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Sex-Specific Stress-Related Behavioral Phenotypes and Central Amygdala Dysfunction in a Mouse Model of 16p11.2 Microdeletion

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ABSTRACT

BACKGROUND: Substantial evidence indicates that a microdeletion on human chromosome 16p11.2 is linked to neurodevelopmental disorders, including autism spectrum disorder (ASD). Carriers of this deletion show divergent symptoms besides the core features of autism spectrum disorder, such as anxiety and emotional symptoms. The neural mechanisms underlying these symptoms are poorly understood.

METHODS: We used mice heterozygous for a deletion allele of the genomic region corresponding to the human 16p11.2 microdeletion locus (i.e., *16p11.2 del/+* mice) and their sex-matched wild-type littermates for the study and examined their anxiety-related behaviors, auditory perception, and central amygdala circuit function using behavioral, circuit tracing, and electrophysiological techniques.

RESULTS: Mice heterozygous for a deletion allele of the genomic region corresponding to the human 16p11.2 microdeletion locus (i.e., *16p11.2 del/+* mice) had sex-specific anxiety-related behavioral and neural circuit changes. Specifically, we found that female, but not male, *16p11.2 del/+* mice showed enhanced fear generalization—a hallmark of anxiety disorders—after auditory fear conditioning and displayed increased anxiety-like behaviors after physical restraint stress. Notably, such sex-specific behavioral changes were paralleled by an increase in activity in central amygdala neurons projecting to the globus pallidus in female, but not male, *16p11.2 del/+* mice.

CONCLUSIONS: Together, these results reveal female-specific anxiety phenotypes related to 16p11.2 microdeletion syndrome and a potential underlying neural circuit mechanism. Our study therefore identifies previously underappreciated sex-specific behavioral and neural changes in a genetic model of 16p11.2 microdeletion syndrome and highlights the importance of investigating female-specific aspects of this syndrome for targeted treatment strategies.

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Autism spectrum disorder (ASD) is a spectrum of neurodevelopmental conditions defined by two major diagnostic criteria: "persistent deficits in social communication and social interaction across multiple contexts" and "restricted, repetitive patterns of behavior, interests, or activities" (1). Patients with ASD commonly have one or more comorbid conditions, including intellectual disability (2–4), attention-deficit/hyperactivity disorder (5–8), obsessive-compulsive disorder (9,10), anxiety (11–13), and depression (13–16), and are at increased risk for suicidality, particularly female patients (17–19).

It is well documented that ASD is about 4 times more common in males than in females with an exception for X-linked syndromes, such as Rett syndrome, which is more common in females (20). There is significant evidence of divergence among core symptoms of ASD based on sex. Specifically, many studies have found reduced severity of repetitive and/or stereotyped behaviors in females with ASD compared with males (21–25). In contrast, females show different social impairments than males (22,26–29). These tend toward more internalizing symptoms and emotional disturbance (30–34). Females with ASD also show increased risk of eating disorders (35), sensory impairments (36), sleep disturbances (37), epilepsy, and learning disorders (38). It has been suggested that females may camouflage their autism phenotypes better than males owing to fewer social impairments and better executive functioning (39) as well as reduced externalizing symptoms (29). One way that emotional phenotypes often manifest is as anxiety disorders. In the general population, females have an increased prevalence of stress-related disorders, such as anxiety, depression, and posttraumatic stress disorder (40–43). Therefore, it is possible that anxiety-like phenotypes may present differently in males and females with ASD.

A major limitation of much of the research in ASD has been its emphasis on males. This is not exclusive to ASD research, as most research is done in male subjects (44). Among neuroscience studies in general, the sex bias of human subjects is approximately 5.5 males for every female and with a ratio much higher among animal studies (45). This bias

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precludes our understanding of autism in females and limits our development of effective treatment strategies. Therefore, we sought to examine whether sex differences exist in stress-related behaviors in a mouse model of ASD. To this end, we used a model that mimics a microdeletion on human chromosome 16p11.2. Notably, this deletion is one of the most common genetic variations found in ASD, accounting for approximately 1% of ASD cases (46–51). Patients with this deletion show repetitive behaviors, hyperactivity, intellectual disability, motor and speech/language delay, and anxiety symptoms (52–56). Of note, individuals carrying the 16p11.2 deletion, including carriers without ASD, often receive diagnoses of anxiety disorders or other mood disorders (57).

The mouse model we used was generated by Horev *et al.* (58), and is one of three independently generated mouse genetic models that mimic the 16p11.2 microdeletion (58–60). These models, which were created by deleting largely similar genomic intervals in mouse chromosome 7 corresponding to the syntenic 16p11.2 microdeletion region in humans, exhibit overlapping phenotypes (58–60). In particular, heterozygous deletion mice—hereafter referred to as *16p11.2 del*/+ mice—in each of these lines share basic phenotypes, such as low body weight and perinatal mortality, and, importantly, show behavioral phenotypes related to the symptoms of human 16p11.2 microdeletion carriers. These phenotypes include increased locomotor activity, stereotyped and repetitive behaviors, sleep deficits, recognition memory deficits, reward learning deficits, and social deficits (58–66).

