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RESEARCH PAPER

Discovery of early life stress interacting and sex-specific quantitative trait loci impacting cocaine responsiveness

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Background and Purpose: Addiction vulnerability involves complex gene X environment interactions leading to a pathological response to drugs. Identification of the genes involved in these interactions is an important step in understanding the underlying neurobiology and rarely have such analyses examined sex-specific influences. To dissect this interaction, we examined the impact of prenatal stress (PNS) on cocaine responsiveness in male and female mice of the BXD recombinant inbred panel.

Experimental Approach: BXD strains were subjected to timed mating and assigned to PNS or control groups. PNS dams were subjected to restraint stress (1-hr restraint, three times daily) starting between embryonic day (E) 11 and 14 and continued until parturition. Adult male and female, control and PNS offspring were tested for locomotor response to initial and repeated cocaine injections (sensitization) as well as cocaine-induced conditioned place preference (CPP).

Key Results: Strain, PNS, and sex interacted to modulate initial and sensitized cocaine-induced locomotion, as well as CPP. Moreover, a quantitative trait locus (QTL) interacting with PNS regulating initial locomotor response to cocaine (chromosome X, 37.91 to 50.95 Mb) was identified. Also PNS-independent, female-specific QTLs regulating CPP (chromosome 11, 65.50 to 81.31 Mb) and sensitized cocaine-induced locomotion (chromosome 16, 95.79 to 98.32 Mb) were identified. Publicly available mRNA expression data were utilized to identify cis-eQTL and transcript covariation with the behavioural phenotype to prioritize candidate genes; including *Aifm1*.

Conclusions and Implications: These QTL encompass genes that may moderate genetic susceptibility to PNS and interact with sex to determine adult responsiveness to cocaine and addiction vulnerability.

LINKED ARTICLES: This article is part of a themed section on The Importance of Sex Differences in Pharmacology Research. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v176.21/issuetoc

1 | INTRODUCTION

Cocaine abuse disorder is a neuropsychiatric condition involving persistent and dysregulated use of cocaine (Volkow, Fowler, & Wang, 2003) that produces health, social, and legal consequences, which collectively pose a major burden on society in the form of elevated health care costs, lost productivity, and increased crime rates (McGinnis & Foege, 1999). Unfortunately, the aetiology of cocaine addiction remains poorly understood. Vulnerability to cocaine addiction appears to result from complex genetic and environmental

Abbreviations: *Aifm1*, apoptosis inducing factor, mitochondria associated 1; alQTLXPNS, QTL by PNS interaction discovered for acute cocaine-induced locomotion; B6, C57BL/6J mouse strain; CPP, conditioned place preference; cppQTL, QTL discovered for conditioned place preference; D2, DBA/2J mouse strain; LRS, likelihood ratio statistic; *Pitpna*, phosphatidylinositol transfer protein, α; PNS, prenatal stress; QTL, quantitative trait locus; *Ripk4*, receptor-interacting serine–threonine kinase 4; sensQTL, QTL discovered for sensitization to cocaine-induced locomotion.

factors; including influences of genetic and gonadal sex (Agrawal et al., 2012; Becker & Koob, 2016; Kerstetter & Kippin, 2011). To date, some genes have been associated with this disorder in human populations, and it is expected that many more have not been identified (Gelernter et al., 2014). More critically, such studies are challenged by the gross limitations of forward genetic approaches in humans, including limited ability to dissect environmental factors and gene X environment interactions, particularly the contribution of environmental factors in utero (Burmeister, McInnis, & Zöllner, 2008; Henriksen, Nordgaard, & Jansson, 2017), and these limitations in turn hinder the development of a mechanistic understanding of aetiology. Here, we dissect the impact of gene-prenatal environmental interactions on cocaine responsiveness of adult male and female mice from the BXD recombinant inbred panel.

Early life stressors, including prenatal stress (PNS), are important aetiological factors for many psychiatric disorders, including cocaine abuse (Enoch, 2011). In preclinical models, PNS increases behavioural and neurochemical responsiveness to cocaine during adulthood, including elevated locomotion, self-administration, resistance to extinction, and augmented cocaine primed reinstatement as well as enhanced dopamine responsiveness (Kippin, Szumlinski, Kapasova, Rezner, & See, 2008; Thomas, Hu, Lee, Bhatnagar, & Becker, 2009). The effects of PNS are also moderated by genetic variants, as the effects of PNS on cocaine responsiveness are dependent on inbred (C57BL/6J vs. DBA/2J) mouse strain (Kippin, Campbell, Ploense, Knight, & Bagley, 2015). These data suggest that genetic variants interact with PNS to alter cocaine responsiveness, including an increase in cocaine reward sensitivity. Identification of these variants will enable the discovery of genes that interact with early life stress to moderate risk of cocaine addiction. In addition, many effects of PNS appear to be sex-dependent (Bale, 2011; Frye, Paris, Osborne, Campbell, & Kippin, 2011; Weinstock, 2011) necessitating investigation of the potential sex specificity of gene by environment interactions that affect cocaine responsiveness.

Substantial and widely available resources, including recombinant inbred panels, allow for preclinical forward genetic studies of cocaine abuse relevant behaviours. Furthermore, these resources can be utilized for quantitative trait locus (QTL) mapping strategies that incorporate early life stress with experimental rigour. To date, forward genetic strategies have identified QTLs for cocaine-related behaviours. The bulk of these efforts have focused on locomotor behaviour; multiple QTLs have been associated with cocaine-induced locomotion, with confirmation of some QTLs by secondary mapping approaches (Boyle & Gill, 2001, 2009; Gill & Boyle, 2003; Jones et al., 1999; Kumar et al., 2013; Phillips, Huson, & McKinnon, 1998; Tolliver, Belknap, Woods, & Carney, 1994). More recently, reward and reinforcement measures, including cocaine conditioned place preference (CPP) and self-administration, have also been characterized in forward genetic approaches (Dickson et al., 2015; Philip et al., 2010). Collectively, progress in identifying genes that modulate cocaine responsiveness will lead to a better understanding of the biological mediation of cocaine addiction. Incorporation of relevant environmental factors promises to expand this knowledge and to identify genes uniquely interacting with environmental factors; thus elucidating the biology of gene by environment interactions that contribute to cocaine abuse vulnerability.

What is already known

- Early life stressors can increase addiction risk.
- Genetics can interact with environmental factors to affect addiction risk.

What this study adds

- Identifies candidate genes that may moderate the effects of prenatal stress on cocaine responsiveness.
- Demonstrates sex as a factor that moderates the effects of early life stress on cocaine responsiveness.

What is the clinical significance

- Genes that moderate early life stress to affect addiction risk are potential targets for pharmacotherapy.

