Published in final edited form as:

Biol Psychiatry. 2017 May 01; 81(9): 757–769. doi:10.1016/j.biopsych.2016.10.021.

Cytoplasmic FMR1-interacting protein 2 is a major genetic factor underlying binge eating

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Abstract

Background—Eating disorders are lethal and heritable; however, the underlying genetic factors are unknown. Binge eating is a highly heritable trait associated with eating disorders that is comorbid with mood and substance use disorders. Therefore, understanding its genetic basis will inform therapeutic development that could improve several comorbid neuropsychiatric conditions.

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FINANCIAL DISCLOSURES

The authors declare no biomedical financial interests or potential conflicts of interest.

Methods—We assessed binge eating in closely related C57BL/6 mouse substrains and in an F₂ cross to identify quantitative trait loci (QTL) associated with binge eating. We used gene targeting to validated candidate genetic factors. Finally, we used transcriptome analysis of the striatum via mRNA sequencing (RNA-seq) to identify the premorbid transcriptome and the binge-induced transcriptome to inform molecular mechanisms mediating binge eating susceptibility and establishment.

Results—C57BL/6NJ but not C57BL/6J mice showed rapid and robust escalation in palatable food consumption. We mapped a single genome-wide significant QTL on chromosome 11 (LOD=7.4) to a missense mutation in cytoplasmic FMR1-interacting protein 2 (*Cytip2*). We validated *Cytip2* as a major genetic factor underlying binge eating in heterozygous knockout mice on a C57BL/6N background that showed reduced binge eating toward a wild-type C57BL/6J-like level. Transcriptome analysis of premorbid genetic risk identified the enrichment terms “morphine addiction” and “retrograde endocannabinoid signaling” whereas binge eating resulted in the downregulation of a gene set enriched for decreased myelination, oligodendrocyte differentiation, and expression.

Conclusions—We identified *Cytip2* as a major significant genetic factor underlying binge eating and provide a behavioral paradigm for future genome-wide association studies in populations with increased genetic complexity.

Keywords

eating disorders; GWAS; binge; anxiety; myelin; Prader-Willi Syndrome

INTRODUCTION

Eating disorders (ED) are among the most lethal psychiatric disorders and exhibit a lifetime prevalence of 1 to 3%(1). Binge Eating Disorder (BED) is frequently associated with severe obesity, metabolic dysfunction, and increased mortality(1, 2). Both genetic and environmental factors contribute to binge eating (BE)(3); however genome-wide association studies (GWAS) in humans are currently limited in their power to detect the contribution of common variants(4). BE, defined by the uncontrolled overconsumption of a large amount of food within a brief time period (usually energy-dense palatable food; PF), is one of the most highly heritable traits associated with ED, including BED(5), Bulimia Nervosa(6) and a subset of cases of Anorexia Nervosa(7). Focusing on the genetic and biological basis of a single trait such as BE that is presumed to comprise less genetic and biological complexity than an aggregate disorder could be a more tractable goal toward gene identification(5, 8) and accelerate the development of new therapeutics.

ED are co-morbid with anxiety traits(9), mood disorders, obsessions/compulsions, impulse-control, and substance use disorders, suggesting shared genetic factors(1). Recent theories of BE have adopted theories of addiction to explain its compulsive basis and the underlying genetic and neural mechanisms(10-12). Compulsive BE shares several features with addiction, including an escalation in consumption, physiological and emotional-affective dependence, cue-induced craving, and relapse(12)

Neurochemical mechanisms of BE converge on activation of the mesocorticolimbic dopamine system(13, 14). Cue-induced craving correlates with BE in humans(15) and changes in extracellular dopamine in the dorsal striatum in response to food stimuli correlate with scores of BE severity in patients with BED(16). The dorsal striatum processes food sensation and reward(17), e.g., the nutritional value of sugar(18), and enkephalin-mediated coding of sensory reward induced by palatable food consumption(19, 20). Furthermore, the volume of the dorsal striatum is reduced in patients with anorexia and bulimia and correlates with predicted sensitivity to reward(21). Finally, recruitment of the dorsal striatum combined with a loss of prefrontal cortical inhibition is hypothesized to mediate a shift to habitual, cue-responsive compulsive-like behaviors associated with addiction(22), including binge eating(10).

Quantitative trait locus (QTL) mapping is a genome-wide, discovery-based approach to uncover novel genetic and biological mechanisms of complex traits such as BE(23-25). In mice, millions of genetic variants typically distinguish commonly used inbred strains(26), highlighting one of the challenges to identifying causal genetic variants. To help overcome this particular challenge, closely related substrains exhibiting extreme phenotypic and little genetic diversity can be employed to facilitate gene mapping(27, 28). C57BL/6 (B6) substrains show robust differences in behavioral responses to drugs of abuse(28) and whole genome sequencing identified only approximately 10,000 single nucleotide polymorphisms that distinguish C57BL/6NJ (B6NJ) and C57BL/6J (B6J) strains(26). This drastically reduced genetic complexity can facilitate identification of causal genetic factors(29). Behavioral differences between B6J and B6NJ combined with whole genome sequence information make these strains an attractive and accessible model for identifying the genetic basis of variation in complex traits. However, whether these strains differ in BE has not been examined.

Here, we developed an intermittent, limited access procedure for BE in a conditioned place preference (CPP) paradigm using outbred CFW mice. We identified a robust difference in BE between B6 substrains and used QTL mapping and gene targeting to identify a major genetic factor associated with BE. Finally, we used striatal transcriptome analysis via mRNA sequencing in a subset of F₂ mice and heterozygous gene knockout mice to gain insight into the premorbid neurobiological mechanisms that bridge genetic variation with BE susceptibility and the neurobiological adaptations induced by BE.

METHODS AND MATERIALS

Mice

All experiments were performed in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Boston University. Swiss Webster (CFW) mice (7 weeks old) were purchased from Charles River Laboratory [CrI: CFW (SW), Wilmington, MA USA] and were used to choose the PF diet (5-TUL; TestDiet) and design the BE and PF-CPP paradigm (Supplementary Information). C57BL/6J (B6J) and C57BL/NJ (B6NJ) mice (7 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME USA). Details

on purchased mice and on B6J $\times$ B6NJ-F₁, -F₂, and heterozygous *Cytip2* knockout mice (*Cytip2*^{N/-}) are provided in the Supplementary Information.

