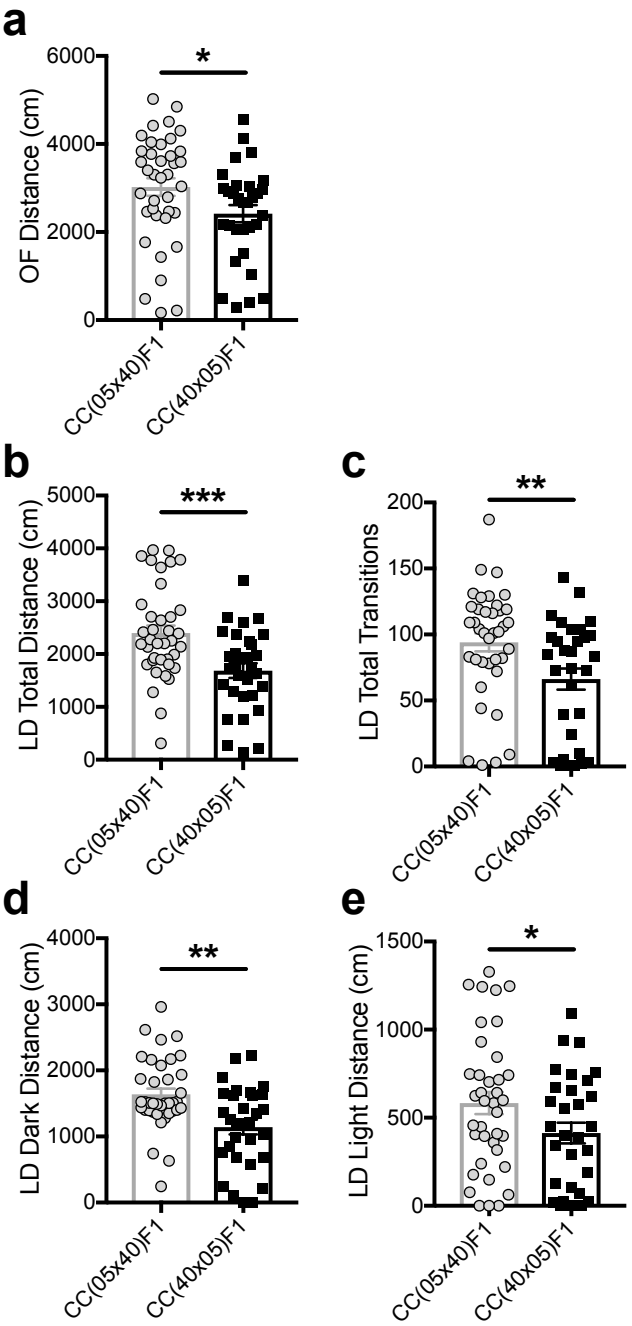
Data points are means of diet exposure groups. Error bars are SEM. There was a significant diet-by-RIX effect on **(a)** body weight at weaning (p=1.5x10^{-7}, q=7.4x10^{-7}) and **(c)** in adulthood (p=0.002, q=0.004). There was a significant overall effect of perinatal diet on **(b)** weight at weaning (p=2.2x10^{-16}, q<2.2=10^{-15}) and **(d)** in adulthood (p=2.2x10^{-16}, q=2.2x10^{-15}). Posthoc analyses revealed that females exposed to PD (N=158) and ME (N=177) weighed significantly less compared to Std (N=170) and VDD (N=180) at both time points (***p<0.001).

Supplemental Figure 3. Parent-of-origin affects locomotion, exploratory behavior and stress response in RIX 06/26 females