The BXD mouse panel is derived from C57BL/6J (B6) and DBA/2J (D2) progenitors (Peirce, Lu, Gu, Silver, & Williams, 2004). The PNS interactions observed in the progenitor strains (Kippin et al., 2015) indicate that the BXD panel is suitable for QTL analyses by PNS interaction mapping strategies. Here, we sought to characterize the effects of PNS on acute cocaine-induced locomotion, locomotor sensitization, and cocaine CPP on multiple BXD strains, including determination of potential sex-specific effects. Between-strain variance in the effects of PNS on these measures was utilized to map for QTLs that interact with PNS to alter cocaine responsiveness. Following identification of positional candidate genes, publicly available strain level mRNA expression in key brain regions was utilized to prioritize candidate genes. These efforts may serve as a preliminary step in identifying genes that interact with PNS to alter cocaine abuse liability.

2 | METHODS

2.1 | Subjects

BXD strains (n = 21; The Jackson Laboratory, Bar Harbor, MI) were housed in a temperature- and humidity-controlled vivarium on a 12-hr light–dark cycle. All mice were maintained on ad libitum mouse chow and water and housed in polycarbonate cages (30 × 8 cm) with wood-chip bedding (SANI-CHIPS, Montville, NJ), a paper nestlet and a red polycarbonate hut at a density of 2-5 mice per cage. All procedures were approved by the University of California at Santa Barbara Institutional Animal Care and Use Committee and conducted in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). Animal studies are reported in compliance with the ARRIVE

guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the *British Journal of Pharmacology*.

2.2 | Breeding

BXD strains were purchased from the Jackson Laboratory to establish a breeding colony in UCSB facilities (see the *x* axes of Figure 1 for strains used in the experiments). The offspring of this colony were used for timed-breeding that generated the subjects for behavioural analyses. Adult males and females, at 8 to 24 weeks of age, were paired for 4 days. Pregnancy was confirmed by weight gain and the dams were assigned to PNS or control conditions. Females that failed to conceive were re-subjected to the breeding procedures in future cohorts. Impregnated females were only used to generate a single litter of offspring, which were used in behavioural experiments. Male breeders were used for multiple cohorts.

2.3 | Maternal restraint stress

PNS was induced by subjecting pregnant mice to repeated restraint stress, 2 weeks following the initial breeding set-up. This corresponded to embryonic Day 11 through 14. The PNS dams were taken from the vivarium into the laboratory and were restrained in 50-ml conical tubes for 1 hr periods, three times a day. Each 1-hr stress session was separated by 1 hr of home cage access. Restraint continued daily until parturition. The control dams were left undisturbed in their home cage and were not removed from the vivarium during pregnancy.

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

2.4 | Cocaine CPP and locomotion

At 9 weeks of age, the PNS and control offspring were subjected to a cocaine CPP protocol. This procedure allows for assessment of the rewarding efficacy of cocaine, as well as exploratory and locomotor measures that include acute and sensitized cocaine-induced locomotion, locomotion in a novel environment, and locomotion after saline injection (Tzschentke, 2007). The CPP procedure involves a 2-compartment chamber (46L × 24W × 18H [cm]) that contains distinct visual (marble and wood coloured wall paper) and tactile cues (textured plexiglass and smooth floor). Initially, mice were placed into the chamber with access to both sides and with no injections (pretest). Time spent in each compartment and locomotion were measured by video tracking with Any-Maze software (Stoelting, Wood Dale, IL, USA). Although, overall, mice spent approximately equal times in both chambers, assignment of cocaine- and saline-paired compartments was biased, with cocaine paired with the un-preferred side as these procedures proved previously to reveal PNS X parental strain interactions (Kippin et al., 2015). Conditioning consisted of four saline and four cocaine once daily sessions, alternating between saline and cocaine across days. The first conditioning day was always saline. Mice were injected with saline (i.p., 10-ml·kg^{-1} body weight) or cocaine (i.p., 10-mg·kg^{-1} body weight, 10-ml·kg^{-1} body weight; which is in the mid-range of the dose–response curve supporting CPP and is sensitive to PNS X progenitor strain interactions; Kippin et al., 2015) and immediately placed into the assigned compartment for 15 min. After completion of conditioning, the mice were placed, without injection, into the chamber with access to both compartments for 15 min (posttest). CPP was measured as the shift in time spent in the cocaine-paired compartment from pretest to posttest. Horizontal distance travelled was measured to quantify locomotion and was tracked during pretest, posttest, and all conditioning sessions. Acute cocaine-induced locomotion was measured as the difference between the first saline-conditioning session and the first cocaine-conditioning session. Cocaine-induced locomotor sensitization was measured as the difference between the fourth and first cocaine-conditioning session. Blinding was not performed because strains can often be distinguished visually, and all testing was performed by automated software, limiting the potential for experimenter bias.

A minimum of four litters were represented in both PNS and control conditions for each strain, with a maximum of three mice per sex selected from each litter. See Table 1 for group sample sizes. These sample sizes are expected to approach the maximum for gains in strain mean and genetic marker correlations for traits of comparable heritability to those in the present paper (Belknap, 1998; Crusio, 2004). Variation in sample size between groups is largely accounted for by differences in breeding performance between strains.

2.5 | Data and statistical analysis

Data were assessed by three-way ANOVA with strain, condition, and sex as factors. Individual mice that were more than two SDs from the group mean (calculated within condition and sex) were excluded. Significant main effects or interactions involving sex were followed by main effects (using the grand mean error) 2-way ANOVA tests within sex. The α level for all analyses was set at 0.05. Heritability was calculated by taking the r^2 term from a one-way ANOVA with strain as a factor within condition and sex. This statistic determines the proportion of variance accounted for by strain and is a measure of broad sense heritability. Two SD outliers were not excluded for heritability estimates. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology.

2.6 | QTL analysis

Strain data for cocaine responsiveness measures including acute locomotion, locomotor sensitization, and CPP were subjected to interval mapping for QTL discovery. Data were uploaded to genenetwork (www.genenetwork.org) and subject to interval mapping by Haley–Knott regression to generate likelihood ratio statistics (LRS) across the entire genome (Haley & Knott, 1992). Significance thresholds for LRS are generated by randomly permuting the strain IDs and means