A few studies examined *16p11.2 del*/+ mice for anxiety- or fear-related behaviors, but with mixed results. When tested in the open field test and elevated plus maze (EPM) test, assays conventionally used to assess anxiety in rodents, these mice do not appear different from wild-type mice (60,61,67) [however, see (68)]. The *16p11.2 del*/+ mice were also examined in fear conditioning paradigms. One study shows that *16p11.2 del*/+ mice have impaired contextual fear conditioning (69), whereas other studies show that *16p11.2 del*/+ mice have normal contextual fear conditioning and normal visually cued fear conditioning (61,67).

Recent studies indicate that environmental factors can exacerbate ASD symptoms and impairments in cognitive and adaptive behaviors in 16p11.2 deletion carriers (70), and *16p11.2 del*/+ mice show altered coping in response to stress compared with wild-type littermates (61,71). In light of these findings and studies showing that males and females can exhibit very different behavioral responses to threats or stress (72,73), we reasoned that under a stressful situation *16p11.2 del*/+ mice may exhibit sex-specific behavioral changes. However, a potential sex-specific effect of the *16p11.2* deletion on anxiety- or fear-related behaviors in mice has not been considered until recently (67). Furthermore, only simple assays, such as the open field test and EPM test, have been used to assess baseline anxiety in *16p11.2 del*/+ mice, which may not be sufficient to reveal potential changes in anxiety or fear processing in response to stress in these mice.

To address these issues, in the current study we examined anxiety-related behaviors under different stress conditions in both male and female *16p11.2 del*/+ mice and their wild-type littermates. We found that female, but not male, *16p11.2 del*/+ mice showed enhanced fear generalization, a hallmark of anxiety disorders (74), after auditory fear conditioning. Furthermore, although at baseline *16p11.2 del*/+ mice were not different from their wild-type littermates in the EPM test, consistent with previous studies (60,61,67), we found that female, but not male, *16p11.2 del*/+ mice showed enhanced anxiety in the EPM test after acute restraint stress. Lastly, we found that these sex-specific behavioral changes were paralleled by an increase in activity in the central amygdala (CeA)—a major limbic structure that regulates anxiety in rodents and primates (75–77)—of female, but not male, *16p11.2 del*/+ mice. Together, our work suggests that 16p11.2 microdeletion differentially affects males and females and may disproportionally disrupt brain functions related to stress regulation in females. These findings provide insight into understanding how ASD may present differently in females at behavioral and neuronal levels and raise the question of whether changes to treatment and diagnostic strategies based on sex should be considered.

METHODS AND MATERIALS

Animals

To breed *16p11.2 del*/+ mice, we used *16p11.2 del*/+ male mice and C57/B6 female mice purchased from the Jackson Laboratory (Bar Harbor, ME) or similar breeders obtained from Pavel Osten's laboratory at Cold Spring Harbor Laboratory. Breeders were housed with a cardboard Bio-Hut (Bio-Serv, Flemington, NJ) under a 12-hour light/dark cycle (7 AM to 7 PM light) with food and water available ad libitum. As *16p11.2 del*/+ mice exhibit postnatal lethality (58), in breeding cages only, standard rodent chow (Purina LabDiet, Gray Summit, MO) was supplemented with DietGel Boost (ClearH_2O, Westbrook, ME), a high-calorie liquid diet that increased survival of *16p11.2 del*/+ pups. Pups were weaned at 3 weeks of age and group housed with wild-type littermates. Mice were genotyped for 16p11.2 microdeletion between 4 and 8 weeks of age with primers provided by Alea Mills' laboratory at Cold Spring Harbor Laboratory.

Mice 2–4 months old were used for all behavioral experiments. Mice 6–10 weeks old were used for all electrophysiology experiments. All experimental mice were housed under a 12-hour light/dark cycle (7 AM to 7 PM light) in groups of 2–5 animals, containing both *16p11.2 del*/+ mice and their wild-type littermates. Food and water were available ad libitum. All behavioral experiments were performed during the light cycle. Littermates were randomly assigned to different groups before experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Cold Spring Harbor Laboratory and performed in accordance with the U.S. National Institutes of Health guidelines.

See Supplemental Methods for details on Behavioral Tasks, Stereotaxic Surgery, and In Vitro Electrophysiology.

Data Analysis and Statistics

Statistical analyses were performed with Prism 7 (GraphPad Software, La Jolla, CA). Normality was tested by D'Agostino-Pearson or Shapiro-Wilk normality tests. Non-normal datasets were log-transformed for normality before statistical testing. All behavioral experiments were controlled by computer systems, and data were collected and analyzed in an automated and unbiased way. Virus-injected animals in which the injection site

was incorrect were excluded. No other animals were excluded. Sex was found to be a significant factor in percent freezing during fear conditioning retrieval ($F_{1,83} = 8.740$, $p = .0041$, two-way repeated measures analysis of variance) (Figure 1). Given this, in addition to our a priori hypothesis that sex differences would be present based on sex bias in the prevalence of human autism and anxiety disorders, all results have been analyzed separately by sex.