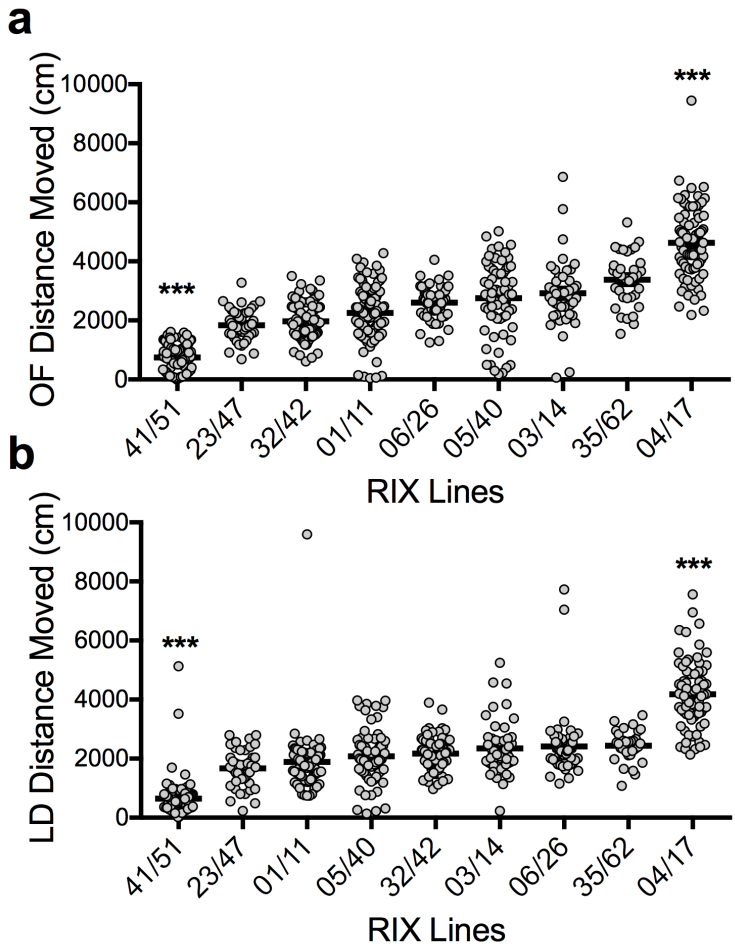
Data points are individual animals with bars indicating means. Error bars are SEM. CC(06x26)F1 females (N=30) showed **(a)** increased total distance (***p=0.001, q=0.01) and **(b)** vertical counts in the OF (***p=0.0002, q=0.004) compared to CC(26x06)F1 females (N=36). **(c)** CC(06x26)F1 females (N=29) had increased basal (**p=0.004, q=0.02) and **(d)** stress-induced CORT levels following restraint stress (**p=0.003, q=0.02), **(e)** but no difference in change in CORT (p>0.05). Data shown in d is also shown in Figure 6a.

Supplemental Figure 4. Parent-of-origin affects locomotor behavior in the OF and LD in RIX 05/40 females



Data points are individual animals with bars indicating means. Error bars are SEM. CC(05x40)F1 females (N=38) exhibited increased locomotion compared to CC(40x05)F1 females (N=31) in **(a)** distance in the OF test (**p*=0.04, *q*=0.14), and the LD test as measured by **(b)** total distance (****p*=0.0002, *q*=0.003), **(c)** total transitions (***p*=0.006, *q*=0.03), (d) distance on the dark side (***p*=0.002, *q*=0.01), and (e) distance on the light side (**p*=0.05, *q*=0.14).

Supplemental Figure 5. RIX significantly affects locomotor behavior in the OF and LD assays



Data from individual females along with RIX mean (black bar) are shown with the RIX lines arranged from lowest to highest distance traveled in the OF and LD tests. There was a significant effect of RIX on (a) total distance in the OF test (p=2.0x10^{-16}, q=3.4x10^{-16}; Ns= 119, 46, 96, 110, 66, 69, 48, 34, 83) and (b) total distance in the LD test (p=2.0x10^{-16}, q=3.4x10^{-16}; Ns= 122, 50, 116, 69, 96, 48, 66, 34, 79). ***posthoc revealed that RIX 41/51 and RIX 04/17 were significantly different from all other lines in both tests (p<0.001).

APPENDIX B

Supplemental Material for Chapter 3

SUPPLEMENTAL METHODS

Fast Scan Cyclic Voltammetry

Carbon-fiber microelectrodes were prepared on the day of FSCV recordings for each animal. Glass-encased electrodes had 70-120 μm of the fiber exposed, and electrodes were calibrated post-experiment as previously described (Robinson *et al*, 2009) to obtain current measurements for known concentrations of dopamine.