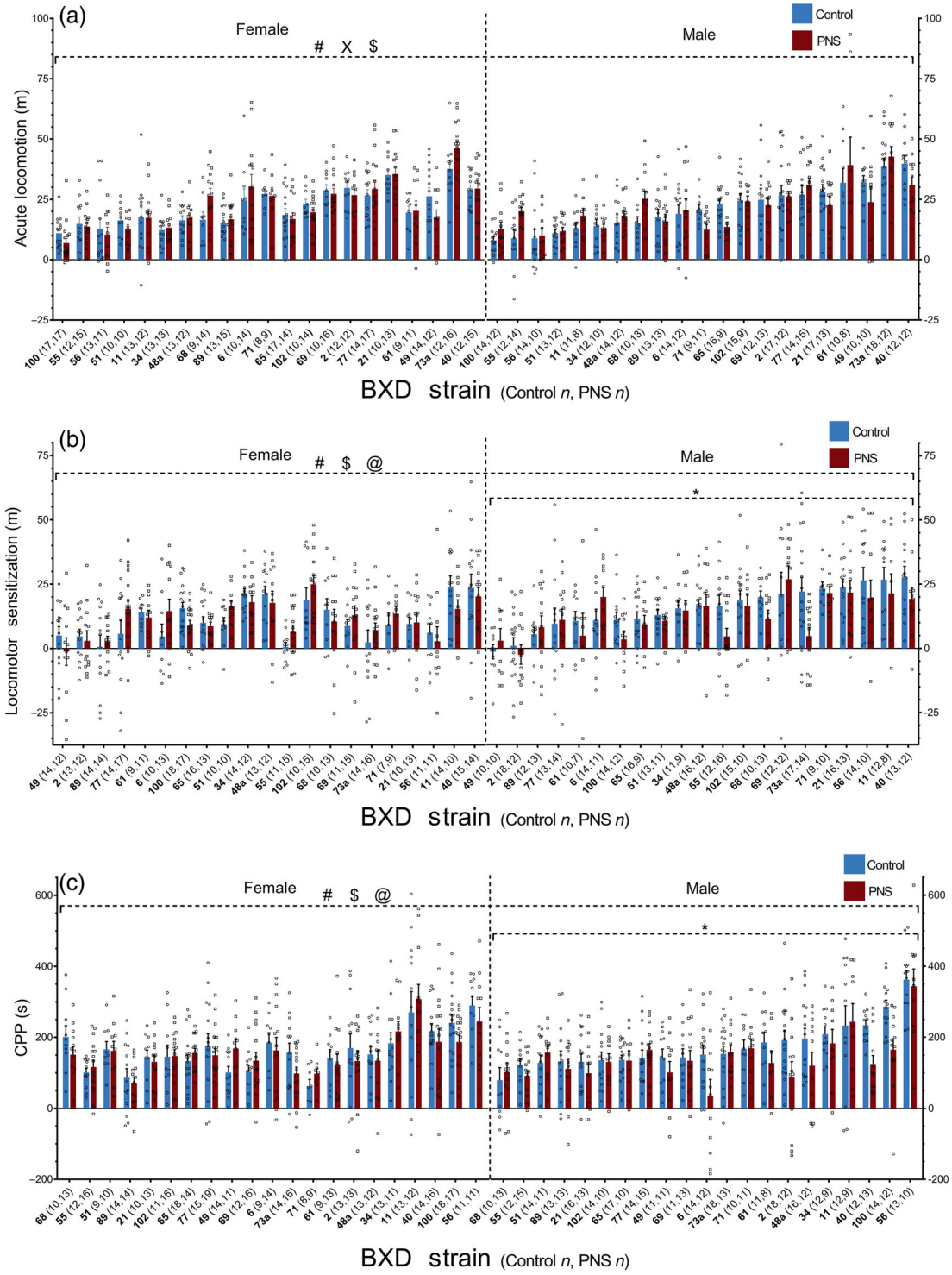

FIGURE 1 Effects of sex, strain, and PNS on cocaine (10-mg·kg^{-1} IP X 4) responsiveness in adult BXD mice. Strains are rank ordered on the *x* axis by the control male means. (a) Acute cocaine-induced locomotion is impacted by sex X strain and PNS X strain interactions. (b) Sensitization of cocaine-induced locomotion is impacted by sex X strain and PNS X sex interactions. Within-sex analysis revealed an effect of PNS in males but not females. (c) Conditioned place preference is impacted by sex X strain and PNS X sex interactions. Within sex analysis revealed an effect of PNS in males but not females. All data are plotted as mean with SEM. # = significant main effect of strain, $ = significant strain by sex interaction, X = significant strain by PNS interaction, @ = significant sex by PNS interaction, * = significant main effect of PNS

1,000 times and then mapping each of those permutations (Churchill & Doerge, 1994). The peak LRS that occurs in 5% of these permutations is used as the significance threshold; this corresponds to a genome wide *P* value of 0.05. Any locus with an LRS that exceeds this threshold was deemed as a significant QTL. A suggestive threshold, which corresponds to a genome-wide *P* value of 0.63, was also

TABLE 1 Group sample sizes

Strain	Control female	Control male	PNS female	PNS male
BXD2	13	18	14	12
BXD6	10	14	14	12
BXD11	14	12	12	9
BXD21	10	17	14	13
BXD34	14	12	13	10
BXD40	15	13	16	13
BXD48a	13	16	12	12
BXD49	14	11	12	11
BXD51	10	14	10	12
BXD55	12	12	16	16
BXD56	13	14	12	10
BXD61	9	11	13	9
BXD65	18	17	14	10
BXD68	10	10	14	14
BXD69	12	12	16	13
BXD71	8	10	9	11
BXD73a	14	18	17	14
BXD77	15	15	19	15
BXD89	14	13	15	13
BXD100	18	15	18	13
BXD102	11	15	16	10

determined. Any locus with an LRS that exceeds this threshold was determined a suggestive QTL. Outlier strain means were determined by Tukey's interquartile range with a 1.5 constant. Where outliers were identified, the values were winsorized (Shete et al., 2004).

QTL confidence intervals were determined by 2-LOD (1 LOD = 4.61 LRS) drop-off intervals from the peak marker, which is estimated to provide greater than 95% coverage (Dupuis & Siegmund, 1999).

2.7 | Analyses of QTL X PNS interactions

QTLs that interact with PNS were determined by subtracting the control mean from the PNS mean for each strain (difference score). The strain difference scores were subjected to interval mapping on genenetwork in order to determine QTL by PNS interactions (Lowry et al., 2013). Conversely, main effect QTLs were determined by adding the PNS and control strain means (sum score). Where sex was found to interact with PNS in the ANOVA results, separate means, difference, and sum scores were calculated and mapped for each sex.

2.8 | Prioritization of positional candidates

Significant QTLs were investigated by determining all genes within the 2-LOD support interval. Candidate genes were prioritized by considering those with cis-eQTL and transcript levels which covary with the behavioural phenotype.