BE and PF-CPP

We chose a BE CPP design in order to examine both the consummatory and the conditioned motivated behaviors associated with BE. The long-term goal is to identify shared and divergent genetic factors that mediate these behaviors using forward genetics. We used a two-chamber conditioned place preference (CPP) design(30) to measure BE and PF-CPP on Day (D)1-D22 (see **Fig.1**). On D23, F₂ mice received a final PF training session and on D24, mice were tested on the elevated plus maze (EPM) for 5 min (Supplementary Information) and sacrificed for collection of striatum punches(31). For the knockout study, *Cytip2*^{N/-} and *Cytip2*^{N/N} mice were tested for compulsive-like eating in the light/dark conflict test on D23 (see below) and sacrificed on D24 (24 h later) for collection of striatum punches(31). Mice homozygous for the *Cytip2* knockout allele are lethal at postnatal day 1.

Light/dark conflict test

The light/dark conflict test was employed on D23 as a measure of compulsive-like eating (32) in *Cytip2*^{N/N} and *Cytip2*^{N/-} mice. One side is black opaque plexiglas whereas the other is transparent and light-exposed with a small doorway allowing access to both sides. The light side is an aversive, bright compartment that the mice normally avoid. We operationalized an increase in PF consumption in the light side in spite of adverse conditions as a construct of compulsive-like eating (32). A porcelain bowl containing PF (5-TUL pellets) was placed into the center of the light side and each mouse was placed on the light side facing the food bowl and the doorway. Time on the light side, amount of PF consumed, and entries were recorded for 30 min.

Behavioral analysis of BE in CFW, B6J, and B6NJ mice

Behavioral analysis was conducted in R (<https://www.r-project.org/>) using mixed-model ANOVAs (Genotype, Treatment, and Sex as independent variables; Day as a repeated measure) with an alpha level of 0.05 to detect main effects and interactions. Primary outcome measures included percent BW consumed and time spent on the PF-paired side. We also determined differences in the slope (rate) of escalation in PF consumption as an additional measure of BE(33). Post-hoc analyses following main effects and interactions were performed using one- or two-way ANOVAs, and Welch's unequal variances t-tests ($p < 0.05$, Bonferroni-corrected for the number of comparisons made).

QTL analysis of B6J $\times$ B6NJ-F₂ mice

F₂ mice were genotyped using a custom Fluidigm genotyping array (South San Francisco, CA, USA) with 96 SNPs(26, 29). QTL analysis was conducted in R/qtl (scanone) using Haley-Knott regression and 1000 permutations to establish significance thresholds ($p < 0.05$, error probability=0.0001)(34). We also analyzed females (N=78) and males (N=78) separately. The marker position (cM) was estimated using the sex-averaged position from the Mouse Map Converter (<http://cgd.jax.org/mousemapconverter>)(35).

Behavioral analysis of *Cytip2*^{N/-} mice

We used a 2 × 2 factorial design to measure PF versus Chow consumption as a function of Genotype (*Cytip2*^{N/N}, *Cytip2*^{N/-}; 59-61 days old on D1). A minimum sample size of N=20 was employed based on 80% power (p<0.05; Supplementary Information). Mice were trained and tested for BE and PF-CPP on D1-D22. On D23, mice were provided 30 min access to PF in the light side of the light-dark box and tested for compulsive-like eating. On D24, mice were sacrificed and striatum punches were harvested for RNA-seq.

Transcriptome analysis via RNA-seq

B6J × B6NJ-F₂ study. A subset of PF-trained F₂ mice were chosen for transcriptome analysis that were homozygous for the wild-type B6J allele (BE-resistant; ^{J/J}) or the mutant B6NJ allele (BE-prone; ^{N/N}) at the *Cytip2* missense mutation (rs240617401) and two flanking markers (rs48169870 and rs6268547) to ensure QTL capture. A sample size of N=8 per genotype was employed (4 females, 4 males; 69-100 days old when sacrificed). Striatum punches were harvested on D24 following EPM testing, stored in RNAlater for 48h, blotted with a kimwipe, and transferred to -80°C. Total RNA was isolated(31, 36) and shipped to the University of Chicago Genomics Core Facility for cDNA library preparation and sequencing(31) (see Figure Legend and Supplementary Information for additional details).

Cytip2^{N/-} transcriptome study. On D24 (24 h post-light/dark test), mice were sacrificed and striatum punches were harvested and stored as described. We sequenced a subset of N=8 mice per Genotype (*Cytip2*^{N/N}, *Cytip2*^{N/-}) per Treatment (PF, Chow; 4 females, 4 males; 82-84 days old when sacrificed) that represented the group averages of compulsive-like eating. Samples were sequenced and analyzed similarly to F₂ mice. For most gene lists, we used a cut-off of 1.1-fold change in expression (FDR< 20%). One exception was made for the small gene list derived from PF-versus Chow-trained wild-type *Cytip2*^{N/N} mice where we employed a cut-off of p<0.0001 (1.1-fold change). We chose a subset of genes for qPCR validation (see below).

Enrichment analysis

We applied Ingenuity Pathway Analysis (IPA) (www.qiagen.com/ingenuity)(37) to three gene lists: 1) F₂ mice (*Cytip2*^{N/N} versus *Cytip2*^{J/J}), 2) effect of Genotype (*Cytip2*^{N/N} versus *Cytip2*^{N/-}), and 3) effect of Treatment (PF versus Chow). We permitted 70 molecules per network and restricted assessment to neural tissues and cell lines. Statistical significance was assessed using Fisher's exact test with correction for multiple testing. We also used Enrichr (<http://amp.pharm.mssm.edu/Enrichr/>) to compute enrichment scores for ranked terms derived from 35 different gene set libraries. Finally, we used Cell Set Enrichment Analysis (CSEA) (<http://genetics.wustl.edu/jdlab/csea-tool-2/>) that utilizes cell type-specific expression datasets to infer cell type enrichment(38).