On the experiment day, animals were anesthetized with urethane (50% w/w in saline, 1.8 g/kg i.p.) and placed in a stereotaxic frame atop a heated pad. Holes were drilled in the skull above the ventral tegmental area (VTA; AP -3.8, ML -0.2), nucleus accumbens (NAc; AP +1.2, ML -1.2), and, contralaterally, two holes were drilled for a reference electrode and screw. The Ag/AgCl reference electrode was lowered and fixed using a screw and dental cement. The stimulating electrode (bipolar, parallel, stainless-steel, 0.2mm in diameter per tip; Plastics One, Roanoke, VA) was lowered -4.0mm from the skull surface into the VTA. Next, the animal and stereotaxic frame were placed in a Faraday chamber and the carbon fiber microelectrode was lowered into the NAc -4.0mm from the dura mater. A triangle waveform potential ramping from -0.4 V to +1.3 V and back at 400 V/s was applied to the carbon-fiber electrode at 60 Hz for 20 minutes to allow the fiber to condition to the tissue environment, then applied at 10Hz for the remainder of the experiment. Thereafter, the evoked dopamine signal was optimized by moving the stimulator and/or carbon-fiber electrode in 100 μM increments, as previously described (Shnitko *et al*, 2016).

Once optimal signal was achieved (signal-to-noise ratio higher than 20), the stimulating and recording electrodes were left in place and measurements of evoked dopamine release were collected using electrical stimulation (24 biphasic, square-wave pulses, 2 ms/phase, 125–185 μA, 60 Hz). All evoked dopamine signals were collected five minutes apart with a duration of 20 s per recording. After stable evoked release was achieved (less than 20% variability), a saline injection was given and three dopamine signal recordings were collected – this constituted the baseline measurement. Next, animals received 20 mg/kg, (IP) or an equivalent volume of saline and evoked dopamine as collected every five minutes for one hour.

FSCV Data Analysis

Electrochemical signals measured upon electrical stimulation were analyzed from the color plots (TarHeel CV 6.0, Department of Chemistry, UNC Chapel Hill) as previously described (Shnitko *et al*, 2016). Color plots were created by plotting current as a function of applied potential (y-axis) over time (x-axis). Background-subtracted cyclic voltammograms were obtained by subtracting voltammograms collected during baseline recording (typically the 12 scans 1.2 s immediately preceding stimulation) from those collected during and after stimulation (Shnitko and Robinson, 2014). An increase in current at approximately 0.65 V versus the Ag/AgCl reference electrode was considered to be due to oxidation of dopamine. This was confirmed by analyzing the background-subtracted cyclic voltammogram averaged over 500 ms around the peak of the oxidation current. Dopamine oxidation current was converted to dopamine concentration, or [DA] in μM, using *in vitro* calibration factors obtained following recording. All statistical analyses of release and uptake were conducted on dopamine concentration.

Dopamine Release and Clearance Parameters

For each animal, traces for baseline dopamine release were created by averaging the concentration-versus-time trace from the three recordings following saline vehicle injection. For animals receiving cocaine injection, the recording with maximal dopamine release was determined and this concentration-versus-time trace was used to assess dopamine release and clearance dynamics following cocaine injection. Kinetics of dopamine release were assessed using metrics adapted from previous studies (Saddoris, 2016; Yorgason *et al*, 2011). In the file with the identified peak cocaine effect, T_{max} was the time (post cocaine) that file was collected and $[DA]_{max}$ was the maximal dopamine concentration observed following electrical stimulation. We also measured the latency from the end of the stimulation to the $[DA]_{max}$ and the full width at half height (FWHH), or the width of the dopamine signal at 50% of $[DA]_{max}$. Values for T80 and T30 were then determined as the time at which the [DA] decayed to 80% or 30% of $[DA]_{max}$, respectively. Lastly, slope was determined by calculating the slope of the line between the T80 and T30 points.

Supplemental Table 1: Brain concentrations of cocaine and metabolites

[ng/g]	Affect Behavior	RIX 41/51 *(n=15)* Mean	RIX 41/51 *(n=15)* StDev	RIX 04/17 *(n=15)* Mean	RIX 04/17 *(n=15)* StDev
Cocaine (COC)	Active	5549.96	2319.64	6358.86	2611.19
Metabolites					
Benzoyl Ecgonine (BZE)	Active	63.69	65.75	62.19	27.28
Norcocaine (NOR)	Inactive	661.58	357.98	647.45	372.67