2.9 | cis-eQTL

QTLminer (genenetwork.org; RRID:SCR_002388) was used to determine all cis-eQTLs in the 2-LOD interval for the following brain regions: whole brain (UTHSC Mouse BXD Whole Brain RNA Sequence [Nov12] RPKM), amygdala (INIA Amygdala Cohort Affy MoGene 1.0 ST [Mar11] RMA), cerebellum (SJUT Cerebellum mRNA M430 [Mar05]), hippocampus (Hippocampus Consortium M430v2 [Jun06]), hypothalamus (INIA Hypothalamus Affy MoGene 1.0 ST [Nov10]), midbrain (VU BXD Midbrain Agilent SurePrint G3 Mouse GE [May12]), neocortex (HQF BXD Neocortex ILM6v1.1 [Dec10v2] RankInv), nucleus accumbens (VCU BXD NA Sal M430 2.0 [Oct07]), pituitary (INIA Pituitary Affy MoGene 1.0ST [Jun12]), prefrontal cortex (VCU BXD Prefrontal Cortex Sal M430 2.0 RMA), striatum (HQF BXD Striatum ILM6.1 [Dec10]), and ventral tegmental area (VCU BXD VTA Saline AffyM430 2.0 [Jun09]). Transcripts with significant cis-eQTLs (genome wide $P < 0.05$) were then checked for genetic correlation with the behavioural phenotype by determining the Pearson's and Spearman rank order correlations. Bonferroni significance levels were determined for total number of transcripts evaluated, for a family-wise significance threshold of 0.05. Transcripts with a cis-eQTL within the 2-LOD interval and levels that correlate with the behavioural phenotype are considered top candidate genes.

Prioritized positional candidate genes were investigated for associations to traits from independent studies in order to elucidate the biological role and identify related affected traits. The ePheWAS tool, available on http://www.systems-genetics.org (Li et al., 2018), was utilized to calculate associations to phenotypes deposited in genenetwork.org. Only brain regions where expression correlated to traits in the current study were assessed. Significance was determined by Bonferroni corrected *P* values.

2.10 | Genetic correlations: Cocaine behaviours

Control data (i.e., derived from subject without PNS exposure) for acute locomotion/sensitization, CPP, and cocaine self-administration (days to acquisition and number of infusions, data collected by Dickson et al., 2015 and available on genenetwork.org) were assessed by Pearson's correlation. Dickson et al. (2015) assessed cocaine self-administration at eight doses. The lowest three doses contain eight strains in common with the present study, limiting statistical power. The top five doses contain 13 strains in common. The strain means for the top five doses correlate strongly to each other; therefore, one dose (1.0 $mg{\cdot}kg^{-1}$ per infusion) was selected for assessment with the present study. Significant correlations suggest common alleles affect these behaviours.

2.11 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to

PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Christopoulos et al., 2017; Alexander, Fabbro et al., 2017).

3 | RESULTS

Overall, PNS interacted with strain and/or sex for almost all outcomes. These were verified by ANOVA and interactions were deconstructed.

3.1 | ANOVA for behavioural measures

3.1.1 | Acute cocaine-induced locomotion

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

The heritability of acute cocaine-induced locomotion for control females is 0.36 and PNS females 0.40. The heritability for control males is 0.36 and PNS males 0.30.

3.1.2 | Locomotion sensitization following repeated cocaine

Sensitization of cocaine-induced locomotion was determined as the difference in locomotion from the first to the last cocaine injection and was analysed by three-way ANOVA. A strain by sex interaction [$F(20, 963) = 2.92$] a PNS by sex interaction [$F(1, 963) = 3.85$] a main effect of strain [$F(20, 963) = 9.28$] and a main effect of sex [$F(1, 963) = 11.79$] were detected. Within-sex analysis revealed a main effect of strain [$F(20, 963) = 4.68$] for females. For males, a main effect of strain [$F(20, 963) = 5.94$] and a main effect of PNS [$F(1, 963) = 4.9$] were detected (Figure 1b).

The heritability of cocaine-induced sensitization for control females is 0.23 and PNS females 0.16. The heritability for control males is 0.21 and PNS males 0.21.

3.1.3 | Conditioned place preference

CPP was calculated as the difference in the time spent in the cocaine-paired compartment between the pretest and posttest and was analysed by three-way ANOVA. A PNS by sex interaction [$F(1, 989) = 4.33$], a strain by sex interaction [$F(20, 989) = 2.16$], a main effect of strain [$F(20, 989) = 12.88$], and a main effect of PNS [$F(20, 989) = 10.07$] were detected. Within-sex analysis revealed a main effect of strain in females [$F(20, 989) = 7.3$]. For males, a main effect of strain [$F(20, 989) = 7.95$], and a main effect of PNS [$F(1, 989) = 13.24$] were detected (Figure 1c).

The heritability of CPP for control females is 0.24 and PNS females 0.16. The heritability for control males is 0.25 and PNS males 0.23.

3.1.4 | Locomotion without cocaine

Locomotion during the pretest and posttest, as well as during the first saline-conditioning trial, was assessed. A three-way ANOVA for pretest locomotion (Genenetwork IDs 20432, 20424, 20425, 20426) revealed a strain by PNS interaction [$F(20, 993) = 2.03$], a strain by sex interaction [$F(20, 971) = 2.9$], a main effect of strain [$F(20, 993) = 43.79$], and a main effect of sex [$F(1, 993) = 26.17$]. Within-sex analysis revealed a main effect of strain for females [$F(20, 993) = 26.59$]. For males, a strain by PNS interaction [$F(20, 993) = 2.21$], and a main effect of strain [$F(20, 993) = 21.56$] were found.

A three-way ANOVA for locomotion during the first saline-conditioning session (Genenetwork IDs 20439, 20436, 20437, 20438) revealed a strain by PNS by sex interaction [$F(20, 971) = 1.82$], a strain by sex interaction [$F(1, 971) = 2.69$], a main effect of strain [$F(20, 971) = 62.98$], a main effect of PNS [$F(1, 971) = 4.19$], and a main effect of sex [$F(1, 971) = 19.38$]. Within-sex analysis revealed a main effect of strain in females [$F(20, 971) = 37.62$]. For males, a strain by PNS interaction [$F(20, 971) = 2.66$], and a main effect of strain [$F(20, 971) = 28.35$] were found.

A three-way ANOVA for locomotion during the posttest (Genenetwork IDs 20427, 20428, 20429, 20430) revealed a strain by sex interaction [$F(20, 983) = 2.26$], a strain by PNS interaction, a main effect of strain [$F(20, 983) = 42.43$], and a main effect of sex [$F(1, 983) = 28.86$]. Within-sex analysis revealed a strain by PNS interaction for females [$F(20, 983) = 1.66$], and a main effect of strain [$F(20, 983) = 26.12$]. For males, a strain by PNS interaction [$F(20, 983) = 1.93$], and a main effect of strain [$F(20, 983) = 18.81$], were found.

Individual difference scores between posttest and pretest distance travelled (Genenetwork IDs 20431, 20432, 20433, 20434) were assessed by three-way ANOVA. A strain by PNS interaction [$F(20, 985) = 1.85$], and a main effect of strain [$F(20, 985) = 18.83$] were found.

3.2 | QTL mapping of behavioural measures

3.2.1 | QTL by PNS interaction

A significant QTL by PNS interaction (across sexes) was detected for acute cocaine-induced locomotion (aIQTLXPNS; genenetwork ID 18710) on chromosome X (LRS = 17.9). The 2-LOD interval is 37.91 to 50.95 mb (see Figure 2). No other significant QTL by PNS interactions were detected.

