Mood Disorder Susceptibility Gene *CACNA1C* Modifies Mood-Related Behaviors in Mice and Interacts with Sex to Influence Behavior in Mice and Diagnosis in Humans

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Background: Recent genome-wide association studies have associated polymorphisms in the gene *CACNA1C*, which codes for $Ca_v1.2$, with a bipolar disorder and depression diagnosis.

Methods: The behaviors of wild-type and *Cacna1c* heterozygous mice of both sexes were evaluated in a number of tests. Based upon sex differences in our mouse data, we assessed a gene × sex interaction for diagnosis of mood disorders in human subjects. Data from the National Institute of Mental Health Genetics Initiative Bipolar Disorder Consortium and the Genetics of Recurrent Early-Onset Major Depression Consortium were examined using a combined dataset that included 2021 mood disorder cases (1223 female cases) and 1840 control subjects (837 female subjects).

Results: In both male and female mice, *Cacna1c* haploinsufficiency was associated with lower exploratory behavior, decreased response to amphetamine, and antidepressant-like behavior in the forced swim and tail suspension tests. Female, but not male, heterozygous mice displayed decreased risk-taking behavior or increased anxiety in multiple tests, greater attenuation of amphetamine-induced hyperlocomotion, decreased development of learned helplessness, and a decreased acoustic startle response, indicating a sex-specific role of *Cacna1c*. In humans, sex-specific genetic association was seen for two intronic single nucleotide polymorphisms, rs2370419 and rs2470411, in *CACNA1C*, with effects in female subjects (odds ratio = 1.64, 1.32) but not in male subjects (odds ratio = .82, .86). The interactions by sex were significant after correction for testing 190 single nucleotide polymorphisms ($p = 1.4 \times 10^{-4}$, 2.1×10^{-4}; $p_{corrected} = .03, .04$) and were consistent across two large datasets.

Conclusions: Our preclinical results support a role for *CACNA1C* in mood disorder pathophysiology, and the combination of human genetic and preclinical data support an interaction between sex and genotype.

Key Words: Animal model, bipolar disorder, CACNA1C, $Ca_v1.2$, gender, major depression, sex differences

Bipolar disorder (BP) and major depressive disorder (MDD) are severe psychiatric disorders affecting about 1% to 3% and 15% of the world's population, respectively, over a lifetime. The two disorders are closely related, as depressive episodes are a core feature of BP, and the illnesses often run together in families (1). Despite being common and often severe illnesses, there is limited knowledge regarding their underlying pathophysiology. It is becoming increasingly clear that gender differences are a critical consideration. For MDD, prevalence rates are approximately twice as high for women as for men, and this difference only arises after puberty (2). In BP, some studies suggest the course of illness is different between genders, with women tending to have more depressive episodes, an increased risk for the rapid cycling form of the illness, and a later age of onset (3,4). While studies have suggested sex differences in genetic risk factors for depression (5) and BP (6), the underlying causes of these sex differences are unclear.

The heritability of BP and depression, ~ 70% to 90% and 40% to 50%, respectively, indicates that genetic variation in particular genes predisposes to the development of these disorders and that understanding the biological significance of this variation will provide insight into pathophysiology (7,8). The results of genome-wide association studies (GWAS) have converged to implicate a small number of genes in BP etiology. One candidate gene that has emerged from some (9,10), though not all (11), BP GWAS is *CACNA1C*. Additional association studies have correlated polymorphisms in *CACNA1C* with depression and schizophrenia (12–17). A recent GWAS meta-analysis of BP and MDD patients reported single nucleotide polymorphisms (SNPs) in *CACNA1C* as the most significant findings, with p values that surpassed genome-wide significance, the strongest being $p = 3.1 \times 10^{-8}$ (18). In addition, reports have associated SNPs in *CACNA1C* with differences in mean gray matter volume (19,20), verbal fluency (21), limbic activity (22), and mediotemporal emotional processing and prefrontocortical working memory processing (66) in healthy human subjects.

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The L-type voltage-gated calcium channel family consists of four distinct isoforms referred to as $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, and $Ca_v1.4$ (23). *CACNA1C* codes for the pore-forming alpha-1C subunit of the $Ca_v1.2$ isoform. In the mouse brain, $Ca_v1.2$ accounts for ~ 85% of the L-type channels, with $Ca_v1.3$ accounting for most of the remainder (24). While it was recently shown that the risk-associated SNP (rs1006737) identified in previous bipolar disorder GWAS is associated with increased expression of *CACNA1C* in human postmortem brains (17) it remains critical to understand how changes in gene expression and protein levels may consequently affect behavior in model systems. We investigated the effects of *Cacna1c* haploinsufficiency on mouse behavior in both sexes in tests with relevance to human mood disorders. Based upon sex differences we observed in our mouse behavioral data, we assessed a gene × sex interaction of SNPs of *CACNA1C* with mood disorders in human subjects across 2021 cases and 1840 control subjects.

Methods and Materials

Animals

Founder mice were obtained from Jackson Laboratories (stock number 005783, Bar Harbor, Maine) that had been backcrossed to C57BL/6J for at least seven generations (see Supplement 1 for additional details).

Behavioral Tests

Mice were tested in the open field, home cage activity, hole board, elevated plus maze, light-dark box, novelty-induced hypophagia, stress-induced hyperthermia, sensitization to *d*-amphetamine, acoustic startle, and forced swim tests (FSTs) using established methods. Minor alterations to the tail suspension tests (TSTs) were required to prevent the tendency of C57BL/6J mice to climb their tails (25). A clear plastic cylinder (4 cm length, 1.5 cm diameter) was placed around their tails. The learned helplessness procedure was performed in a Coulbourn Mouse Shuttle Cage (Coulbourn Instruments, Whitehall, Pennsylvania) and consisted of three stages: stage 1: 120 inescapable shocks, each 15 seconds at .3 mA with a 15-second intertrial interval; stage 2: 30 trials, 15-second shocks, .3 mA, average intertrial interval of 20 seconds (in trials P1–P5, gate opened when the shock began; in trials 1–25, gate opened 3 seconds following shock); stage 3: retest. See Supplement 1 for additional details for all behavioral tests.