RESULTS

Female-Specific Increase in Fear Generalization in *16p11.2 del/+* Mice

One hallmark of anxiety disorders is fear generalization (74). Fear generalization can be assessed in mice using a fear conditioning paradigm with a discrimination component (see Supplemental Methods), in which mice are trained to associate one auditory stimulus (conditioned stimulus [CS]) (CS+) with a foot shock (unconditioned stimulus [US]), while a different auditory stimulus (CS−) is presented without the shock. In a fear retrieval test 24 hours following the conditioning, freezing in response to the CS+ and freezing in response to the CS− are measured and used to calculate a discrimination index, which is the difference between an animal's average freezing to the CS+ and to the CS−, normalized to the sum of the two measurements.

Interestingly, we found that during a habituation session before the conditioning, female *16p11.2 del/+* mice showed a small (10%–20%) but robust increase in freezing to the auditory stimuli compared with their wild-type littermates (Figure 1A, left). Male *16p11.2 del/+* mice did not show such a change (Figure 1A, right). However, we did not observe a significant difference in freezing during the first tone presentation in the subsequent conditioning session (i.e., before mice received any shocks) between genotypes for either the female or the male mice (Figure 1B, D), suggesting that the enhanced freezing in *16p11.2 del/+* female mice during habituation may be related to the fact that the auditory stimuli were novel to the animals.

After fear conditioning and on memory retrieval, both female and male *16p11.2 del/+* mice showed levels of freezing similar to those of their wild-type littermates in response to the CS+ (Figure 1B, D), consistent with previous findings that *16p11.2 del/+* mice have intact fear conditioning (61,67). Surprisingly, however, female, but not male, *16p11.2 del/+* mice showed increased freezing to the CS− compared with wild-type littermates (Figure 1B, D), resulting in reduced levels of fear discrimination in female, but not male, *16p11.2 del/+* animals (Figure 1C, E). In addition, we found that female, but not male, *16p11.2 del/+* mice showed enhanced reactions to foot shocks compared with wild-type mice, as measured by enhanced movement velocity and distance immediately following shocks of varying intensities (Figure 2). These results suggest that female *16p11.2 del/+* mice have enhanced fear generalization following fear conditioning, which could result from heightened alertness (as indicated by increased freezing during habituation) and/or an increase in sensitivity to aversive stimuli (as indicated by increase reactivity to foot shocks).

16p11.2 del/+ Mice Have Normal Auditory Perception

An alternative explanation for the enhanced fear generalization in female *16p11.2 del/+* mice is that these mice have an impairment in auditory processing, such that they cannot effectively discriminate between a 4-kHz tone and a 12-kHz tone, which were used as CS+ and CS−, respectively, during fear conditioning. To test this possibility, we trained a new cohort of mice, including *16p11.2 del/+* mice and their wild-type littermates, in an auditory two-alternative choice task that depended on discriminating between a 4-kHz tone and a 12-kHz tone (Figure 3A) (see Supplemental Methods) (78). Both female and male *16p11.2 del/+* mice learned the two-alternative choice task at a rate similar to that of their wild-type littermates (Figure 3B, C). In fact, male *16p11.2 del/+* mice tended to be faster than wild-type mice in learning the task (Figure 3C), though this difference did not reach significance. In addition, the performance of female and male *16p11.2 del/+* mice in discriminating a series of sounds with frequencies ranging from 4 to 12 kHz (Figure 3D–F and H–J), or with different intensities (Figure 3G, K), was indistinguishable from their wild-type littermates. These results indicate that *16p11.2* microdeletion does not affect auditory perception in mice, ruling out the possibility that the enhanced fear generalization in female *16p11.2 del/+* mice is confounded by an impairment in auditory processing in these mice.

Stress Induces an Increase in Anxiety in Female *16p11.2 del/+* Mice

In fear conditioning, mice receive electric shocks as the aversive US, which is a significant stressor to animals. Therefore, the enhanced fear generalization in female *16p11.2 del/+* mice after fear conditioning points to a possibility that these animals are prone to stress-induced anxiety. To further test this possibility, we sought to examine anxiety-like behaviors in mice subjected to a different stressor. For this purpose, we used physical restraint (see Supplemental Methods), a well-characterized stress induction procedure in rodents that has been shown to affect the function of the CeA (79,80). As described previously (81), animals were physically restrained in a well-ventilated 50-mL conical tube for 2 hours in a dark, sound-attenuated box. The animals were tested 24 hours later in the EPM (see Supplemental Methods). We found a significant interaction between sex and genotype in the time spent in the open arms (Figure 4A) and significant effects of sex on movement velocity (Figure 4B) and distance traveled (Figure 4C). Post hoc analysis revealed that the stressed female *16p11.2 del/+* mice spent significantly less time in the open arms of the EPM compared with their female wild-type littermates (Figure 4A). We did not find any change in time spent in the open arms in male *16p11.2 del/+* mice.