3.2.2 | Sex-specific, PNS-independent QTLs

A sex-specific main effect QTL was detected for female CPP (cppQTL; genenetwork ID 18691 females, 18705 males), on chromosome 11 (LRS = 17.9). The 2-LOD interval is 65.50 to 81.31 mb (see Figure 3 a,b). A sex-specific main effect QTL was detected for female locomotion sensitization (sensQTL; genenetwork ID 18695 females, 18709 males), on chromosome 16 (LRS = 19.7). The 2-LOD interval is 95.79

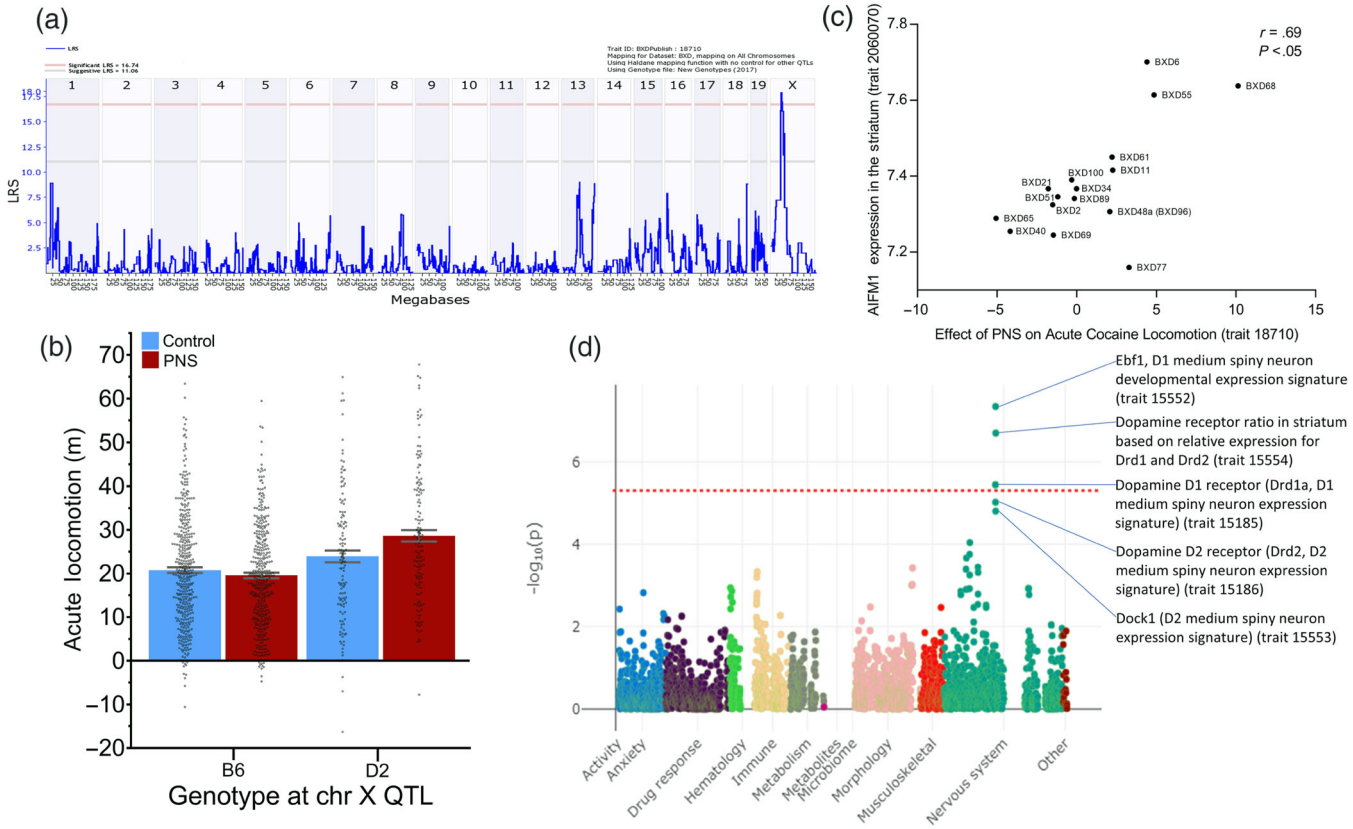

FIGURE 2 PNS interacting QTL impact acute cocaine-induced locomotion. (a) A significant QTL by PNS interaction on chromosome X. (b) Effects of genotype at the peak marker and PNS on acute locomotion, plotted as mean with SEM (B6 control n = 403, B6 PNS n = 380, D2 control n = 125, D PNS n = 142). (c) Scatter plot of PNS strain effects on acute cocaine-induced locomotion and striatal expression of *Aifm1*. (d) Association of striatal *Aifm1* expression with striatal D_1/D_2 receptor expression

to 98.32 (see Figure 3c,d). No other significant main effect QTLs were detected. For suggestive QTLs, see Table 2.

3.3 | Positional candidate genes

3.3.1 | cis-eQTL for acute locomotor QTL by PNS

The 2-LOD interval for alQTLXPNS contains 65 transcripts. Two of these transcripts were found to have a cis-eQTL in one or more brain regions. One of these cis-eQTL transcripts (apoptosis inducing factor, mitochondrion associated 1 [*Aifm1*]) has expression levels that correlate with acute locomotion strain difference scores at Bonferroni-corrected (0.05/2) significance levels in the midbrain, amygdala, and hypothalamus. Significant correlations were also found in the striatum and neocortex; however, these regions demonstrate suggestive cis-eQTL. See Table 3.

ePheWAS analysis of *Aifm1* expression identified associations between striatal expression of *Aifm1* and striatal D_1/D_2 receptor expression as well as markers of D_1 and D_2 medium spiny neuron cells (see Figure 2d).

3.3.2 | cis-eQTL for CPP QTL

The 2-LOD interval for cppQTL contains 373 transcripts; 138 of these map a cis-eQTL in one or more brain regions. Of these cis-eQTL transcripts, 10 demonstrate Bonferroni-corrected (.05/138) significant correlations to female CPP strain means. However, eight of these transcripts have one or more single nucleotide polymorphisms SNP(s) in the probe target region and were excluded from consideration. These SNPs may affect probe hybridization and produce false positive cis-eQTLs. See Table 3.

3.3.3 | cis-eQTL for sensitization QTL

The 2-LOD interval for sensQTL contains 30 transcripts. Of these transcripts, six map a cis-eQTL in one or more brain regions. Of these cis-eQTL transcripts, one (***Ripk4***) demonstrates a Bonferroni-corrected (.05/5) significant correlation to female sensitization strain means in the hippocampus. Receptor-interacting serine–threonine kinase 4 (*Ripk4*) also correlates at uncorrected significance in the ventral tegmental area and neocortex. See Table 3.