Statistical analysis of the mouse behavioral data was performed using GraphPad Prism Version 5 (Graphpad Software, San Diego, California). Statistics used were two-tailed *t* test or repeated measure two-way analysis of variance (ANOVA), either paired or unpaired dependent upon the experimental design, and Bonferroni post hoc test was performed when indicated. Evaluation of equal variances was performed using Bartlett's test. Detailed statistical results for mouse behavioral data are included in Table S1 in Supplement 1. Data are reported as mean ± SEM and $p < .05$ was considered significant. Male and female mice were assessed on separate days and therefore (because of likely effects of day) direct statistical comparison between sexes was not made.

Human Genetics Cohorts

Data were collected by the National Institute of Mental Health Genetics Initiative Bipolar Disorder Consortium (NIMH-BP) (26) and the Genetics of Recurrent Early-Onset Major Depression Consortium (GenRED) (27). The NIMH-BP SNP genotyping was performed on 1001 BP cases and 1034 unrelated control subjects as part of the Genetic Association Information Network Bipolar Initiative and Bipolar Genome Study (11). In a separate effort, genome-wide SNP genotyping was performed on 1020 MDD cases from the GenRED sample and 1636 unrelated control subjects (15). Genotyping in both samples was performed using the Affymetrix Genome-wide Human SNP Array 6.0 (Affymetrx, Santa Clara, California) and we used the cleaned genotype data from each sample. Quality control measures and analytic methods are described in Supplement 1. We combined the data from the NIMH-BP and GenRED samples at the genotype level in a mega-analysis using PLINK (28), keeping only those SNPs common to both datasets. In addition, 830 control subjects were common to both NIMH-BP and GenRED and were included in the dataset only once. For the analysis of rs2370411 and rs2370419 by substudy, unique control subjects were apportioned to each substudy to avoid overlap. We extracted 190 SNPs located in the gene *CACNA1C* or within 10 kilobases (kb) of the gene boundaries for analysis. All alleles are presented in the forward strand orientation.

Results

Baseline Behaviors, Activity, and Exploration

As homozygous deletion of *Cacna1c* results in embryonic lethality in mice (29), we conducted studies with heterozygous (*Cacna1c*$^{+/-}$ [HET]) *Cacna1c* knockout mice and compared these animals with their wild-type (*Cacna1c*$^{+/+}$ [WT]) littermates. Western blot analysis confirmed that heterozygous knockout of *Cacna1c* results in a significant decrease in levels of $Ca_v1.2$ without a change in $Ca_v1.3$, which is the most likely protein to compensate for such changes (Figure S1 in Supplement 1). As expected, whole-cell voltage-clamp recordings made from cornu ammonis 1 pyramidal cells in ex vivo hippocampal slices revealed that the fraction of the current sensitive to nimodipine, a dihydropyridine L-type calcium channel blocker, was significantly larger in cells from WT mice than in cells from HET mice ($p < .05$) (Figure S1 in Supplement 1). Due to the widespread expression of *Cacna1c* in the mouse brain and thus the potential for nonspecific effects on behavior, baseline sensory motor function was assessed in male and female mice. We found no significant differences between genotypes in motor coordination and motor learning as assessed on the repeated accelerating rotarod, olfaction as assessed in the hidden cookie test, pain sensitivity in the hot plate test, or muscle strength in the hanging wire test (Figure S2 in Supplement 1).

We assessed general locomotor activity in a 50 × 50 cm open field in both male and female mice (Figure 1A,B). Repeated measure two-way ANOVA indicated no significant difference in open field locomotion between male WT and HET mice. However, female HET mice displayed a subtle, yet statistically significant, decrease in locomotor activity in the open field ($p < .05$). Bartlett's test of equal variances revealed no significant differences in the variance between male and female mice of both genotypes, suggesting estrous cycle was not influencing behavior (Table S1 in Supplement 1).

To assess baseline activity in a nonnovel environment, we tracked home cage activity in both male and female mice. Mice were single-housed for at least 1 week before home cage activity was monitored by an infrared tracking system during the 12 hours corresponding to the dark cycle. Neither male nor female HET mice displayed significant differences in home cage locomotion compared with WT mice (Figure 1C,D). The hole board test measures exploratory behavior and previous work has reported that lithium, one of the most effective mood-stabilizing medications, decreases hole poke behavior in mice (30). We found both male ($p < .05$) and female ($p < .05$) HET mice displayed a significantly lower number of hole pokes during a 5-minute test (Figure 1E,F).