We also examined anxiety levels in naïve mice using the EPM test. Compared with naïve female or male wild-type littermates, naïve female or male *16p11.2 del/+* mice did not show any change in the time spent in the open arms (Figure 4D), movement velocity (Figure 4E), or distance traveled (Figure 4F). This

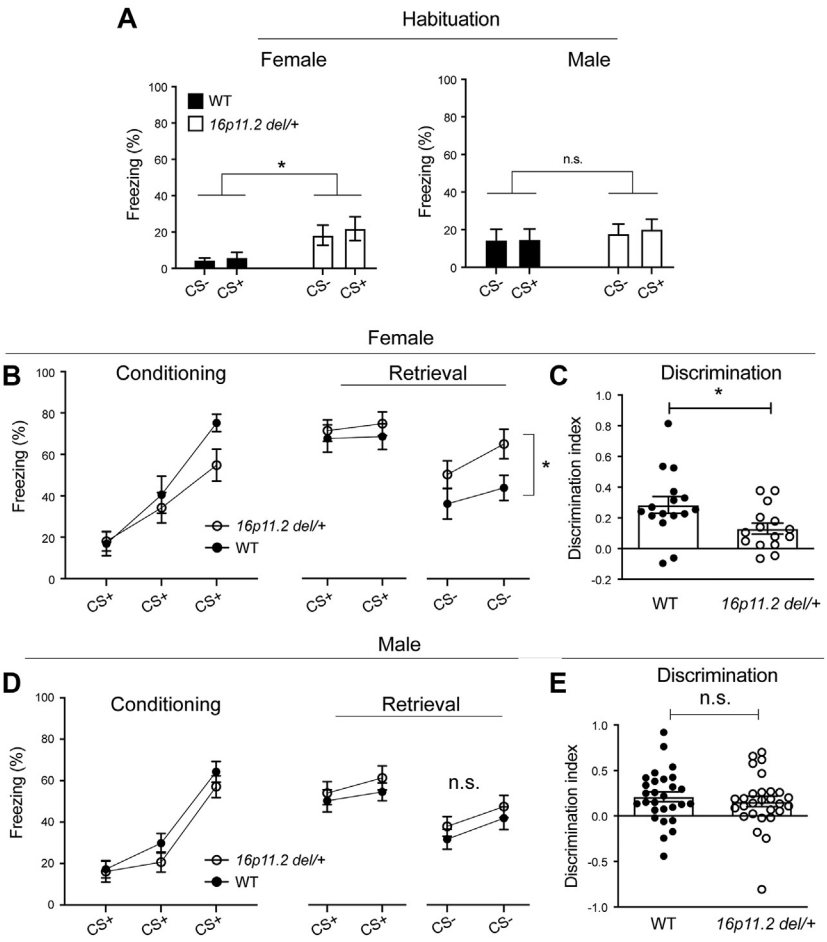

Figure 1. Female *16p11.2 del/+* mice exhibit fear generalization following fear conditioning. **(A)** Freezing behavior of male and female *16p11.2 del/+* mice and their respective WT littermates in response to CS+ and CS− during habituation (female [*16p11.2 del/+*, $n = 15$, WT, $n = 16$]: $F_{1,29} = 6.023$, $p = .0204$; male [*16p11.2 del/+*, $n = 28$, WT, $n = 28$]: $F_{1,54} = 0.3433$, $p = .5604$; $^*p < .05$; two-way analysis of variance with repeated measures). **(B)** Freezing to each stimulus presentation during conditioning and retrieval for female mice (conditioning: $F_{1,29} = 1.419$, $p = .2432$; CS+ retrieval: $F_{1,29} = 0.4314$, $p = .5165$; CS− retrieval: $F_{1,29} = 5.765$, $p = .0230$; $^*p < .05$; two-way analysis of variance with repeated measures and post hoc Sidak test). **(C)** Discrimination index $[(CS^+ - CS^-)/(CS^+ + CS^-)]$ for female mice ($^*p = .0192$, Mann-Whitney *t* test). **(D)** Freezing to each stimulus presentation during conditioning and retrieval for male mice (conditioning: $F_{1,54} = 0.9938$, $p = .3233$; CS+ retrieval: $F_{1,54} = 0.6327$, $p = .4298$; CS− retrieval: $F_{1,54} = 0.8779$, $p = .3530$; two-way analysis of variance with repeated measures). **(E)** Discrimination index for male mice ($p = .3742$, Mann-Whitney *t* test). Data are presented as mean ± SEM. CS, conditioned stimulus; n.s., not significant; WT, wild-type.

result is consistent with previous findings (60,61,67). Together, our results indicate that female *16p11.2 del/+* mice have increased susceptibility to stress-induced anxiety.

16p11.2 del/+ Mice Have CeA Dysfunction

Previous studies have revealed that the CeA is particularly responsive to stress and is a major contributor to anxiety-related behaviors (75–77). Therefore, we examined whether the *16p11.2* microdeletion affects CeA neuronal function in a sex-specific manner. We recorded miniature excitatory postsynaptic currents (mEPSCs)—a measurement of total excitatory synaptic drive onto the recorded neurons—from CeA neurons in acute brain slices prepared from female or male *16p11.2 del/+* mice as well as their female or male wild-type littermates (Figure 5A). We found significant effects of sex and genotype on mEPSC frequency in randomly recorded CeA neurons (Figure 5B–D). Post hoc analysis revealed that female mice with 16p11.2 microdeletion specifically had increased mEPSC frequency compared with female wild-type littermates. There was no difference in mEPSC amplitude between genotypes or sexes (Figure 5E). These results indicate that female, but not male, *16p11.2 del/+* mice have enhanced excitatory synaptic drive onto CeA neurons.