RESULTS

BE and PF-CPP in B6 substrains

The BE protocol and PF-CPP apparatus are shown in **Fig.1A** and **Fig.1B**. Results from CFW mice in diet selection and the BE PF-CPP design are shown in **Fig.S1** and **Table S1**.

CFW mice showed a robust escalation in PF consumption that correlated with PF-CPP for the 5-TUL diet (**Table S1**). Because B6 substrains are an efficient tool for QTL mapping of complex behaviors induced by substances of abuse(29), we next tested for B6 substrain differences in the BE PF-CPP paradigm. We found robust differences in BE whereby B6NJ mice showed a greater and more rapid escalation of PF consumption than B6J (**Fig.1C,D**) in the absence of any difference in PF-CPP (**Fig.1E,F**) or correlation with consumption (**Table S2**). There were no strain differences in body weight during PF training (**Fig.1G**). Importantly, BE in binge-prone B6NJ mice was specific for PF because there was less initial and escalated intake with 20 mg Chow pellets lacking sucrose (**Fig.S2**).

QTL mapping in F₂ mice

We next mapped the genetic basis of BE susceptibility in a B6J × B6NJ-F₂ cross. **Table S3** lists the SNP markers. We identified a single QTL on chromosome 11 for summed PF consumed that peaked at the *Cytip2* missense mutation (46,222,615 bp; mm10, rs240617401; **Fig.2A,B**). The allelic effect was in the same phenotypic direction as the parental strains (*Cytip2*^{N/N} > *Cytip2*^{J/J}), was consistent across D2-D16, and was less robust during later training days (D18-D23; **Fig.2C,D**).

We estimated environmental variance using phenotypic variance of B6J, B6NJ, and F₁ mice (**Fig.S3**)(29). The *Cytip2* locus accounted for 16% of the phenotypic and 33% of the genetic variance. The estimated narrow-sense heritability of the QTL for total PF consumption was 49%(29). The QTL was more robust in females (**Fig.2B,E,F**) and peaked on D2. A second, distal QTL was identified in females on D9 and D11 (**Fig.2E**). In males, a single QTL was centered near *Cytip2* but no individual day was significant (**Fig.2G,H**).

Female F₂ mice consumed more PF and escalated more rapidly than males (**Fig.S4A,B**) without any difference in PF-CPP (**Fig.4C,D**). Females also showed an increase in freezing episodes in the EPM relative to males (**Fig.S4E,F**). No sex difference was observed in a separate cohort of F₂ mice that previously received saline injections during CPP(30) (**Fig.S4G,H**).

BE and compulsive-like eating in *Cytip2*^{N/N} and *Cytip2*^{N/-} mice

Because the QTL for PF consumption peaked near the *Cytip2* missense mutation (*Cytip2*^{N/N}) associated with cocaine behavior in B6 substrains(29) and because the mesocorticolimbic dopaminergic circuitry is crucial for BE(13), we hypothesized that *Cytip2*^{N/N} is a major genetic factor underlying BE. *Cytip2*^{N/-} mice (**Fig.S5A**) showed reduced PF but not Chow consumption (**Fig.3A,B**) toward a wild-type B6J-like level (**Fig. 1C; Fig.2D**). No difference in CPP was observed (**Fig.S5**). Similar effects of Genotype on BE were observed in females and males (**Fig.S6**); thus, we collapsed across sex for behavioral and transcriptome analyses. Slope analysis identified a greater y-intercept in PF-trained *Cytip2*^{N/N} versus *Cytip2*^{N/-} mice ($m=0.173$ vs. 0.063 ; $F_{1,11}=18.1$; $p=0.001$). For Chow groups, the slopes did not differ from zero ($p>0.05$), indicating specificity for PF. Importantly, *Cytip2*^{N/-} mice showed reduced compulsive-like eating and concomitant behaviors relative to *Cytip2*^{N/N} mice (**Fig.3C-F**).

Transcriptome of binge-prone *Cytip2^{N/N}* F₂ mice versus binge-resistant *Cytip2^{J/J}* F₂ mice

To gain insight into the neurobiological mechanisms that bridge *Cytip2* polymorphisms with BE, we sequenced striatal RNA from a subset of binge-prone *Cytip2^{N/N}* and binge-resistant *Cytip2^{J/J}* F₂ mice based on D2 PF consumption (**Fig.S7A**), a phenotype with a highly significant QTL (**Fig.2C,E**). We obtained an average of 50 million reads per sample and identified 576 differentially expressed genes (338 downregulated genes, 238 upregulated genes with 1.1-fold change (FDR<0.2; **Fig.4A; Table S4**). The top GO terms were associated with synaptic components and plasticity, behavior, axonogenesis, ion channels, transporters, and glutamate receptors (**Table 1A; Table S11A**). Top KEGG terms included “endocannabinoid signaling”, “morphine addiction”, and “nicotine addiction” as well as GABAergic, glutamatergic, and dopaminergic, and cholinergic synapses (**Table 1A; Table S11A**).

Transcriptome analysis of compulsive-like eating in *Cytip2^{N/N}* and *Cytip2^{N/-}* mice

Analysis of select F₂ mice (all PF-trained) precluded us from isolating the effect of Genotype versus Treatment on the transcriptome. Thus, we examined the premorbid transcriptome (effect of Genotype) and the transcriptome associated with compulsive-like eating (effect of Treatment). Striatal mRNA was sequenced from a subset (N=8) of representative mice from the four groups assayed for compulsive-like eating (**Fig.S7B**). We obtained an average of 45 million reads per sample and identified 224 genes for the main effect of Genotype (**Fig.4A; Table S5**) and 42 genes for the main effect of Treatment (**Fig. 4A; Table S6**). Because only *Cytip2^{N/N}* mice showed compulsive-like eating, we also included differentially expressed genes between PF-versus Chow-trained *Cytip2^{N/N}* mice ($p < 0.0001$) for a total of sixty-one genes in enrichment analysis of Treatment (**Fig.4A; Table S7,S8**).