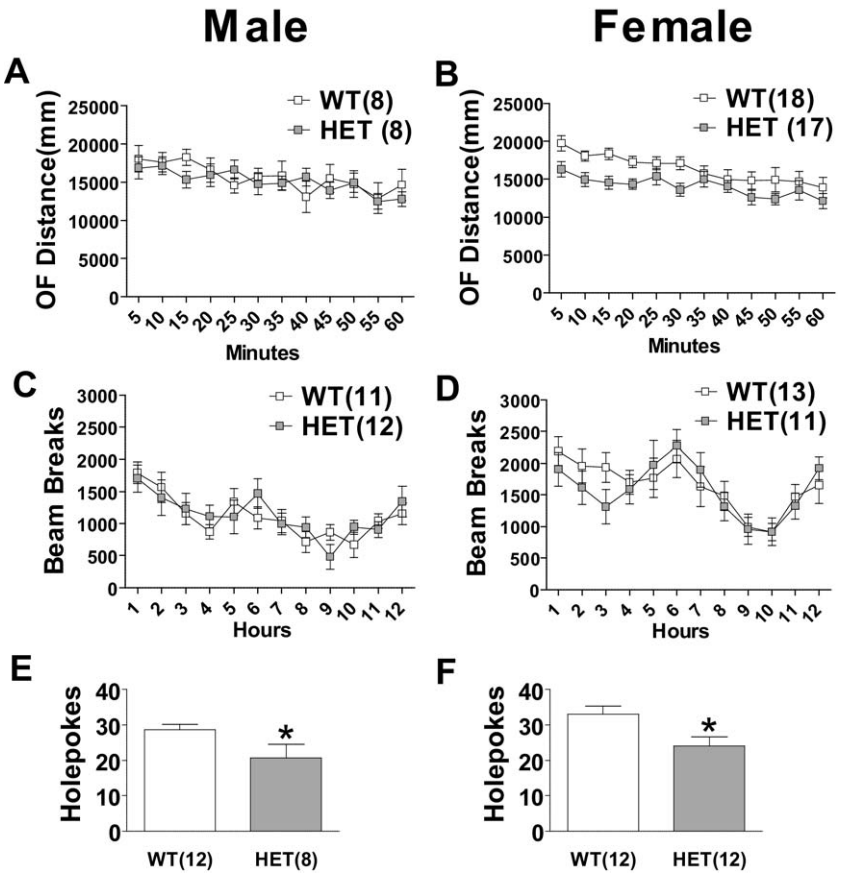

Figure 1. Effect of heterozygous (*Cacna1c*$^{+/-}$ [HET]) *Cacna1c* knockout on activity and exploration. **(A)** Male HET mice did not differ significantly from wild-type (*Cacna1c*$^{+/+}$) mice in open field locomotion. **(B)** In female mice, genotype had a significant effect on locomotion in the open field ($p < .05$). Neither male **(C)** nor female **(D)** HET mice were significantly different from wild-type mice in home cage activity. **(E)** Male and **(F)** female HET mice had lower exploratory activity on the hole board test. Data are expressed as mean ± SEM. *$p \leq .05$. HET, heterozygous *Cacna1c* knockout (*Cacna1c*$^{+/-}$) mice; OF, open field; WT, wild-type (*Cacna1c*$^{+/+}$) mice.

Anxiety/Risk-Taking Behavior

We explored possible differences between WT and HET mice in multiple tests of anxiety and risk-taking behavior. We tested WT and HET mice of both sexes in a 100 × 100 cm open field, which is more sensitive to thigmotaxis than a smaller arena, over a 10-minute period (Figure 2A,B). Female, but not male, HET mice showed significantly decreased center time ($p < .001$). This increased thigmotaxis observed in female, but not male, HET mice suggests an increased anxiety or a decreased risk-taking phenotype. The elevated plus maze explores the anxiety and risk taking associated with entering an exposed, raised platform. Over 5 minutes, we measured time spent on the open arms in WT and HET mice (Figure 2C,D). In female mice only, HET mice spent significantly less time in the open arm ($p < .001$), indicating lower risk taking or greater anxiety; however, female HET mice also had a significant decrease in number of total arm entries ($p < .001$), indicating lower overall activity. We also tested WT and HET mice in the light-dark box (Figure 2E,F). Increased time spent in the aversive light compartment is used as an inverse measure of anxiety and is related to risk taking. In female mice only, HET mice spent significantly less time in the light area compared with WT mice ($p < .05$). Additionally, in both male and female mice, genotype had no significant effect on time for first emergence to the light area or total number of crosses.

We assessed the behavior of mice in two additional tests, novelty-induced hypophagia and stress-induced hyperthermia, that do not rely upon the animals entering an exposed area from a safer enclosed area. The novelty-induced hypophagia test assesses anxiety by comparing changes in latency to consume a familiar, palatable liquid between subjects' home cage and a novel cage (31). In both male and female mice, a repeated measure two-way ANOVA revealed a significant increase in latency to consume the liquid in the novel cage ($p < .001$) but no significant effect of genotype and no significant interaction (Figure 2G,H).

The physiological response to a stressful or anxiety-provoking event is an increase in body temperature and this increase is attenuated by anxiolytics (32). A repeated measure two-way ANOVA revealed both male and female WT and HET mice had a significant increase in temperature after stress ($p < .001$), but there was no significant effect of genotype and no significant interaction (Figure 2I,J).

Response to *d*-Amphetamine and Acoustic Startle

Amphetamine-induced hyperlocomotion is a model of the hyperactivity associated with bipolar mania; this hyperactivity is attenuated by lithium in both humans and mice (33–36). We conducted amphetamine sensitization in three stages: habituation, sensitization to *d*-amphetamine, and long-term sensitization or challenge (37). In male mice, both WT and HET mice habituated to the same level of locomotion with saline injections between the first and last days (Figure 3A). During *d*-amphetamine sensitization, repeated measure two-way ANOVA revealed a significant effect of day ($p < .001$) and a significant effect of genotype on distance traveled ($p < .05$). Between days 18 and 39, a repeated measure two-way ANOVA revealed a trend for effect of genotype ($p = .063$), indicating a long-lasting attenuation in response to *d*-amphetamine in male HET mice. In female mice, HET mice started at and habituated to a significantly lower level of locomotion compared with WT mice with a significant effect of day ($p < .001$) and genotype ($p < .001$) on distance traveled and a trend for an interaction between genotype and day ($p = .056$) (Figure 3B). In female mice,