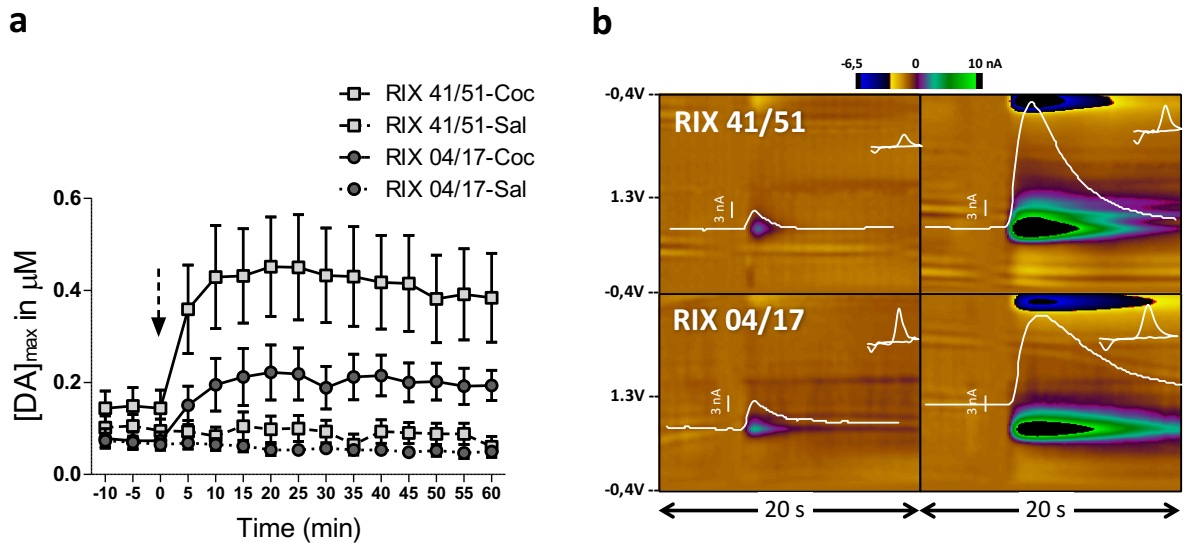
Affect behavior refers to whether or not the drug or metabolite has been shown to have an effect on behavior phenotypes (i.e., psychomotor activation).

Supplemental Table 2. Monoamine tissue content in dorsal striatum and nucleus accumbens