For the Genotype $\times$ Treatment interaction, we identified five genes (*Rn18s*, *Rn45s*, *Lars2*, *Glul*, *Gjb6*, and *Gstm5*). The behavioral interaction was driven by compulsive-like eating in *Cytip2^{N/N}* mice (**Fig.3D**); thus, we also examined pairwise differential gene expression between PF-trained *Cytip2^{N/-}* versus *Cytip2^{N/N}* mice (**Table S9**) and identified 44 unique genes (**Table S10**; see IPA network in **Fig.S9**).

For the effect of Genotype, there was extensive overlap with the F₂ gene list (**Fig.4A**). Importantly, the direction and degree of differential expression in the *Cytip2^{N/N}* genotype versus the *Cytip2^{J/J}* or *Cytip2^{N/-}* genotype were highly correlated (**Fig.4B**), indicating that like behavior, *Cytip2^{J/J}* and *Cytip2^{N/-}* alleles induced functionally similar effects on gene transcription relative to *Cytip2^{N/N}*. This correlation was not driven by the fold-change cut-off (**Fig.S8**).

Enrichment analysis for the effect of Genotype (*Cytip2^{N/-}* relative to *Cytip2^{N/N}*) identified GO terms in **Table 1B** (see also **Table S11B**). Top canonical IPA pathways included Gi, cAMP, GPCR, and GABA receptor signaling ($-\log P=4.72-9.34$). Top upstream regulators included HTT, L-dopa, CNTF, Foxp1, and ethanol ($p=1.46 \times 10^{-12}$ – 1.46×10^{-6}). Notably, CNTF (ciliary neurotrophic factor) treatment can suppress food intake, induce weight and fat loss, and improve metabolic syndrome(39) and was associated in binge-resistant

Cytip2^{N/-} mice with downregulation of *Chat*, *Drd2*, *Pde1b*, *Penk*, *Ppp1r1b*, and *Tac1* and upregulation of *Vip* (Table S5). The top IPA network was Behavior, Endocrine System Development and Function, Molecular Transport where dopamine was an enriched IPA molecule for *Ppp1r1b* (DARPP-32), *Drd2*, *Penk*, *Chrm4*, *Gpr22*, *Adcy5*, *Kcnab3*, *Pde1b*, and *Hrh3* (Fig.4C). Finally, CSEA identified the highest enrichment for genes in D1 and D2 striatal spiny neurons (Fig.4E).

For the effect of Treatment (PF relative to Chow), GO terms are listed in Table 1C (see also Table S11C). TCF7L2 was the top IPA upstream regulator ($p=1.09 \times 10^{-23}$) with 21 downregulated genes (see the TCF7L2 node in Fig.4D). TCF7L2 (T-Cell Specific, HMG-Box) is a transcription factor that regulates peptide secretion and is associated with type 2 diabetes, dietary preference, obesity, blood glucose homeostasis, metabolic syndrome, and BE in bipolar disorder(40, 41). The top IPA network for Treatment included 30 downregulated genes enriched for “Cellular Development, Nervous System Development and Function, Tissue Morphology” (Fig.4D). Strikingly, most of these genes were enriched for myelination, axon ensheathment, glial cell and oligodendrocyte formation and differentiation (Table 1C; Table S11C). None of these genes were identified in binge-resistant *Cytip2*^{N/-} mice or from the effect of Genotype (Fig.4A), indicating specificity for BE. In support, CSEA identified an enrichment of genes in oligodendrocytes and progenitors (Fig.4F). Thus, the transcriptional profile of BE implicates decreased oligodendrocytes and myelination as a neurobiological consequence of BE. We validated a subset of genes for the effect of Genotype and Treatment via qPCR (Table S12,S13; Fig.S10).

DISCUSSION

We exploited the large phenotypic and small genetic diversity of B6 substrains to identify *Cytip2* as a major genetic factor underlying BE (Figs.1-3). Our study was facilitated by whole genome sequencing of B6 substrains(26, 29, 42) and the identification of the same locus and gene associated with psychostimulant-induced neurobehavioral plasticity(29). The difference in direction of the effect of the *Cytip2*^{N/N} allele on BE (increase) versus psychostimulant behavior (decrease) could potentially indicate differences in underlying neurobiological mechanisms. However, it should be noted that the cocaine results were obtained from experimenter-delivered drug administration whereas the BE results were obtained from voluntary PF consumption.

Several observations suggest that *Cytip2* is associated with the immediate consummatory response to PF that drives BE behavior. First, B6 substrain differences in PF-CPP were not significant (Fig.1E,F). Second, there was no correlation between consumption and PF-CPP in F₂ mice (Table S2). Third, no significant QTL for PF-CPP was identified. Fourth, there was no effect of the *Cytip2*^{N/-} allele on PF-CPP (Fig.S5). Finally, the largest effect of the *Cytip2* locus on PF consumption was observed on D2 (Fig.2E).

The *Cytip2*^{N/N} allele is associated with decreased protein stability (29) which represents just one molecular correlate associated with this mutation. If considered in isolation, a decrease in protein stability suggests a loss-of-function and the predicted phenotype should be similar to the *Cytip2*^{N/-} allele. However, *Cytip2*^{N/N} codes for a S968F missense mutation(29) that is

translated and could modulate other molecular functions (protein-protein interactions) to affect neural development and behavior.

The *Cytip2*^{N/N} mutation was associated with decreased cocaine-induced locomotor stimulation, dendritic spine number, and glutamatergic neurotransmission in the nucleus accumbens that were partially reversed in *Cytip2*^{N/-} mice toward a *Cytip2*^{J/J}-like level(29). The BE phenotype and the transcriptome of *Cytip2*^{N/N} mice were also partially reversed in *Cytip2*^{N/-} mice toward a *Cytip2*^{J/J}-like level (**Fig.3; Fig.4B**). Enrichment analysis of *Cytip2*^{N/-} versus *Cytip2*^{N/N} genotypes identified “channel regulator activity”, “glutamate receptor binding”, “ionotropic glutamate receptor binding” as the top three molecular GO terms and “glutamatergic synapse” as the top KEGG term (**Table 1B; Table S11B**). Interestingly, the glutamatergic system may be an effective target for BE (43, 44). CYFIP1/2 is a component of the WAVE regulatory complex(45) and also associates with FMRP(46). Both WAVE and FMRP regulate dendritic spine morphology and synaptic plasticity(45, 47). CYFIP modulates protein interactions in the FMRP-ribosome complex(48) to regulate activity-dependent synaptic translation and plasticity. In addition to glutamatergic transmission, the premorbid *Cytip2* genotype also perturbed gene sets enriched for the KEGG terms “morphine addiction”, and “retrograde endocannabinoid signaling” (Table 1B). The overlap of these top enrichment terms in two independent transcriptome studies (**Table 1A,B**) provided additional support that premorbid differential gene expression within these gene sets could bridge *Cytip2* genotype with susceptibility to BE.