We recently identified a pathway from the CeA to the globus pallidus externa (GPe) (82), which originates predominantly from somatostatin-expressing CeA neurons that play an essential role in fear learning and anxiety (75,83,84). This pathway conveys information of the US and is critical for learning in fear conditioning (82). Importantly, optogenetic activation of the CeA-GPe pathway increases fear generalization whereby animals increase their freezing to CS−. Therefore, we sought to determine whether the GPe-projecting CeA neurons are affected by the 16p11.2 microdeletion. To this end, we used a retrograde labeling strategy whereby fluorescently conjugated CTB (cholera toxin subunit B) was injected in the GPe to label the GPe-projecting CeA neurons (Figure 6A) (see Supplemental Methods). Three days after the CTB injection, we recorded mEPSCs selectively from the CTB-labeled GPe-projecting CeA neurons in acute brain slices prepared from female or male *16p11.2 del/+* mice and their respective wild-type littermates (Figure 6A, B). Again, we found a significant interaction between sex and genotype whereby females with 16p11.2 microdeletion exhibited increased mEPSC frequency compared with wild-type littermates (Figure 6D, E). Thus, our results indicate that the *16p11.2* microdeletion caused a female-specific enhancement of

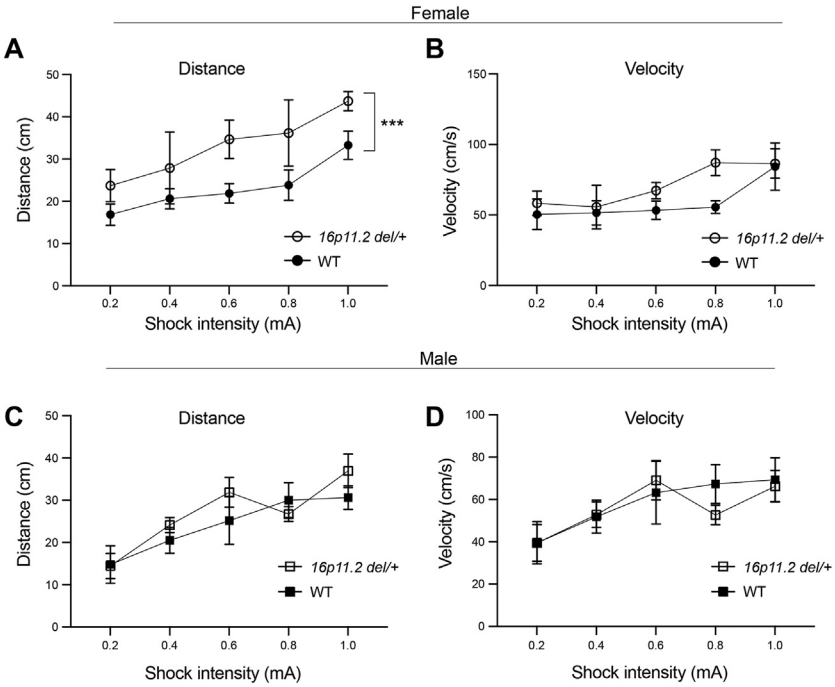

Figure 2. Female *16p11.2 del/+* mice show enhanced reactivity to foot shock. **(A)** Distance traveled during 2-second shock presentations for female mice ($F_{1,50}$ = 14.94, p = .0003; ***$p < .001$; two-way ANOVA; *16p11.2 del/+*, n = 4, WT, n = 8). **(B)** Movement velocity during 2-second shock presentations for female mice ($F_{1,50}$ = 2.596, p = .1135; two-way ANOVA). **(C)** Distance traveled during 2-second shock presentations for male mice ($F_{1,50}$ = 1.410, p = .2407; two-way ANOVA; *16p11.2 del/+*, n = 7, WT, n = 5). **(D)** Movement velocity during 2-second shock presentations for male mice ($F_{1,50}$ = 0.1467, p = .7033; two-way ANOVA). Data are presented as mean ± SEM. ANOVA, analysis of variance; WT, wild-type.

excitatory synaptic drive onto CeA neurons and moreover suggest dysfunction in the somatostatin-expressing CeA neurons in the CeA-GPe pathway as a potential mechanism for the increased stress susceptibility and fear generalization identified in female *16p11.2 del/+* mice.

DISCUSSION

Our results indicate that female, but not male, *16p11.2 del/+* mice have increased susceptibility to anxiety-like phenotypes following stressful life events, revealing a previously underappreciated sex-specific effect in the modulation of behavior by 16p11.2 microdeletion. Furthermore, we identify that CeA dysfunction, in particular CeA dysfunction in the CeA-GPe circuit, may underlie the female-specific behavioral phenotypes caused by the 16p11.2 microdeletion. Notably, the CeA-GPe pathway originates predominantly from somatostatin-expressing CeA neurons (82), which have been shown to have an essential role in fear learning and anxiety (75,83–86). Our results thus pinpoint a precise cellular and circuit substrate as a potential target of future therapeutics for anxiety symptoms.

These findings are consistent with the vast literature that females with ASD show distinct behavioral symptoms compared with males (22,26–29), in particular, more internalizing symptoms and emotional disturbances (30–34). Our findings are also consistent with the notion that in the general population, females have an increased prevalence of stress-related disorders, such as anxiety, depression, and posttraumatic stress disorder (40–43). Our study thus urges a careful examination of anxiety and other emotional symptoms as well as functional changes in the amygdala–basal ganglia circuits in 16p11.2 microdeletion carriers, in particular in female carriers. In general, our study also urges sex-specific diagnostic and treatment strategies for ASD.