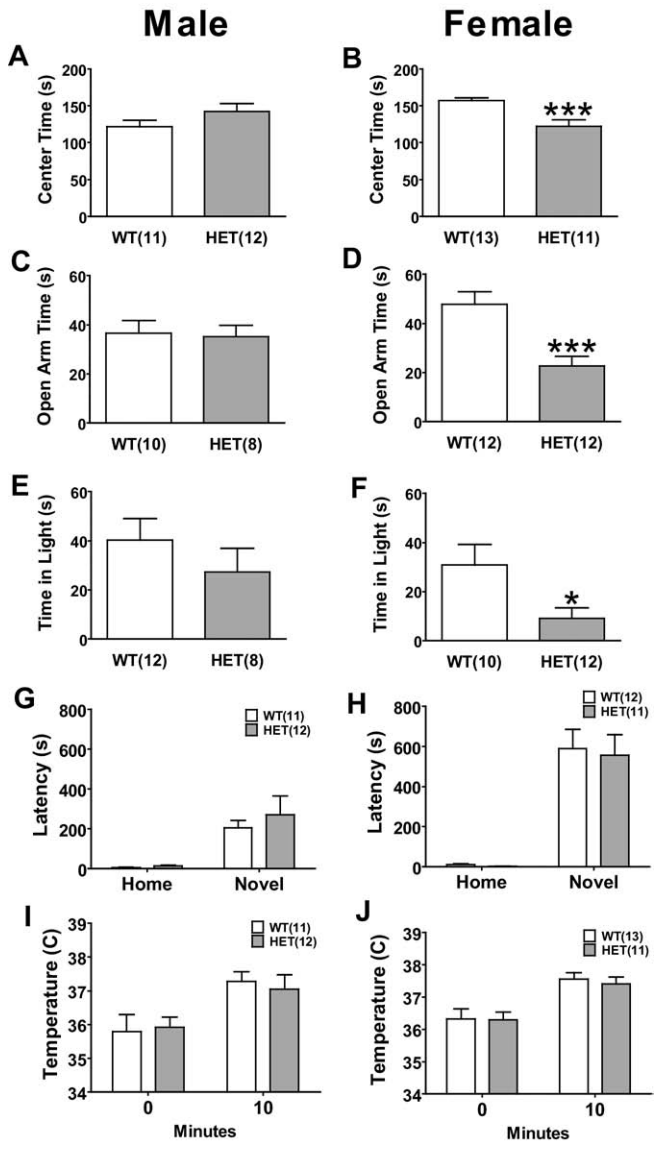

Figure 2. Effect of heterozygous (*Cacna1c*$^{+/-}$ [HET]) *Cacna1c* knockout on risk-taking and anxiety-like behavior. **(A)** Male HET mice had no significant difference in center time in a large open field. **(B)** Female HET mice spent less time in the center of a large open field. **(C)** There was no significant difference in time spent on the open arms of the elevated plus maze in male HET mice. **(D)** Female HET mice spent less time on the open arms of the elevated plus maze. **(E)** There was no significant difference in the time male HET mice spent in the light compartment of the light-dark box. **(F)** Female HET mice spent less time in the light compartment of the light-dark box. There was no significant effect of genotype in either **(G)** male or **(H)** female mice to modify latency to drink in the novel cage when compared with wild-type (*Cacna1c*$^{+/+}$) mice. There was no significant effect of genotype in either **(I)** male or **(J)** female subjects to modify hyperthermia in response to stress. Data are expressed as mean ± SEM. *$p \leq .05$; ***$p < .001$. HET, heterozygous *Cacna1c* knockout (*Cacna1c*$^{+/-}$) mice; WT, wild-type (*Cacna1c*$^{+/+}$) mice.

there was a significant effect of genotype on locomotion response to *d*-amphetamine during both sensitization days and challenge days 18 and 39 ($p < .01$).

Changes in acoustic startle response can be induced by pharmacological (38) or genetic manipulations of the dopaminergic system (39). Changes in acoustic startle response habituation have also been reported in humans during the manic phase of BP (40). Mice were tested on their response to startle pulse intensities of 80, 90, 100, 110, and 120 dB. In male mice, we found no significant difference between genotypes at all startle intensities (Figure 3C). In female mice, a repeated measure two-way ANOVA revealed a significant interaction between genotype and startle intensity ($p < .05$) and a Bonferroni post hoc test indicated that HET mice startled significantly less at the 120 dB pulse (Figure 3D; $p < .05$). The normal startle response of female HET mice at intensities between 80 and 110 dB suggest that the attenuated startle at 120 dB is not due to a nonspecific effect of *Cacna1c* haploinsufficiency on hearing.

Tests of Antidepressant Efficacy

Previous studies indicate that administration of L-type calcium channel antagonists to rodents results in antidepressant-like effects in the forced swim test and tail suspension test (41). Similarly, lithium has been reported to decrease immobility time in these tests (30,33,42). In both male and female mice, HET mice spent significantly less time immobile than WT mice in the FST ($p < .05$) (Figure 4A,B). Similarly, in the TST, both male and female HET mice had significantly less immobility time ($p < .05$) (Figure 4C,D).

Learned Helplessness Model of Depression

The learned helplessness test is a validated model of depression-like behavior (43). Twenty-four hours following exposure to inescapable/uncontrollable shock, we tested WT and HET mice in a shuttle box to measure their motivation to escape shock. We measured number of escape failures, defined as failure to escape and thus terminate shock, both 24 hours after induction of the learned helplessness effect and then 7 days after first testing. In trials P1 to P5, shock onset and gate opening occurred simultaneously. In subsequent trials, 1 to 25, gate opening occurred 3 seconds after shock onset. In female mice, there was a significant effect of genotype ($p < .05$) on number of escape failures in trials 1 to 25 on both days (Figure 5B,D). Female HET mice showed fewer escape failures. Male HET mice did not significantly differ from WT mice on either day (Figure 5A,C). There was no significant difference between genotype in trials P1 to P5 in either sex for either day. Female, but not male, HET mice also displayed significantly lower mean escape latencies ($p < .05$) for trials 1 to 25 on both days (Figure S3 in Supplement 1). In addition, there was no significant difference in escape latencies between naive WT and HET mice not exposed to inescapable shock 24 hours before testing (Figure S3 in Supplement 1). Together, these data suggest that in female mice, *Cacna1c* haploinsufficiency attenuates the development of depressive-like behavior due to uncontrollable shock.