Female F₂ mice were more prone to BE (**Fig.2; FigS4A,B**)(49-51) which is consistent with human studies(52) and could potentially be explained by the sweetened PF diet(51, 53), neurodevelopmental sex differences in the food reward circuitry and response to PF(54), activational effects of gonadal steroids(50, 52, 55), impulsivity(56), and/or neurobehavioral plasticity that underlies negative reinforcement(57). We did not observe sex differences in PF-CPP in F₂ mice (**Fig.S4C,D**); however, we did observe an increase in freezing episodes in female F₂ mice at 24 h post-BE that was specific to PF training (**Fig.S4F-H**). We previously observed an increase in freezing episodes in a place conditioning paradigm that correlated with conditioned place aversion induced by blockade of endogenous opioid signaling(30).

Cytip2^{N/-} mice showed reduced compulsive-like eating compared to *Cytip2*^{N/N} mice as indicated by decreased PF consumption in the light side (**Fig.3**) - an aversive, bright environment that mice normally avoid. Compulsive-like PF consumption was associated with a downregulation of a large set of myelination genes in the striatum (**Table 1C; Table S11C; Fig.4D**). Brain imaging studies in patients with bulimia nervosa found a reduction in white matter integrity in pathways linking frontal and temporoparietal cortices and limbic regions, including the fornix, internal capsule, corpus callosum, sub-insula, and corona radiata(58, 59). Furthermore, loss of white matter integrity correlated with the number of BE episodes(58), indicating a potential link between reduced myelination and increased symptomatic severity. White matter pathology was also observed in the brains of anorexic patients exhibiting restricted food intake(60, 61). Furthermore, rodents restrict chow intake between intermittent binge episodes of limited access to PF(62-64). Thus, although we did not measure home cage chow intake, the reduced expression of myelination genes could

ultimately be due to repeated cycles of self-induced food restriction of home cage chow. Future studies will assess myelin-associated protein levels during binge withdrawal versus the binge-sated state to determine the persistence of the proposed decrease in myelination. Directly targeting oligodendrocytes in preclinical models will reveal the potential role of demyelination versus remyelination in development versus recovery from BE and could represent a new therapeutic avenue for ED.

Previous studies reported B6 substrain differences in weight gain from palatable food diets(65). A recent study using an N₂ cross found weak evidence for an association of a SNP near *Cytip2* (rs13481014; chr. 11, 48 Mb) with high fat diet-induced obesity and lower leptin levels(66). Our BE procedure did not induce weight gain and there were no strain differences in body weight (**Fig.1G**). However, the differences in BE suggest that the *Cytip2* locus could be associated with diet-induced obesity and metabolic dysfunction in B6 substrains under conditions of prolonged PF access that induce weight gain(66, 67).

It is worth noting that *CYFIP1* (homolog of *CYFIP2*; 88% similarity in amino acid sequence) is deleted or imprinted in patients with Type I Prader-Willi Syndrome (PWS), a neurodevelopmental disorder defined by extreme hyperphagia(68) and a hyper-responsive reward system to food-associated cues(69). Type I deletions comprise 40% of PWS deletions and can be associated with more severe neurobehavioral pathology, including an increase in compulsive behavior(70, 71). Based on our identification of *Cytip2* for BE (**Fig.3**), the shared neurobiological function of CYFIP proteins(47) and the association of *CYFIP1* with PWS, one hypothesis is that *CYFIP1* polymorphisms also affect BE and hyperphagia.

We did not observe any Genotype by Sex interactions in behavior with our sample sizes and the effects of the *Cytip2* locus (F₂ study) and genotype (knockout study) on behavior were similar between females and males. Combining sexes clearly improved our power to detect the *Cytip2* QTL (**Fig.2A,B**) and changes in the transcriptome. Future studies in additional crosses should be powered to detect sex-specific QTLs and Genotype × Sex interactions in BE that likely exist in genetically more complex populations. Furthermore, because our transcriptome analysis was limited by the number of samples, brain regions, cell types, and time points that could be examined, a comprehensive transcriptomic study of BE is warranted to further our understanding of the dynamic neurobiological mechanisms influencing susceptibility versus establishment and persistence of BE.

We identified *Cytip2* as a major genetic factor in preclinical BE. These results suggest that *CYFIP2* and possibly *CYFIP1* could be associated with maladaptive feeding in humans. Our success in QTL mapping and gene validation provides a clear rationale for expanding QTL mapping studies of BE to genetically diverse rodent populations and reference panels. These efforts will inform human GWAS of ED and could reveal new therapeutic directions to pursue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

These studies were supported by R21DA038738 (C.D.B.), R03DA038287 (C.D.B.), R00DA029635 (C.D.B.), F31DA040324 (N.Y.), R01DA030425 (P.C.), T32GM008541 (L.R.G., N.Y., and A.B.), Burroughs Wellcome Fund Transformative Training Program in Addiction Science #1011479 (N.Y. and J.W.), and Boston University's Undergraduate Research Opportunities Program (K.I.L. and K.P.L.). We thank Dr. Kenneth Albrecht and Gregory Martin at the Boston University Transgenic and Genome Engineering Core Facility for assistance in re-derivation of *Cyfp2*^{N/-} mice. We thank Dr. Joseph Dougherty for his advice on CSEA.