DORSAL STRIATUM												
	RIX 41/51						RIX 04/17					
	Basal			Cocaine-Induced			Basal			Cocaine-Induced		
[ng/mg]	*F (n=2)*	*M (n=4)*	*Both (n=6)*	*F (n=4)*	*M (n=5)*	*Both (n=9)*	*F (n=4)*	*M (n=4)*	*Both (n=8)*	*F (n=3)*	*M (n=4)*	*Both (n=7)*
Dopamine (DA)	43.8±0.8	50.9±32	48.6±25.1	50.4±18	107.0±41	81.8±43	53.2±4.6	45.0±41	49.1±27	69.8±41	51.5±6	59.4±26
DOPAC (D)	50.5±0.2	42.6±31	45.2±24.7	33.0±7.9	24.4±17	28.2±14	49.5±3.4	31.0±22	40.3±17	18.9±6.1	29.9±10	25.2±10
HVA	17.4±1.6	18.5±11	18.1±8.8	21.6±3.2	21.8±4.9	21.7±4**	16.5±1.0	10.7±6.6	13.6±5.3	15.1±2.0	14.6±3.7	14.8±3**
3-MT	7.1±0.6	6.6±5.1	6.8±3.9^{+}	9.2±2.4	12.2±2.0	10.8±2.6^{+}	8.5±1.1	5.4±6.4	7.0±4.5	9.8±9.2	10.1±1.4	10.0±5.4
(D+HVA+3MT)/DA	1.7±.03	1.6±0.7	1.6±0.6	1.4±0.4	0.68±0.6	0.98±0.6	1.4±0.2	1.4±0.8	1.4±0.5	0.84±0.6	1.1±0.4	0.97±0.4
Serotonin (5-HT)	3.4±0.4	3.8±1.1	3.7±0.9*	1.9±0.5	4.5±2.7	3.4±2.4	2.5±0.8	1.8±1.2	2.1±1*	4.3±2.2	2.5±0.5	3.3±1.6
5-HIAA	9.7±1.8	9.5±2.3	9.6±2$^{**,+++}$	6.4±0.8	6.1±1.3	6.2±1^{+++}	7.3±1.2	5.5±1.6	6.4±1.6**	4.3±3.8	6.2±1.2	5.4±2.5
5-HIAA/5-HT	2.9±0.9	2.6±0.7	2.7±0.7	3.4±0.7	1.0±0.1	2.2±1.4	3.0±0.6	2.0±0.2	2.6±0.7	0.79±0.7	2.6±0.9	1.8±1.2
Norepinephrine	0.58±0.8	0.46±0.9	0.50±0.8	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.54±0.7	0.27±0.5	0.0±0.0	0.28±0.3	0.16±0.3
NUCLEUS ACCUMBENS												
	RIX 41/51						RIX 04/17					
	Basal			Cocaine-Induced			Basal			Cocaine-Induced		
[ng/mg]	*F (n=2)*	*M (n=3)*	*Both (n=5)*	*F (n=4)*	*M (n=5)*	*Both (n=9)*	*F (n=4)*	*M (n=3)*	*Both (n=7)*	*F (n=2)*	*M (n=4)*	*Both (n=6)*
Dopamine (DA)	20.6±0.8	29.7±24	26.1±17.4	42.1±28	48.9±20.8	45.9±23	24.0±9.1	15.5±21	20.4±14	30.2±23	19.0±14	22.7±16.2
DOPAC (D)	44.6±3.2	35.1±27	38.9±19.7^{+}	28.4±3.4	10.2±6.1	18.3±11^{+}	44.8±21	9.0±5.3	29.5±25	26.5±18	27.8±7.8	27.4±10.2
HVA	15.1±0.4	12.2±8.8	13.4±6.4	16.0±3.2	9.3±3.0	12.3±4.5	14.3±6.6	4.7±0.5	10.2±6.9	10.6±3.1	10.5±4.5	10.5±3.8
3-MT	4.2±0.3	5.2±4.6	4.8±3.3	8.0±4.0	6.9±3.1	7.4±3.3	4.4±1.5	1.2±2.1	3.0±2.3	5.7±8.1	5.3±5.4	5.4±5.5
(D+HVA+3MT)/DA	3.1±.004	1.9±0.3	2.4±0.7	2.2±2.2	0.54±0.1	1.3±1.6	2.6±0.4	2.3±1.8	2.5±1.1	2.3±2.2	2.8±2.2	2.6±1.4
Serotonin (5-HT)	3.0±0.1	4.6±1.9	4.0±1.6	2.6±1$^{\#\#\#}$	8.4±1.9$^{\#\#\#}$	5.8±3.4**	3.2±0.6	3.5±2.4	3.3±1.4	2.5±3.5	1.9±1.2	2.1±1.9**
5-HIAA	9.6±1.6	8.6±1.3	9.0±1.3	8.7±2.8	5.8±2.1	7.1±2.7	10.7±2.3	9.5±3.8	10.2±2.8	6.7±1.2	8.9±3.1	8.1±2.7
5-HIAA/5-HT	3.1±0.4	2.0±0.6	2.5±0.8	4.5±3.5	0.69±0.2	2.4±2.9	3.4±0.4	4.0±2.8	3.7±1.7	1.2±na	5.5±2.4	4.7±2.9
Norepinephrine	0.26±0.4	0.54±0.5	0.43±0.4**	1.1±2.3	2.5±4.4	1.9±3.5	2.1±2.1$^{\#}$	7.6±2.5$^{\#}$	4.5±3.6**	0.0±0.0	3.0±2.1	2.0±2.2