Analysis of Human Genetic *CACNA1C* Data for Female-Specific Association

Given the sex-specific findings in some of our mouse behavioral studies, we hypothesized that in human mood disorders, sex-specific genetic association would be observed for SNPs in *CACNA1C*. We examined our data from the GWAS of the NIMH-BP (11) and GenRED samples (15). We utilized a combined dataset that included 2021 mood disorder cases (1223 female and 798 male subjects) and 1840 control subjects (837 female and 1003 male subjects) in a mega-analysis and focused our examination on 190 SNPs genotyped in *CACNA1C* and within 10 kb of the gene (Figure 6A). The 15 *CACNA1C* SNPs showing nominally significant interactions with sex are listed in Table 1 (full results are shown in Table S2 in Supplement 1). Of these, rs2370419 showed an effect in female subjects (odds ratio [OR] = 1.64; $p = 4.1 \times 10^{-4}$) but not in male subjects (OR = .82;

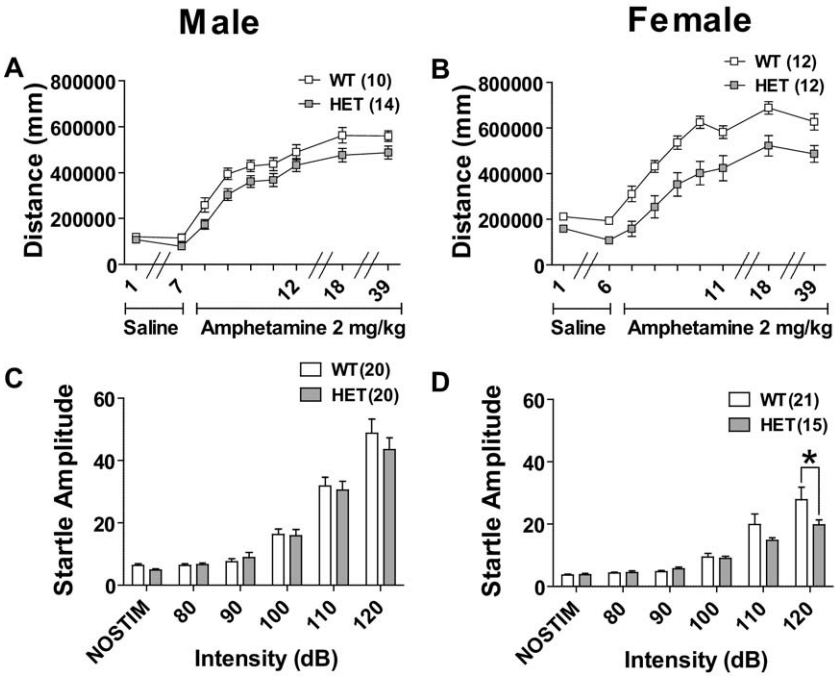

Figure 3. Effect of heterozygous (*Cacna1c*$^{+/-}$ [HET]) *Cacna1c* knockout on response to *d*-amphetamine and acoustic startle response. **(A)** Male wild-type (*Cacna1c*$^{+/+}$) and HET mice habituated at the same level of locomotion during saline injection days 1 to 7. There was a significant effect of genotype to modify sensitization to *d*-amphetamine over days 8 to 12 in male mice ($p < .05$). In male mice, there was a trend for an effect of genotype to modify long-term sensitization to *d*-amphetamine on day 39 ($p = .063$) but not day 18. **(B)** Female HET mice had lower locomotion during the saline habituation phase days 1 to 6 ($p < .001$). In female HET mice, there was a significant effect of genotype on sensitization to *d*-amphetamine ($p < .001$). There remained a significant effect of genotype when female mice were re-challenged with *d*-amphetamine sensitization days 18 and 39 ($p < .001$). **(C)** Male HET mice showed no significant difference in response to multiple acoustic startle intensities. **(D)** Female HET mice had significantly lower acoustic startle response at 120 dB. Data are expressed as mean ± SEM. *$p \leq .05$ Bonferroni posttest. HET, heterozygous *Cacna1c* knockout (*Cacna1c*$^{+/-}$) mice; NOSTIM, no acoustic stimulation; WT, wild-type (*Cacna1c*$^{+/+}$) mice.

$p = .16$), with an interaction that remained significant after correction for testing 190 SNPs ($p = 1.4 \times 10^{-4}$; $p_{corrected} = .03$) (Figure 6B). A second SNP, rs2370411, also showed an interaction with sex that remained significant after correction ($p = 2.1 \times 10^{-4}$, $p_{corrected} = .04$), and an association with mood disorder in female subjects (OR = 1.32, $p = 1.9 \times 10^{-4}$) but not in male subjects (OR = .86, $p = .07$) (Figure 6C). These effects were consistent across the two datasets (NIMH-BP and GenRED), with the minor alleles associated with increased risk of illness in female subjects, but not male subjects, in each set. These two SNPs are both intronic, are located ~ 142 kb from each other, and are in modest linkage disequilibrium (LD) with an $r2 = .22$ (D′ = .85). Their minor allele frequencies, by sex, are shown in Table 2. Of note, Table S2 in Supplement 1 shows results for the overall male + female analyses, in which the strongest result yielded a *p* value of .0051, an order of magnitude less strong than the best female-specific *p* value.

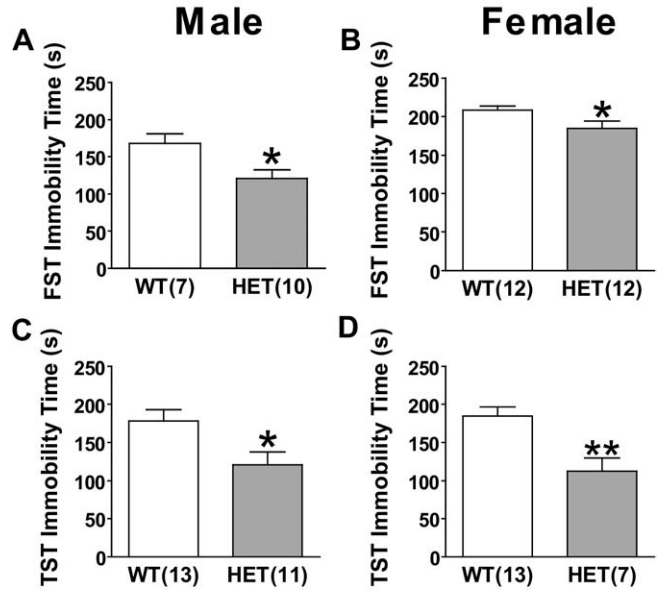

Figure 4. Effect of heterozygous (*Cacna1c*$^{+/-}$ [HET]) *Cacna1c* knockout in tests of antidepressant efficacy. **(A)** Male HET mice spent significantly less time immobile on the forced swim test. **(B)** Female HET mice spent significantly less time immobile on the forced swim test. **(C)** Male HET mice spent significantly less time immobile in the tail suspension test. **(D)** Female HET mice spent significantly less time immobile in the tail suspension test. Data are expressed as mean ± SEM. *$p \leq .05$; **$p < .01$. FST, forced swim test; HET, heterozygous *Cacna1c* knockout (*Cacna1c*$^{+/-}$) mice; TST, tail suspension test; WT, wild-type (*Cacna1c*$^{+/+}$) mice.