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Figure 1. Escalation of PF consumption in B6NJ but not B6J mice

Mice were video recorded and video tracked in unlit sound attenuating chambers for preference for the PF-paired side (right side). On Day (D)1, mice were assessed for initial preference whereby mice were placed into the left side and provided free access to both sides (30 min) that contained clean, empty, porcelain food dishes via an open entryway for 30 min. For PF training days [Day (D)2, D4, D9, D11, D16, D18, and D23 (F₂ only)], mice were provided 20 mg 5-TUL pellets in a non-porous, porcelain dish in the far right-hand corner of the PF-paired side. The 5-TUL pellets are similar in nutrient composition to the home cage diet but also contain sucrose to increase palatability. Mice were confined to the PF-paired side and allowed to consume the pellets for 30 min. The pellets were weighed immediately prior to and following the 30 min session. For the “No PF” training sessions (D3, D5, D10, D12, D17, and D19), mice were confined to the left side and provided a clean, empty porcelain dish. Control mice were presented with custom-designed Chow-like pellets (20 mg; no sucrose) on the right side. We quantified the amount of food consumed as percent body weight (BW). Mice were assessed for PF-CPP on Day (D)8, D15, and D22 in the same manner as D1. **(A):** D = Day of protocol. CPP = Day of CPP assessment. PF = palatable food (20 mg pellets of 5-TUL diet) were provided on the right side for 30 min. “No PF” = empty dish was provided on the left side for 30 min. “Home cage” = mice were left undisturbed in their home cage in the vivarium. **(B):** Dimensions and texture of the CPP apparatus. Time spent on the PF-paired side (right side) and time spent within the PF-paired triangle of the right side were examined. White circles denote the porcelain food bowls that contained food or no food. The two horizontal lines in the middle represent the entryway between each side that was open only during CPP assessment. **(C):** Assessment of B6 substrain differences in PF consumption (% body weight; BW) indicated a main effect of

Substrain ($F_{1,189}=84.42$; $p=2.0 \times 10^{-16}$) and a Substrain $\times$ Day interaction ($F_{6,189}=3.45$; $p=0.0028$). B6NJ mice ($N=15$; 8 females, 7 males) showed significantly greater PF consumption than B6J mice ($N=8$ females, 7 males) on D4 through D23 ($t_{19,22}=3.30-5.79$; $*p<0.007$; corrected 0.05 level for 7 comparisons). The strain difference in summed PF consumed (% BW) across training days is shown in the inset ($t_{21}=4.73$; $p=0.0001$). **(D):** Slope analysis across PF training days indicated a steeper escalation in PF consumption in B6NJ versus B6J mice ($F_{1,10}=63.2$; $p<0.0001$). **(E):** For PF-CPP, there was no effect of Strain ($F_{1,108}<1$), no effect of Day ($F_{3,108}=2.07$; $p=0.11$), and no interaction ($F_{3,108}=1.04$; $p=0.38$). **(F):** When considering time spent in the PF triangle, there was no effect of Substrain ($F_{1,108}<1$) and an effect of Day ($F_{3,108}=3.20$; $p=0.03$). **(G):** In considering differences in body weight between strains, there was no effect of Strain ($F_{1,189}=2.24$; $p=0.14$), no effect of Day ($F_{6,189}<1$), and no interaction ($F_{6,189}<1$).

Figure 2. QTL near *Cyfip2* on chromosome 11 in B6J x B6NJ-F₂ mice

The F₂ dataset and R code for QTL analysis are available at github (https://github.com/camronbryant/QTL_BingeEating_B6Substrains). The phenotype of B6J x B6NJ-F₁ mice (Fig.S3) versus parental strains (Fig.1C,D) suggested that the B6NJ allele was semi-dominant(29). (A,B): A single genome-wide significant QTL for summed PF consumed (% BW) was identified on chromosome 11 that peaked at the location of the *Cyfip2* missense mutation (rs240617401; 28 cM; 46,222,615 bp; mm10; LOD=7.4; bolded uptick on x-axis of QTL plots) previously identified for cocaine behavioral sensitivity (29). The 1.5-LOD support interval was defined by the nearest markers flanking the peak (rs48169870, rs13481117) and spanned 31 Mb-79 Mb. We considered females and males combined (black trace), females only (red trace), or males only (blue trace) in the analysis. The significance thresholds are represented by the horizontal lines which were nearly identical for the three separate analyses and thus overlay one another. (C,D): The time course of the effect plot for the *Cyfip2* marker (rs240617401; bolded uptick) across PF training days indicated that the most significant LOD score and largest separation of genotypes in PF consumption occurred during the earlier training days (D2-D11; red, orange, yellow, green, light blue). (E,F): For F₂ females (N=78), the effect plot for the *Cyfip2* marker (bolded uptick on x-axis) indicated that the QTL peaked early on D2 (red) and that a second, more distally localized QTL emerged on D9 and D11 at 60 cM (green, yellow). Coding and structural variants on distal chromosome 11 identified via whole genome sequencing include an initiator codon variant in *Stxbp4* (syntaxin binding protein 4; 90 Mb) located at 90 Mb and an intronic, retrotransposon insertion in *Rptor* (regulatory associated protein of MTOR complex 1; 120 Mb)(26, 42, 72). (G,H): For F₂ males (N=78), the QTL traces and effect plots indicated suggestive peaks located near *Cyfip2* (bolded uptick on x-axis), none of which were

genome-wide significant. Six missing genotypes at rs240617401 were imputed for QTL analysis (A,C,E). The effect plots represent phenotypic data from the 150 out of 156 called genotypes, including N=33 *Cytip2^{J/J}* (16 females, 17 males), N=85 *Cytip2^{J/N}* (43 females, 42 males), and N=32 *Cytip2^{N/N}* (17 females, 15 males). Data are presented as the mean $\pm$ S.E.M. The significance thresholds (horizontal lines) in panels C, E, and G represent D2 which was the highest (most conservative) threshold across all days.