Three lines of evidence suggest that heightened alertness or an increase in sensitivity to aversive stimuli, or to the stimuli signaling potential threat, may underlie the increased susceptibility to anxiety-like phenotypes in female *16p11.2 del/+* mice following stressful experiences. First, *16p11.2 del/+* mice, especially females, show increased freezing when they are exposed to an unfamiliar sound, which is a sign of uncertainty or potential danger. Second, female *16p11.2 del/+* mice have enhanced reactivity to foot shocks. Third, CeA neurons in female *16p11.2 del/+* mice have enhanced sensitivity to excitatory inputs. This enhanced sensitivity may lead to heightened alertness or attention, as the CeA has been implicated in selective processing of salient information (87,88).

The CeA has central roles in the generation of fear and anxiety states (75–77,83,84,89–102). In parallel, amygdala dysfunction has been implicated in the pathogenesis of ASD. Abnormal developmental trajectory of the amygdala has been observed in ASD (103). Brain imaging studies indicate that the amygdala is enlarged precociously in children with autism (104,105) and that amygdala enlargement in autistic children is associated with anxiety symptoms (106). In addition, cellular changes in the amygdala have been reported in ASD (103). In a recent study (82), we found that a subpopulation of neurons in the CeA sends direct projections to the GPe, and the CeA-GPe pathway conveys US information and controls learning during fear conditioning. In the current study, we found