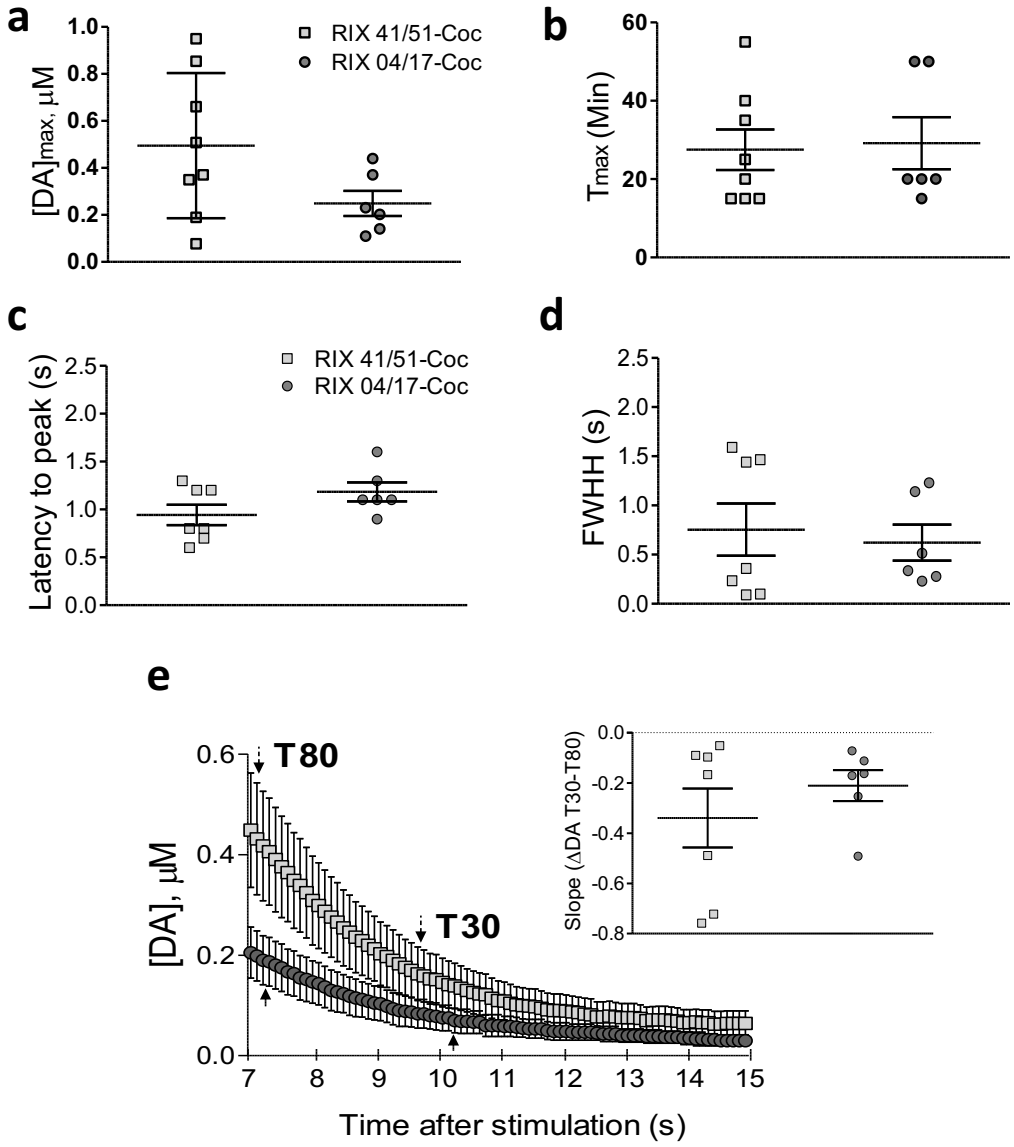
*RIX effect within treatment group; #sex effect within RIX and treatment group; +Effect of treatment within RIX

Supplemental Figure 1. Cocaine enhanced electrically-evoked dopamine release in the nucleus accumbens of RIX 41/51 and RIX 04/17 anesthetized mice



(a) Data shown in Figure 4b, plotted in 5-min increments. Cocaine (Coc) increased evoked DA release in both strains. Dashed arrow indicates Coc or saline (SAL) injection. **(b)** Individual recordings illustrating the effects of 20 mg/kg Coc IP on electrically-evoked DA release in the nucleus accumbens by electrical stimulation of the ventral tegmental area, 10 minutes after vehicle (left panels) and 20 minutes after Coc (right panels), in urethane anesthetized RIX 41/51 (top) and RIX 04/17 (bottom) mice. DA was monitored using fast-scan cyclic voltammetry at carbon-fiber microelectrodes. The color plots show current (color) at applied potential (y-axis) over time (x-axis). Overlaid are the concentration-versus time traces for the current at the oxidation potential of DA, as well as the background-subtracted cyclic voltammograms that confirm the current is dopaminergic.

Supplemental Figure 2. Kinetic factors of dopamine release after 20 mg/kg cocaine in RIX 41/51 and RIX 04/17 anesthetized mice



The recording with the peak effect of cocaine (Coc) on $[DA]_{max}$ was identified and characterized, as described in the supplemental methods, by the following parameters: **(a)** $[DA]_{max}$ at the peak Coc response, **(b)** the time of the peak Coc response (T_{max}), **(c)** the latency from the end of the stimulation to the $[DA]_{max}$, **(d)** full width at half height (FWHH), and **(e)** the slope of DA clearance after evoked release. None of these parameters significantly differed between strains. Arrows in **(e)** indicate the average time of T80 and T30 for each strain. Data are expressed mean ± SEM (n=6-8/group).

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