Figure 3. Reduced BE in *Cyfip2*^{N/N} mice

(A): In examining changes in food consumption across days, there was an effect of Genotype ($F_{1,623}=42.45$, $p=1.5 \times 10^{-10}$), Treatment ($F_{1,623}=181.4$, $p<2.0 \times 10^{-16}$), a Genotype $\times$ Treatment interaction ($F_{5,623}=19.59$, $p=1.13 \times 10^{-5}$), and a Treatment $\times$ Day interaction ($F_{5,623}=9.78$, $p=5.25 \times 10^{-9}$). To reveal the source of the interactions, two-way ANOVA for each day identified significant Genotype $\times$ Treatment interactions on D2, D9, and D16 ($F_{1,105}=7.39$, 6.76 , 4.71 , $p=0.007$, 0.011 , 0.032) that were in part explained by increased PF versus Chow consumption in *Cyfip2*^{N/N} mice but not *Cyfip2*^{N/-} mice on D2 ($t_{50}=3.11$; $\%p<0.0125$; Bonferroni-corrected for four comparisons) and by increased PF consumption in *Cyfip2*^{N/N} versus *Cyfip2*^{N/-} mice on D9 ($t_{39}=3.19$; $\%p<0.0125$). No differences in Chow pellet consumption were observed between *Cyfip2*^{N/N} and *Cyfip2*^{N/-} mice across any training day ($p>0.0125$). **(B):** For total food consumption, there was an effect of Genotype (Geno; $F_{1,150}=11.29$, $p=0.001$), Treatment (Tx; $F_{1,105}=49.87$, $p=1.85 \times 10^{-10}$), and an interaction ($F_{1,105}=5.35$, $p=0.02$). Both *Cyfip2*^{N/N} and *Cyfip2*^{N/-} mice showed greater PF consumption compared to their respective Chow groups ($t_{33}=5.6$, 4.32 , $\%p<0.0125$). Furthermore, *Cyfip2*^{N/N} mice consumed more PF than *Cyfip2*^{N/-} mice ($t_{43}=2.75$, $\%p<0.0125$) but not Chow ($t_{29}=1.0$, $p>0.0125$). **(C):** Dimensions of light/dark conflict test of compulsive-like PF consumption provided in the center of the light side. **(D):** In measuring compulsive-like PF consumption in the light/dark conflict test, there was a main effect of Genotype ($F_{1,105}=14.83$, $p=0.0002$) and Training Treatment ("Train."; $F_{1,105}=5.14$, $p=0.025$), but no interaction ($F_{1,105}=3.06$, $p=0.08$). Only the PF-trained *Cyfip2*^{N/N} mice consumed more PF than their Chow-trained *Cyfip2*^{N/N} controls ($\%t_{39}=3.68$; $p<0.0125$). There was no difference in PF consumption in Chow-trained *Cyfip2*^{N/N} versus Chow-trained *Cyfip2*^{N/-} mice ($t_{47}<1$), demonstrating a selective reduction of compulsive-

like PF consumption in *Cytip2^{N/-}* mice. **(E,F):** In examining concomitant percent time in the light side and the number of entries into the light side during compulsive-like PF consumption, there were significant Genotype $\times$ Training Treatment interactions ($F_{1,105}=4.23, 5.48$; $p=0.04, 0.02$) that were explained by PF-trained *Cytip2^{N/N}* mice exhibiting an increase in time spent on the light side and a decrease in the number of entries to the light side relative to Chow-trained *Cytip2^{N/N}* mice ($t_{38-43}=2.67, 2.63$, $*p<0.0125$) whereas Chow-versus PF-trained *Cytip2^{N/-}* mice did not differ from their Chow-trained *Cytip2^{N/-}* controls ($t_{58-60}<1$).

Figure 4. Effect of Genotype and Treatment on the striatal transcriptome

The F₂ dataset is available on Gene Expression Omnibus (GEO): (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=kdcviyasnmxfen&acc=GSE84252>). The dataset for *Cyfp2*^{N/N} and *Cyfp2*^{N/-} mice is available at GEO (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ixioxekcdrcxtqt&acc=GSE84329>). (A): Venn diagram indicates the number of differentially expressed genes and their overlap between F₂ versus knockout analysis from the main effect of *Cyfp2* Genotype (GENO: *Cyfp2*^{N/-} versus *Cyfp2*^{N/N}) or Treatment (TX: PF vs. Chow). 224 genes from the main effect of GENO and 61 unique genes from the main effect of TX (green-colored numbers) were included in subsequent enrichment analyses. (B): Correlation of log₂ fold-change in gene expression from the 84 overlapping genes identified in *Cyfp2*^{N/N} mice from the F₂ study (relative to *Cyfp2*^{J/J}; x-axis) versus the knockout study (relative to *Cyfp2*^{N/-}; y-axis). (r=0.76; r²=0.58; F_{1,82}=115.2; p=2.7 × 10⁻¹⁷). (C): The top IPA network for the effect of GENO is shown (enrichment score = 36). Green = downregulated in binge-resistant *Cyfp2*^{N/-} mice (or, i.e., upregulated in binge-susceptible *Cyfp2*^{N/N} mice). Red = upregulated in binge-resistant *Cyfp2*^{N/-} mice (or, i.e., downregulated in binge-susceptible *Cyfp2*^{N/N} mice). (D): The top IPA network for the effect of Treatment is shown (enrichment score = 28). Green = downregulated in PF-trained mice. (E,F): CSEA results for the effect of Genotype (E) or Treatment (F) are presented as bullseye plots for each cell type. The topographical plot represents the hierarchical clustering of each cell type based on the relative gene expression profile(38). The specificity index probability (pSI) statistic(73) was previously used to identify cell-specific reference gene lists that were used to build the bullseye plots. The size of the hexagon for each cell type is scaled to the number of transcripts expressed in that particular cell type relative to the other cell types. The bullseye

plot is divided into four separate rings. The outer ring represents the reference gene set with the least stringent threshold of enrichment for that particular cell type ($p < 0.05$) whereas the inner ring (the bullseye) represents the reference gene set with the most stringent threshold of enrichment for that particular cell type ($p < 1 \times 10^{-4}$). The degree of enrichment of one's own experimentally derived gene list for the reference gene list within each ring is color-coded based on the p-value derived from a Fisher's exact test. Enrichment signals originating from the outer rings are more likely to contain genes that are somewhat more promiscuously expressed across different cell types whereas enrichment signals located at the center bullseye represent genes that are the most exclusive for that particular cell type(38). For the effect of Genotype (E), only D1 and D2 striatal spiny neurons contained a strong enrichment signal within all rings of the two cell types (including the bullseye) which indicates an enrichment for gene sets that are most exclusive for D1 and D2 striatal spiny neurons. In contrast, only the outer rings of other cell types contained enrichment signals (cortical, cerebellar, cholinergic, and serotonergic neurons and cones) which are more likely to be driven by somewhat more promiscuously expressed genes across different cell types. Enrichment in deep cortical layers could also reflect an imprecision in the dissection punches. (F): For the effect of Treatment, the enrichment signals were exclusive to oligodendrocyte progenitors and oligodendrocytes.