3.3.4 | Genetic correlations: Cocaine behaviours

A significant correlation was detected between sensitization and CPP, and sensitization and cocaine self-administration infusions (data collected by Dickson et al., 2015). A significant correlation was detected between CPP and days to meet self-administration acquisition (data collected by Dickson et al., 2015). See Table 4.

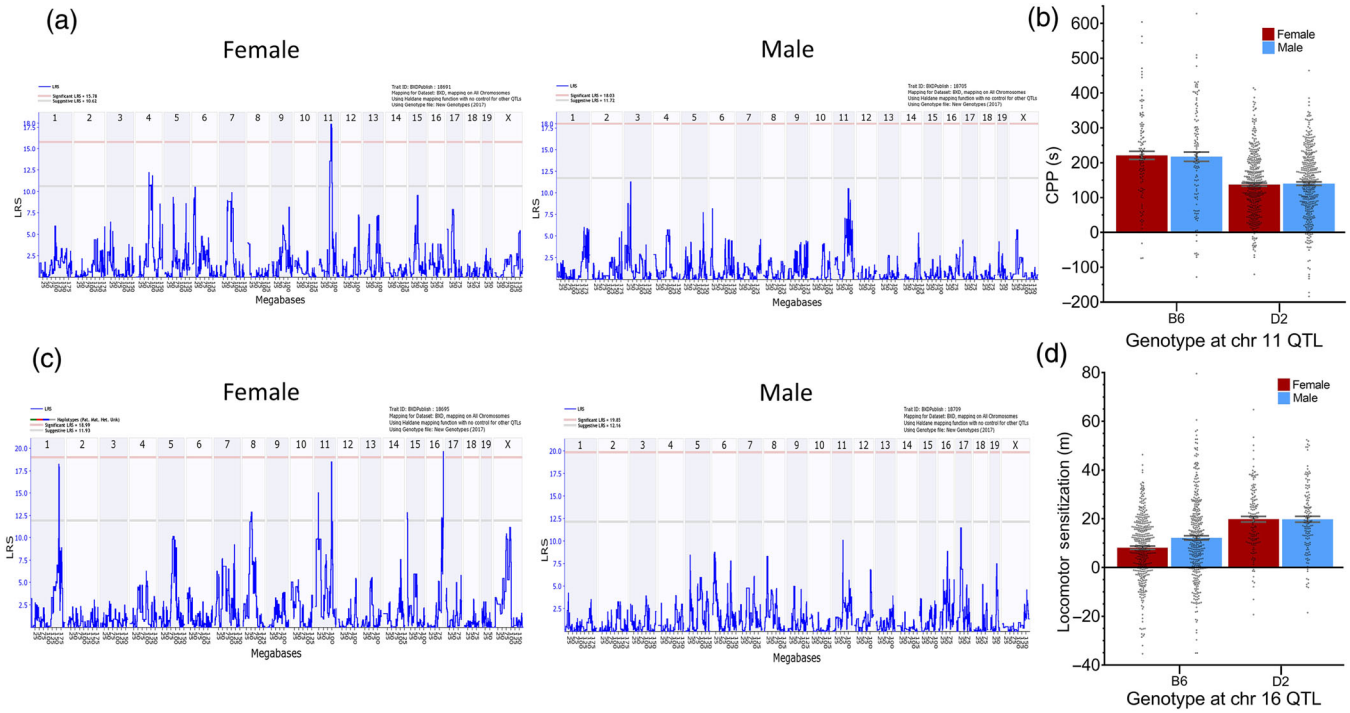

FIGURE 3 Sex-specific QTLs impacting conditioned place preference (CPP) and sensitization of cocaine-induced locomotion in females. (a) A significant main effect QTL for female CPP on chromosome 11. (b) Effects of genotype at the peak marker and sex on CPP, plotted as mean with SEM (B6 female *n* = 114, B6 male *n* = 118, D2 female *n* = 411, D2 male *n* = 409). (c) A significant main effect QTL for female sensitization on chromosome 16. (d) Effects of genotype at the peak marker and sex on sensitization, plotted as mean with SEM (B6 female *n* = 387, B6 male *n* = 378, D2 female *n* = 145, D2 male *n* = 137)

TABLE 2 Suggestive QTL X PNS interactions and PNS-independent QTLs

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

4 | DISCUSSION

We examined the interactions of early environmental stress, sex, and genetic factors in determining cocaine responsiveness in adult mice of the BXD mouse recombinant inbred reference panel by subjecting pregnant dams to restraint to induce PNS. PNS interacted with strain and sex to impact the psychostimulant and rewarding effects of cocaine. A PNS by QTL interaction was discovered for the acute psychostimulatory effects of cocaine and, subsequently, a promising candidate gene was identified at this locus. Furthermore, sex-specific and PNS-independent QTLs were discovered for CPP and sensitization of cocaine's psychostimulatory effects.

The literature to date generally indicates that PNS increases cocaine responsiveness across a variety of measures (Kippin et al., 2008; Thomas et al., 2009). However, the present PNS X BXD data suggest a more complex relation between early life stress, addiction-related behaviour, sex, and genetics. For acute cocaine-induced locomotion, PNS increases responsiveness to a variety of psychostimulants, including cocaine, in outbred rats (Kippin et al., 2008; Morley-Fletcher et al., 2004) and to cocaine in B6, but not

TABLE 3 Transcripts within 2-LOD QTL intervals with cis-eQTL and strain expression levels that correlate to the behavioural phenotype strain difference or sum scores