Discussion

Our data indicate that in male and female mice, *Cacna1c* haploinsufficiency is associated with decreased exploratory behavior, decreased hyperlocomotion in response to amphetamine, and antidepressant-like behavior in the FST and TST. There are also sex differences in the effects of *Cacna1c* haploinsufficiency: female mice exhibit more robust attenuation of amphetamine-induced hyperlocomotion than male mice and, unlike male mice, display an attenuated acoustic startle response and reduced development of learned helplessness, along with phenotypes of increased anxiety or decreased risk taking. Existing genetic animal models of mania-related behaviors display hyperactivity, increased risk taking or decreased anxiety, increased responsiveness to amphetamine, and increased acoustic startle response (39,44–50). On the other hand, lithium treatment and a model of lithium action, the glycogen synthase kinase-3β heterozygous knockout mouse, result in decreased immobility time in the FST and TST, decreased exploratory behavior in the hole board test, and decreased response to amphetamine (30,33,42,51,52). Taken together with this evidence, our data suggest that *Cacna1c* haploinsufficiency in mice results in a resilient, or mood stabilized, phenotype that is modified by sex for some but not all behaviors.

It remains unclear why some behaviors are modified by sex, while others are not. In particular, both male and female HET mice showed antidepressant-like behavior in the TST and FST, but only female HET mice are resistant to depression-like behavior in the learned helplessness test. This may be related to the fact that the TST

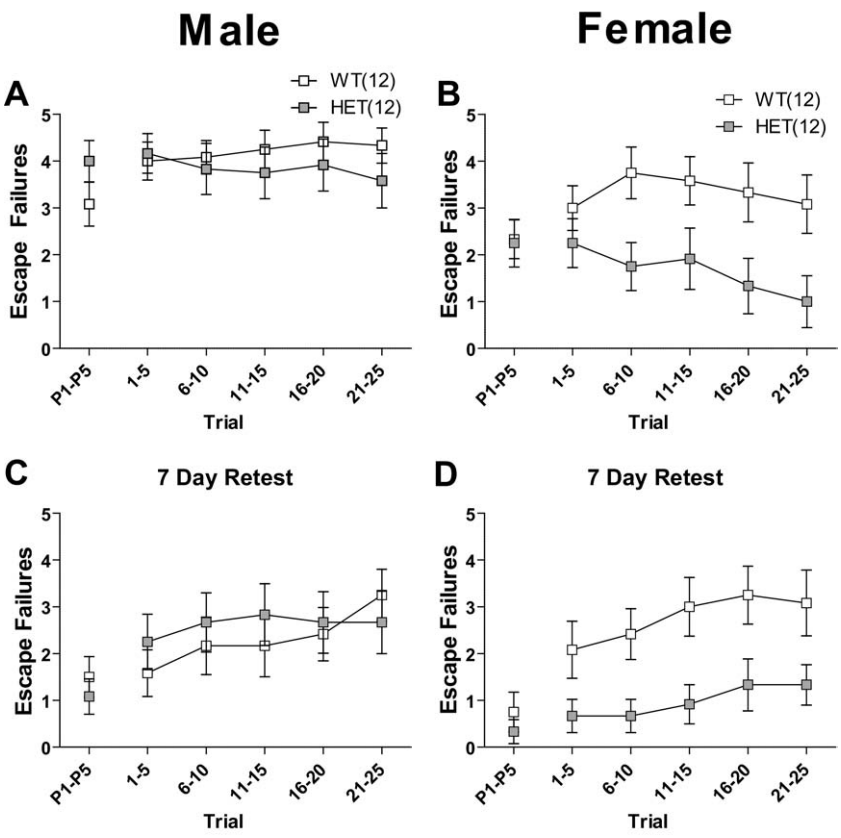

Figure 5. Effect of heterozygous (*Cacna1c*$^{+/-}$ [HET]) *Cacna1c* knockout on depression-like behavior in the learned helplessness model. Mice were tested 1 and 7 days after induction of learned helplessness with .3 mA inescapable shocks. In trials P1 to P5, shock start and gate opening were simultaneous. In trials 1 to 25, a delay of 3 seconds was imposed between shock start and gate opening. In male mice on days 1 **(A)** and 7 **(C)**, there was no significant effect of genotype in trials P1 to P5 and in trials 1 to 25. In female mice on days 1 **(B)** and 7 **(D)**, there were no significant differences in performance in trials P1 to P5, but there was a significant effect of genotype in trials 1 to 25 ($p < .05$). Data are expressed as mean ± SEM. HET, heterozygous *Cacna1c* knockout (*Cacna1c*$^{+/-}$) mice; P1–P5, Pretrial 1-Pretrial 5; WT, wild-type (*Cacna1c*$^{+/+}$) mice.

and FST we used are models of antidepressant efficacy, while the learned helplessness model has better validity as a measure of depression. However, overall, there is an imprecise relationship between mouse behavioral tests and human psychiatric conditions.