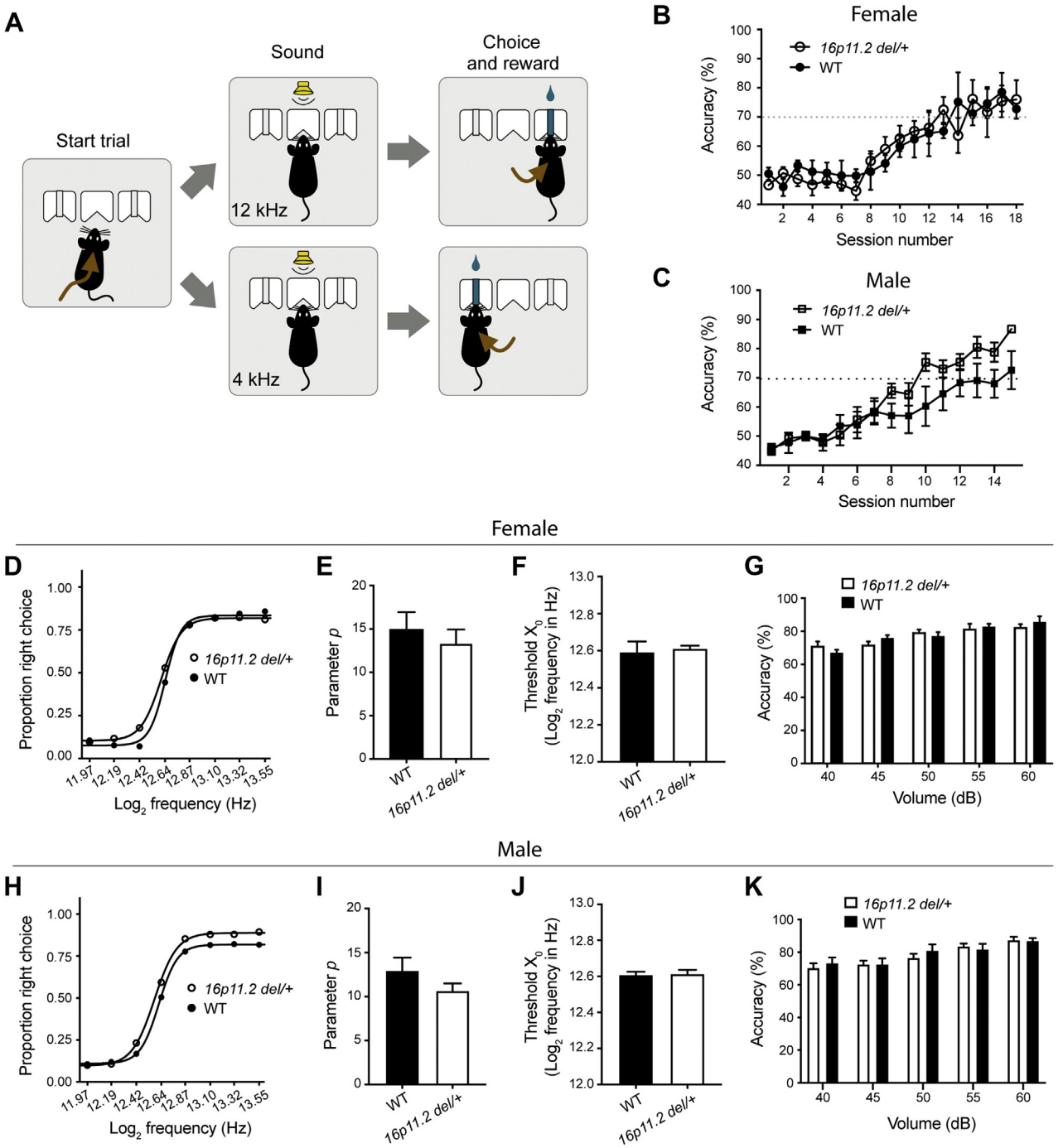

Figure 3. *16p11.2 del/+* mice have normal auditory perception. **(A)** Schematic of the behavioral task. **(B)** Performance levels across training for female mice ($F_{1,8}$ = 0.005112, p = .9448; two-way ANOVA; *16p11.2 del/+*, n = 7, WT, n = 3). **(C)** Performance levels across training for male mice ($F_{1,14}$ = 2.557, p = .1321; two-way ANOVA; *16p11.2 del/+*, n = 9, WT, n = 7). **(D)** Psychometric response curve for frequencies between 4 and 12 kHz (in $\log_2$ values) for female mice. **(E)** Quantification of the slope of the psychometric curve (parameter p) for female mice (p = .5878, t test). **(F)** Quantification of the median threshold, X_0, from the psychometric function for female mice (p = .6465, t test). **(G)** Average performance levels at 4 and 12 kHz for stimuli volume between 40 and 60 dB for female mice ($F_{1,8}$ = 0.04474, p = .8378; two-way ANOVA with repeated measures). **(H)** Psychometric response curve for frequencies between 4 and 12 kHz (in $\log_2$ values) for male mice. **(I)** Quantification of the slope of the psychometric curve (parameter p) for male mice (p = .1713, t test). **(J)** Quantification of the median threshold, X_0, from the psychometric function for male mice (p = .8607, t test). **(K)** Average performance levels at 4 and 12 kHz for stimuli volume between 40 and 60 dB for male mice ($F_{1,14}$ = 0.0173, p = .8972; two-way ANOVA with repeated measures). All data are presented as mean ± SEM. ANOVA, analysis of variance; WT, wild-type.