	Transcript cis-eQTL							Covariation		
QTL	Gene	Probe	Position (mb)	Region	*n*	cis-eQTL	LRS	*n*	Pearson *r*, *P* value	Spearman *r*, *P* value
alQTLxPNS	AIFM1	10604405	ChrX: 48.474944	Amygdala	50	ChrX: 47.144891	25.9	13	**0.61, 0.0239**	0.56, 0.0452
		10604405	ChrX: 48.474944	Hypothalamus	50	ChrX: 48.026885	35.3	13	**0.80, 0.0006**	0.60, 0.0268
		A_55_P2002864	ChrX: 48.474961	Midbrain	37	ChrX: 47.144891	65.5	10	**0.71, 0.019**	0.43, 0.223
		ILM2060070	ChrX: 48.504847	Neocortex	72	ChrX: 50.523592	14.1	16	**0.55, 0.0245**	0.49, 0.0561
		ILM2060070	ChrX: 48.504847	Striatum	75	ChrX: 48.504847	13.3	16	**0.69, 0.0024**	**0.61, 0.0107**
cppQTL	PITPNA	1423283_at	Chr11: 75.625491	Hippocampus	99	Chr11: 77.197052	22.9	16	−0.67, 0.0043	−0.71, 0.0020
		A_55_P2166773	Chr11: 75.626667	Midbrain	37	Chr11: 72.859313	27.3	10	−0**.95, 3.4E-5**	−0.92, 0.0005
		1423283_at	Chr11: 75.625491	NAc	36	Chr11: 76.607143	17.6	6	−0.93, 0.0078	−0.89, 0.0333
	SAT2	10377560	Chr11: 69.622052	Amygdala	50	Chr11: 69.788406	136	13	**0.85, 0.0002**	0.85, 0.0004
		1430318_at	Chr11: 69.623689	Hippocampus	99	Chr11: 69.788406	131	16	0.60, 0.0153	0.38, 0.1447
		10377560	Chr11: 69.622052	Hypothalamus	50	Chr11: 69.788406	142	13	0.80, 0.0010	0.75, 0.0044
		A_55_P2345425	Chr11: 69.622212	Midbrain	37	Chr11: 69.788406	133	10	0.74, 0.0154	0.64, 0.0545
		A_55_P2047345	Chr11: 69.623810	Midbrain	37	Chr11: 69.788406	143	10	0.73, 0.0165	0.40, 0.2568
		10377560	Chr11: 69.622052	Pituitary	52	Chr11: 69.788406	119	14	0.78, 0.0009	0.75, 0.0033
		ILM5860731	Chr11: 69.622901	Striatum	75	Chr11: 72.494723	29.3	16	0.53, 0.03367	0.59, 0.0186
sensQTL	RIPK4	1418487_at	Chr16: 97.741954	Hippocampus	99	Chr16: 97.356657	27.6	16	**0.65, 0.0065**	**0.68, 0.0037**
		ILM360019	Chr16: 97.742194	Neocortex	72	Chr16: 97.496522	25.2	16	0.44, 0.0907	0.57, 0.0249
		1418488_s_at	Chr16: 97.742021	VTA	37	Chr16: 96.501253	15.7	6	0.87, 0.0256	0.89, 0.0333

Note. Bold text = significant at Bonferroni-corrected threshold.

TABLE 4 Within and across-study genetic correlations for cocaine behaviours

		CPP	Acute locomotion	Sensitization	IVSA acquisition (days)	IVSA infusions
CPP	*r*		−0.181	**0.495**	**0.684**	0.165
(genenetwork ID 18748)	*P* value (*n*)		0.434 (21)	**0.023 (21)**	**0.009 (13)**	0.59 (13)
Acute locomotion	*r*	−0.27		−0.145	−0.175	0.044
(genenetwork ID 18744)	*P* value (*n*)	0.237 (21)		0.529 (21)	0.569 (13)	0.887 (13)
Sensitization	*r*	**0.462**	−0.031		0.021	**0.808**
(genenetwork ID 18750)	*P* value (*n*)	**0.035 (21)**	0.896 (21)		0.964 (13)	**0.001 (13)**
IVSA acquisition (days)	*r*	**0.667**	−0.096	0.133		−0.215
(genenetwork ID 18492)	*P* value (*n*)	**0.013 (13)**	0.755 (13)	0.664 (13)		0.201 (37)
IVSA infusions	*r*	0.133	0.15	**0.675**	−0.112	
(genenetwork ID 18502)	*P* value (*n*)	0.664 (13)	0.625 (13)	**0.011 (13)**	0.508 (37)	

Note. Bold text indicates significance ($P < 0.05$). Lower left half are Pearson's correlations, upper right are Spearman's rank order correlations. IVSA measures were collected by Dickson et al. (2015). All other measures come from the present study.

D2, mice (Kippin et al., 2015). In the present data, the effect of PNS on acute cocaine-induced locomotion is strain-dependent and may vary in the direction of change across strains. For example, the strain mean for PNS-exposed males of the BXD55 strain demonstrates an approximate 50% increase in acute locomotion relative to the control group, while PNS-exposed males of BXD77 demonstrate an approximate 50% reduction. Moreover, we identified a novel QTL interacting with PNS on this measure. Similarly, PNS in outbred rats and B6 mice increases the level of locomotor response observed following repeated psychostimulant exposure (Henry et al., 1995; Kippin et al., 2015, 2008), with one study reporting a female-specific augmentation of sensitization to repeated cocaine (Thomas et al., 2009). Again, the direction of the PNS effects on sensitization in the present data set varied across the strains affected, with most strains demonstrating

attenuation in males, but no significant effect of PNS was detected in females. Lastly, CPP is increased by PNS in B6 mice (Kippin et al., 2015) and outbred rats (Pastor, Pallarés, & Antonelli, 2018), but across the BXD panel examined herein, PNS largely reduced CPP scores in males of affected strains, with only a small PNS effect being observed in females. Collectively, these results indicate that there are probably genetic variants that interact with PNS to augment these traits while other variants interact with PNS to suppress these traits. Moreover, sex appears to be an important moderator of PNS effects, with males demonstrating higher sensitivity to the effects of PNS on many traits, which is generally consistent with findings from experiments using outbred rodent species (Bale, 2011; Frye et al., 2011; Weinstock, 2011).

4.1 | QTl mapping: QTL X PNS interactions

Mapping for strain difference scores revealed a QTL by PNS interaction across sexes for alQTLxPNS on the X chromosome. The D2 genotype at the peak marker associates with a PNS-induced increase in locomotion (see Figure 2b). The 2-LOD interval spans 13.25 mb and contains 65 genes. Positional candidate genes were prioritized by evaluation of cis-eQTL (Table 3). The gene *Aifm1* is located within the 2-LOD interval and was found to have cis-regulated transcripts in multiple brain regions, with expression levels that correlate with strain-difference scores for acute cocaine-induced locomotion. The *Aifm1* gene encodes for an apoptosis inducing factor isoform that is linked to induction of apoptosis after cellular injury (Sevrioukova, 2011). Several lines of evidence suggest that deficiencies in apoptosis inducing factor may have protective effects against neuronal insults (Matsumori et al., 2005; Sun, Zhang, Wang, Blomgren, & Zhu, 2012), including glutamate-mediated excitotoxicity (Öxler, Dolga, & Culmsee, 2012; Wang et al., 2004) suggesting a causal role for apoptosis inducing factor in excitotoxic cell death. Although there is no previous evidence to directly associate apoptosis inducing factor with PNS, an increase in apoptosis may have a role in the development of PNS (Huang et al., 2016; Kim et al., 2015; Kurek et al., 2016; Qulu, Daniels, & Mabandla, 2015; Tobe et al., 2005). In the present study, there is a positive association between genetically-moderated brain *Aifm1* levels and PNS effects on acute locomotion, as indicated by a comparison of the data from the present study with mRNA expression data on genenetwork.org. One plausible explanation is that increased *Aifm1* levels sensitize mice to the apoptotic consequences of PNS.