The behavior of female HET mice in the elevated plus maze, light-dark box, and open field could be interpreted as either increased anxiety or decreased risk taking. Indeed, these are well-validated tests of anxiety-like behavior, which also involve a significant risk component. This distinction is important, as increased risk taking, but not decreased anxiety, is associated with the manic phase of BP (45,53). Several lines of evidence oppose the conclusion that female HET mice are more anxious. First, we did not observe increased anxiety-like behavior in HET mice in the novelty-induced hypophagia and stress-induced hyperthermia tests. While the elevated plus maze, light-dark box, and open field thigmotaxis all assess exploration into a naturally dangerous, open area as a measure of anxiety, the novelty-induced hypophagia and stress-induced hyperthermia tests do not rely on entry into potentially dangerous areas and thus may be less related to taking risks. Second, increased acoustic startle is associated with other measures of anxiety, and increased startle responses can be decreased by administration of anxiolytic drugs (54,55); we observed decreased acoustic startle in female HET mice.

Female HET mice also displayed decreased locomotion in the open field, which may confound some of our results. There was no significant difference in home cage locomotion in either male or female HET mice when compared with their WT littermates. Similarly, the lack of difference in the accelerating rotarod test of coordination or the hanging wire test, as well as the increase in activity observed in the FST and TST, suggests that there are no innate motor deficits in these mice. However, due to baseline differences in open field locomotion, the effects of genotype on amphetamine-induced hyperlocomotion in female mice should be interpreted with caution.

Because we observed sex differences in behavioral alterations in *Cacna1c* haploinsufficient mice, we hypothesized that *CACNA1C* genotype may interact with sex to influence a mood disorder diagnosis in humans. We found evidence for a female-specific association of two intronic SNPs, with the minor alleles being more prevalent in mood-disordered women than in control women. rs2370411 is within ~ 25 to 50 base pairs of a region with high regulatory potential (56) and rs2370419 is within ~ 800 base pairs of such a region, while numerous variants in additional high regulatory potential regions are in LD with both SNPs. We also note that the minor allele frequencies in control women were lower than in control men. This could be the result of random fluctuation, though it could also be related to our screened control sample. Because we excluded 35% of control subjects due to evidence of mood disorders, the remainder might be depleted for mood disorder susceptibility alleles, a phenomenon that would be sex-specific in the case of alleles conferring sex-specific disease vulnerability. Consistent with earlier work (10), we did not observe a robust sex-specific effect for rs1006737, the SNP previously most strongly implicated in BP, although we did see a nominally significant one, as shown in Table 1. The SNP rs1006737 is located roughly midway between the two SNPs that we have highlighted and is in partial LD with them (with rs2370419: $r^2 = .096$, D′ = .81; with rs2370411: $r^2 = .033$, D′ = .26).

Previous GWAS reports in mood disorders have also shown strong associations with rs10848632 (9), rs10774037 (10), rs1024582 (10), and rs11614275 (15). These SNPs are all located within 97 kb of each other in the same intron of *CACNA1C*, again located between the two SNPs we have highlighted, rs2370419 and rs2370411. Given that all of these SNPs are located within a large block of linkage disequilibrium encompassing ~ 150 kb, it is possi-

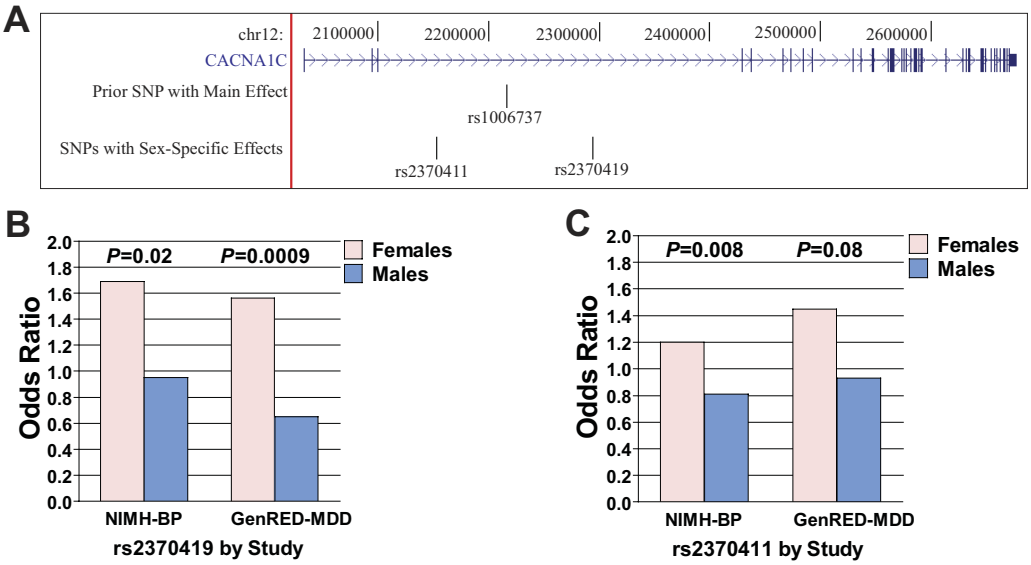

Figure 6. Two human intronic *CACNA1C* single nucleotide polymorphisms show sex-specific association with mood disorders. **(A)** *CACNA1C* is a 644.7 kilobase (kb) gene located on chromosome 12 at 2032677 base pair to 2677376 base pair (hg18). Rs2340419 and rs2370411 are located within intron 3, which is 329 kb long. They are 142 kb from each other. Rs1006737, the most significant result in a prior bipolar disorder genome-wide association studies meta-analysis (10), is also in intron 3, located between rs2370411 and rs2370419. **(B)** Results by study for rs2370419. National Institute of Mental Health Genetics Initiative Bipolar Disorder Consortium: female odds ratio 1.69, male odds ratio, .95. Genetics of Recurrent Early-Onset Major Depression Consortium-major depressive disorder: female odds ratio 1.56, male odds ratio .65. Both studies showed nominally significant *p* values for the interaction of sex and diagnosis. Control subjects were nonoverlapping. **(C)** Results by study for rs2370411. National Institute of Mental Health Genetics Initiative Bipolar Disorder Consortium: female odds ratio 1.20, male odds ratio, .81. Genetics of Recurrent Early-Onset Major Depression Consortium-major depressive disorder: female odds ratio 1.45, male odds ratio .93. Both studies showed nominally significant *p* values for the interaction of sex and diagnosis. Control subjects were nonoverlapping. GenRED, Genetics of Recurrent Early-Onset Major Depression Consortium; MDD, major depressive disorder; NIMH-BP, National Institute of Mental Health Genetics Initiative Bipolar Disorder Consortium.

ble that the varied association signals in different samples all reflect a single regulatory element in the region, which has both sex-independent and sex-dependent variants within it. Alternatively, there could be multiple regulatory elements within this block. Meta-analysis of data on SNPs across this region and dissection of potential sex-specific effects using a very large combined mood disorders set will soon be possible due to the efforts of the Psychiatric GWAS Consortium (57).