Table 1

Enrichment analyses of (A) binge-prone *Cytip2*^{N/N} F₂ mice versus binge-resistant *Cytip2*^{J/J} F₂ mice, (B) the effect of Genotype (*Cytip2*^{N/-} mice versus *Cytip2*^{N/N} mice), and (C) the effect of Treatment (PF versus Chow).

	Term	Overlap	P	FDR
A: Binge-prone <i>Cytip2</i>^{N/N} F₂ mice versus binge-resistant <i>Cytip2</i>^{J/J} F₂ mice				
GO: Biological Process				
GO: 0007268	synaptic transmission	60/434	7.5E-21	2.4E-17
GO: 0007610	behavior	49/494	4.1E-12	6.5E-09
GO: 0032940	secretion by cell	41/415	2.9E-10	3.1E-07
GO: 0007626	locomotory behavior	25/183	3.2E-09	2.6E-06
GO: 0044708	single-organism behavior	35/362	1.0E-08	6.6E-06
GO: Cellular Component				
GO: 0044456	synapse part	42/395	1.9E-11	6.2E-09
GO: 0034702	ion channel complex	31/258	6.5E-10	1.1E-07
GO: 1902495	transmembrane transporter complex	31/286	6.2E-09	6.8E-07
GO: 1990351	transporter complex	31/291	9.0E-09	7.4E-07
GO: 0030425	dendrite	27/236	2.2E-08	1.4E-06
GO: Molecular Component				
GO: 0022836	gated channel activity	31/323	3.3E-07	2.2E-04
GO: 0015267	channel activity	35/427	1.8E-06	3.9E-04
GO: 0022803	passive transmembrane transporter activity	35/427	1.8E-06	3.9E-04
GO: 0005216	ion channel activity	33/396	2.6E-06	4.2E-04
GO: 0022838	substrate-specific channel activity	33/406	4.2E-06	5.6E-04
KEGG ID				
hsa04723	retrograde endocannabinoid signaling	21/101	1.1E-10	2.6E-08
hsa04727	GABAergic synapse	19/88	5.2E-10	6.0E-08
hsa05032	morphine addiction	18/91	5.1E-09	3.9E-07
hsa04724	glutamatergic synapse	19/114	2.2E-08	1.3E-06
hsa04713	circadian entrainment	17/95	5.1E-08	2.4E-06
B: Effect of Genotype (<i>Cytip2</i>^{N/-} mice versus <i>Cytip2</i>^{N/N} mice)				
GO: Biological Process				
GO: 0007610	behavior	31/494	1.3E-14	1.3E-11
GO: 0044708	single-organism behavior	27/362	1.6E-14	1.3E-11
GO: 0007268	synaptic transmission	29/434	2.2E-14	1.3E-11
GO: 0030534	adult behavior	14/141	1.7E-09	7.3E-07
GO: 0007626	locomotory behavior	15/183	4.9E-09	1.7E-06
GO: Cellular Component				
GO: 0044456	synapse part	23/395	1.4E-10	2.9E-08

	Term	Overlap	P	FDR
GO: 0043025	neuronal cell body	16/270	8.9E-08	8.5E-06
GO: 0044297	cell body	17/315	1.2E-07	8.5E-06
GO: 0005887	integral component of plasma membrane	30/1066	2.1E-06	9.4E-05
GO: 0097060	synaptic membrane	13/228	2.3E-06	9.4E-05
GO: Molecular Component				
GO: 0016247	channel regulator activity	8/102	3.3E-05	6.5E-03
GO: 0035254	glutamate receptor binding	5/29	3.6E-05	6.5E-03
GO: 0035255	ionotropic glutamate receptor binding	4/17	7.9E-05	7.5E-03
GO: 0005249	voltage-gated potassium channel activity	7/86	8.3E-05	7.5E-03
GO: 0004714	transmembrane receptor protein tyrosine kinase activity	6/65	1.4E-04	1.0E-02
KEGG				
hsa04724	glutamatergic synapse	13/114	3.1E-10	4.2E-08
hsa05032	morphine addiction	11/91	4.7E-09	3.2E-07
hsa04723	retrograde endocannabinoid signaling	11/101	1.3E-08	5.8E-07
hsa04725	cholinergic synapse	10/111	3.2E-07	8.8E-06
hsa05030	cocaine addiction	8/49	8.5E-08	2.9E-06
C: Effect of Treatment (PF versus Chow)				
GO: Biological Process				
GO: 0042552	myelination	6/59	6.4E-08	2.6E-05
GO: 0008366	axon ensheathment	6/62	8.5E-08	2.6E-05
GO: 0007272	ensheathment of neurons	6/62	8.5E-08	2.6E-05
GO: 0021762	substantia nigra development	4/45	1.9E-05	4.5E-03
GO: 0010001	glial cell differentiation	4/61	6.0E-05	9.8E-03
GO: Cellular Component				
GO:0043209	myelin sheath	3/17	3.7E-05	4.1E-03
GO:0005902	microvillus	4/61	6.1E-05	4.1E-03
GO:0097386	glial cell projection	2/9	5.8E-04	1.9E-02
GO:0044224	juxtaparanode region of axon	2/9	5.8E-04	1.9E-02
GO:0033270	paranode region of axon	2/11	8.2E-04	2.1E-02
MGI Mammalian Phenotype 4				
MP0000920	abnormal myelination	9/134	3.6E-10	8.6E-08
MP0002882	abnormal neuron morphology	14/1006	8.3E-07	9.9E-05
MP0003634	abnormal glial cell	7/231	7.2E-06	5.7E-04
MP0002066	abnormal motor capabilities	14/1482	7.4E-05	4.4E-03
MP0000762	abnormal tongue morphology	4/77	1.1E-04	5.4E-03