Furthermore, ePHeWAS analysis of *Aifm1* indicates a relationship with striatal dopamine D_1 and D_2 receptor expression (see Figure 2d). Increased *Aifm1 RNA* expression is associated with increased D_1 and decreased D_2 receptor RNA expression, suggesting that *Aifm1* expression levels may modify the expression of D_1 and D_2 receptors. D_1 receptors moderate cocaine-induced locomotor activity, as well as the rewarding effects of psychostimulant drugs (Hummel & Unterwald, 2002). Conversely, low D_2 receptor expression is associated with psychostimulant drug abuse liability (Dalley et al., 2007; Lee et al., 2009). Therefore, *Aifm1* interactions with PNS may cause downstream effects that include modification of this critical balance of D_1 and D_2 receptors and subsequent enhancement of cocaine abuse risk.

4.2 | PNS-independent, sex-specific QTLs

In addition to the PNS interacting QTL for acute cocaine-induced locomotion, the present study identified two novel PNS-independent and sex-specific QTLs for cocaine-induced locomotor sensitization and for cocaine-induced CPP. These QTLs are likely to provide further insight into the genetic influence on cocaine addiction and may reveal sex-interacting genes that contribute to the relatively high cocaine responsiveness observed in female humans and laboratory animals; supporting the notion that women exhibit elevated cocaine addiction vulnerability (Becker & Koob, 2016; Carroll & Anker, 2010; Sanchis-Segura & Becker, 2016).

A main effect QTL for female CPP scores was discovered on chromosome 11. The 2-LOD interval is large and encompasses 372 genes. Ten transcripts were found to have cis-eQTL and correlate with female CPP scores, but eight of these transcripts have SNPs in the probe target regions. The genes phosphatidylinositol transfer protein, α (*Pitpna*) and spermidine/spermine N1-acetyl transferase (*Sat2*) do not have SNPs in the probe target region and can be considered as prioritized candidate genes. *Pitpna* is involved in transport of phospholipids and PLC signalling (Tilley et al., 2004). *Pitpna* null mutants have extensive phenotypes, including CNS and behavioural abnormalities (Alb et al., 2003). However, *Pitpna* has not been directly implicated in drug abuse phenotypes. *Sat2* is a rate limiting enzyme that converts spermidime to spermine (Pegg, Seely, Pösö, della Ragione, & Zagon, 1982). Spermine may interact with the cocaine-binding site on the dopamine transporter to inhibit cocaine binding, and spermine levels are increased in the cerebellum after cocaine exposure (Ritz, Mantione, & London, 1994; Shimosato, Watanabe, Marley, & Saito, 1995). Overall, these two genes are novel candidates for further investigation for their roles in cocaine reward, including the potential sex-specific effects of elevated female cocaine reward.

Sensitization to cocaine-induced locomotion in females was also associated with a main effect QTL, in which the 2-LOD interval contains 30 genes. One transcript from this interval, *Ripk4*, demonstrates cis-eQTL and a significant correlation with female sensitization strain means. This transcript also has cis-eQTL and correlations significant at uncorrected levels in the ventral tegmental area and neocortex. *Ripk4* has been implicated in kerotinocyte differentiation (Holland et al., 2002), but there is no evidence implicating *Ripk4* in cocaine-related behaviours, or behaviour/CNS phenotypes in general. In addition, a suggestive QTL for female sensitization was discovered on chromosome 11 (LRS = 18.5) with a 2-LOD interval of 95.6 to 98.6 mb, in which the B6 allele at this locus associates with increased sensitization. A QTL for methamphetamine and opioid locomotion was discovered on chromosome 11, with an interval of 84 to 96 mb (Bryant et al., 2009; Bryant, Kole, Guido, Sokoloff, & Palmer, 2012). Here, the B6 allele increases locomotion relative to the A/J allele. Because confidence intervals overlap, and the B6 allele has the same

direction of effect, the QTL discovered in the present study and that discovered by Bryant et al. (2012) may be detecting the same variant(s).

4.3 | Genetic correlations: Cocaine reward, reinforcement, and psychomotor behaviour

Cocaine locomotion, CPP, and self-administration behaviours are often studied with the intent of elucidating the neurobiology of cocaine abuse disorder. It is important to consider the relationships between these behaviours, including genetic relationships, in order to evaluate the implications of data produced from these various phenotypes. Dickson et al., 2015 has reported a genetic correlation between cocaine-induced locomotion sensitization and the number of cocaine self-administration infusions. Furthermore, a self-administration QTL overlaps with a cocaine sensitization QTL on chromosome 11 (Dickson et al., 2015; Kumar et al., 2013). Both studies also hit on the same candidate gene (*Cyfip2*) by different methods, and Kumar et al., 2013 experimentally validated the role of this gene in cocaine locomotion sensitization. The present study has further supported the relationship between sensitization and self-administration, with the present data for locomotor sensitization demonstrating a robust correlation with cocaine infusions during self-administration (see Table 4), as reported in Dickson et al. (2015).

Sensitization also associates with CPP. However, CPP does not associate with self-administration infusions, suggesting unique shared variance between these reward/reinforcement-related measures and sensitization. CPP does associate with days to acquire self-administration (see Table 4). This relationship suggests that strains with higher CPP scores require more days to acquire self-administration and unexpectedly suggests alleles that facilitate cocaine CPP may also delay acquisition of self-administration. Dickson et al. (2015) report that cocaine CPP collected by Philip et al. (2010) does not associate with self-administration acquisition; however, this CPP was induced by a relatively low cocaine dose (3 mg·kg^{-1}). Furthermore, the CPP data of Philip et al. 2010 does not correlate with the present CPP data (obtained using a 10 mg·kg^{-1} dose), suggesting different alleles may be involved in moderating the effects of different cocaine doses on CPP.

In summary, this study advances a model system to dissect the complex interactions between genes, sex, and environment. Specifically, utilizing the BXD mouse panel, we observed that sex, strain, and early environmental stress (i.e., PNS) interacted and affected cocaine-induced locomotion and reward, indicating a suitable model for locating sex-specific and PNS interacting alleles. Specifically, in females, QTLs were discovered for both CPP and sensitization. A QTL by PNS interaction for acute cocaine locomotion was detected on the X chromosome. Prioritization of positional candidate genes indicated that *Aifm1* is a likely candidate moderator for the effect of PNS on cocaine responsiveness. Towards developing a mechanistic explanation of addiction vulnerability, proximal factors such as maternal stress response and impact on subsequent maternal-offspring interactions will need to be elucidated and, although beyond the scope of the present report, we are currently examining these factors as a consequences of PNS X strain interactions. Overall, the present findings indicate that an examination of the genetic contributors to sex- and early-life stress-dependent factors is a potentially fruitful avenue for understanding initial drug response and, perhaps, addiction vulnerability.

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AUTHOR CONTRIBUTIONS

J.R.B., K.K.S., and T.E.K. contributed to experimental design and drafting the manuscript. J.R.B. conducted experimental procedures and analysed the data.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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