A limitation of our study is that we did not investigate the molecular mechanism through which *Cacna1c* haploinsufficiency exerts its effects on behavior. While we identified significant reductions in $Ca_v1.2$ protein levels and dihydropyridine sensitive calcium

Table 1. Single Nucleotide Polymorphisms in *CACNA1C* with Nominally Significant Sex-Specific and Interaction Results in a Combined Bipolar Disorder and Major Depression Dataset

			Female (n = 2060)		Male (n = 1801)		Interaction	
SNP	BP Location[a]	Minor Allele	OR[b]	p[c]	OR[b]	p[c]	OR[b]	p[c]
rs2370419	2294118	A	1.64	.00041	.82	.16	.47	.00014
rs2370411	2152186	C	1.32	.00019	.86	.071	.67	.00021
rs7295089	2310725	C	1.25	.015	.87	.13	.7	.0058
rs17801265	2468876	A	1.19	.052	.86	.11	.56	.0062
rs4765966	2615463	A	1.24	.13	.74	.035	.7	.0065
rs11062241	2456847	T	1.53	.046	.79	.25	.77	.018
rs2239016	2153653	G	.89	.1	1.13	.11	.47	.026
rs104629	2625616	A	1.13	.35	.72	.024	.49	.027
rs1006737	2215556	A	1.17	.025	.94	.36	.8	.029
rs4765905	2219845	C	1.16	.031	.93	.34	.64	.03
rs2239015	2153456	T	.9	.11	1.12	.13	.82	.031
rs758170	2231721	T	1.15	.041	.93	.34	1.24	.039
rs4765663	2049021	C	1.06	.53	.8	.019	.83	.039
rs2238043	2145924	A	1.18	.015	.96	.59	1.25	.04
rs882194	2220713	G	1.06	.37	.88	.071	.83	.048

BP, base pair; OR, odds ratio; SNP, single nucleotide polymorphism.

[a]All base pair locations are on chromosome 12 from National Center for Biotechnology Information Build 36/hg18. All single nucleotide polymorphisms listed here are intronic.

[b]Calculated under an additive model for the minor allele.

[c]Uncorrected *p* value

Table 2. Minor Allele Frequencies by Sex in Cases and Control Subjects for Two Intronic *CACNA1C* Single Nucleotide Polymorphisms

	rs2370419		rs2370411	
	Case	Control	Case	Control
Female				
Combined[a] ($n = 2060$)	.075	.049	.276	.225
NIMH-BP ($n = 929$)	.077	.048	.275	.237
GenRED ($n = 1131$)	.073	.049	.276	.213
Male				
Combined[a] ($n = 1801$)	.062	.075	.236	.259
NIMH-BP ($n = 930$)	.068	.072	.236	.277
GenRED ($n = 871$)	.051	.077	.237	.245

GenRED, Genetics of Recurrent Early-Onset Major Depression Consortium; NIMH-BP, National Institute of Mental Health Genetics Initiative Bipolar Disorder Consortium.

[a]National Institute of Mental Health Genetics Initiative Bipolar Disorder Consortium and Genetics of Recurrent Early-Onset Major Depression Consortium samples combined.

current in male mice (Figure S1 in Supplement 1), it is possible that such measures are modified by sex. $Ca_v1.2$ is important in modifying the effects of synaptic activity on cell survival, synaptic plasticity, and gene expression (58). Previous work has linked $Ca_v1.2$ to mitogen-activated protein kinase pathway activation (59) and increased immediate early gene expression (60), and a proteolytically cleaved C-terminal fragment of the channel acts as a transcription factor (61). Intracellular calcium levels regulated specifically by $Ca_v1.2$ influence a number of intracellular signaling cascades that modify expression of calcium-dependant genes including Bcl-2, brain-derived neurotrophic factor, and c-fos (62). Other studies have focused on the role of $Ca_v1.2$ in learning and memory using conditional knockout mice (63,64). While a conditional knockout approach may better isolate $Ca_v1.2$ function in adult neurons, the heterozygous knockout model used in our study likely more closely mimics a possible clinical situation, where a nearly complete absence of $Ca_v1.2$ function is unlikely. In addition, $Ca_v1.2$ messenger RNA levels increase in the ventral tegmental area following repeated amphetamine administration, which may be related to the attenuated response to amphetamine observed in *Cacna1c* HET mice (65). We further note that significantly increased *CACNA1C* messenger RNA levels were seen in postmortem brains from BP patients in the array collection of the Stanley Medical Research Institute (http://www.stanleygenomics.org). This is consistent with a recent report by Bigos et al. where they observed that a bipolar disorder and schizophrenia risk associated SNP (rs1006737) predicts increased expression of *CACNA1C* mRNA in the human brain (66). Relevant to the sex differences we observed, physiological levels of estrogen potentiate L-type calcium channels in vitro (66), and some of the neuroprotective effects of estrogen on glutamate-induced cell death have been linked to L-type calcium channels (67).

Overall, our behavioral results provide evidence that decreases in $Ca_v1.2$ function may play a protective role in the development of mood disorders, and our human genetic and preclinical data support an interaction between *CACNA1C* and sex. Further research is needed to investigate the mechanisms by which $Ca_v1.2$ produces its sex-dependent spectra of behavioral effects.

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Supplementary material cited in this article is available online.

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