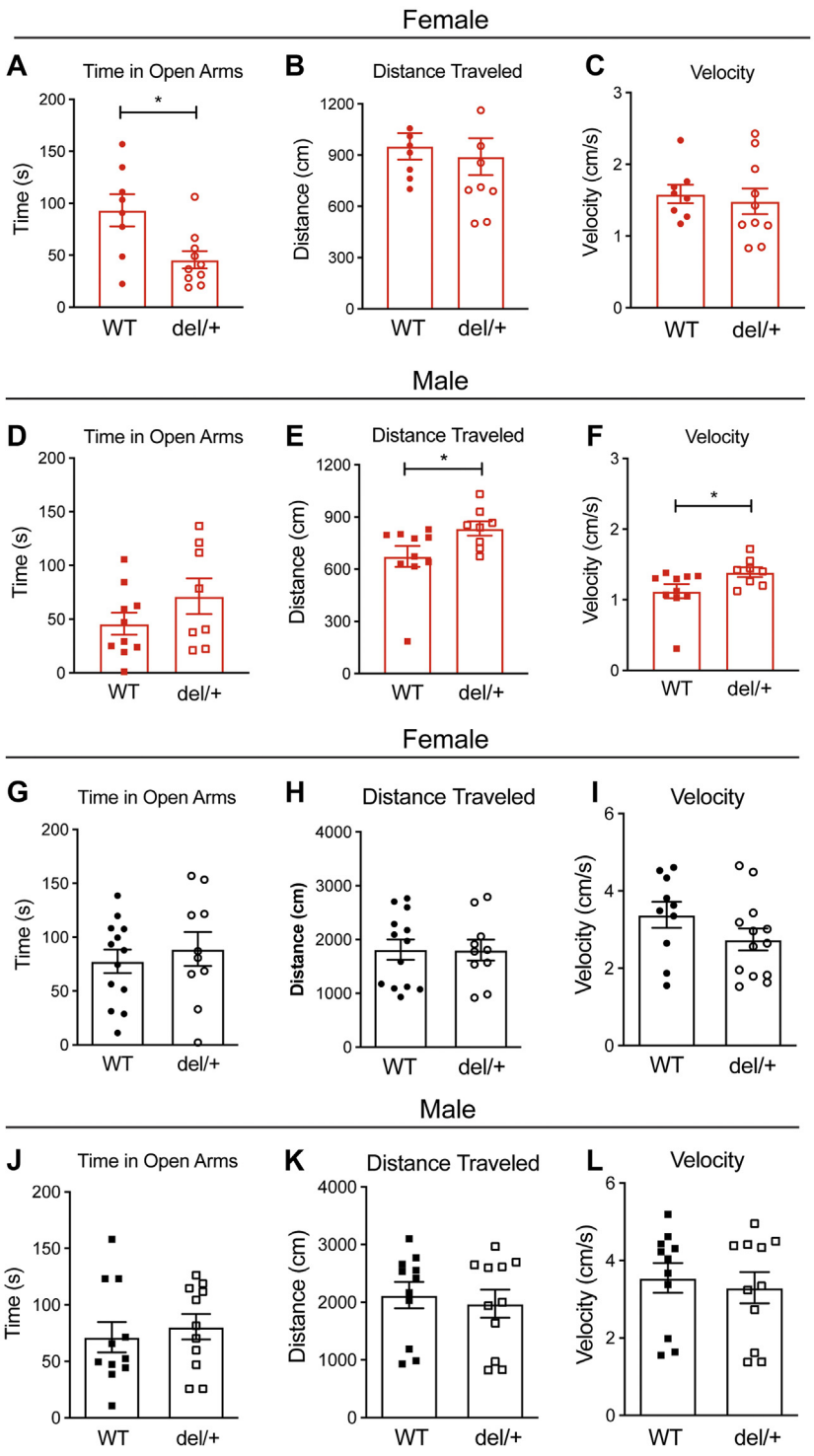

Figure 4. Female *16p11.2 del/+* mice exhibit enhanced stress-induced anxiety-like behavior. **(A)** Time spent in the open arms of the EPM for female mice 24 hours after stress exposure ($p = .0266$; $^*p < .05$; Mann-Whitney *t* test; female *16p11.2 del/+*, $n = 10$, female WT, $n = 8$). **(B)** Distance traveled in the EPM for female mice 24 hours after stress exposure ($p = .4082$; Mann-Whitney *t* test). Same mice as in panel **(A)** were used. **(C)** Movement velocity in the EPM for female mice 24 hours after stress exposure ($p = .4082$; Mann-Whitney *t* test). Same mice as in panel **(A)** were used. **(D)** Time spent in the open arms of the EPM for male mice 24 hours after stress exposure ($p = .3154$; Mann-Whitney *t* test; male *16p11.2 del/+*, $n = 8$, male WT, $n = 10$). **(E)** Distance traveled in the EPM for male mice 24 hours after stress exposure ($p = .0343$; $^*p < .05$; Mann-Whitney *t* test). Same mice as in **(D)** were used. **(F)** Movement velocity in the EPM for male mice 24 hours after stress exposure ($p = .0266$; $^*p < .05$; Mann-Whitney *t* test). Same mice as in **(D)** were used. **(G)** Time spent in the open arms of the EPM for female naïve mice ($p = .5629$; Mann-Whitney *t* test; female *16p11.2 del/+*, $n = 10$, female WT, $n = 13$). **(H)** Distance traveled in the EPM for female naïve mice ($p = .7844$; Mann-Whitney *t* test). Same mice as in panel **(G)** were used. **(I)** Movement velocity in the EPM for female naïve mice ($p = .1306$; Mann-Whitney *t* test). Same mice as in panel **(G)** were used. **(J)** Time spent in the open arms of the EPM for male naïve mice ($p = .6522$; Mann-Whitney *t* test; male *16p11.2 del/+*, $n = 11$, male WT, $n = 11$). **(K)** Distance traveled in the EPM for male naïve mice ($p = .6522$; Mann-Whitney *t* test). Same mice as in panel **(J)** are used. **(L)** Movement velocity in the EPM for male naïve mice ($p = .6522$; Mann-Whitney *t* test). Same mice as in panel **(J)** are used. Data are presented as mean ± SEM. EPM, elevated plus maze; WT, wild-type.

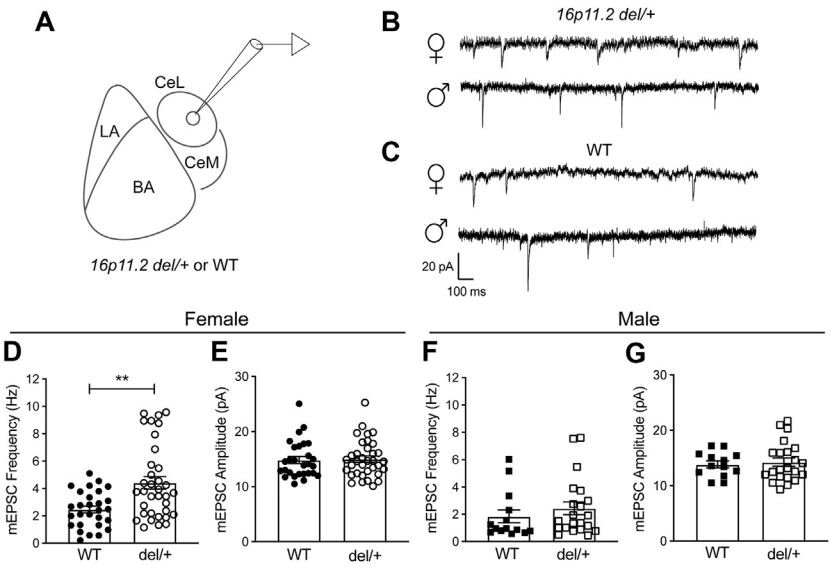

Figure 5. Female *16p11.2 del/+* mice have increased excitatory synaptic transmission onto CeA neurons. **(A)** Schematic of the experimental design. **(B, C)** Representative mEPSC traces from CeA neurons recorded from male and female *16p11.2 del/+* **(B)** and WT **(C)** mice. **(D)** Quantification of mEPSC frequency for CeA neurons in female mice ($p = .0029$; $^{**}p < .01$; Mann-Whitney *t* test; female *16p11.2 del/+*, $n = 35$ cells from 4 mice, female WT, $n = 28$ cells from 3 mice). **(E)** Quantification of mEPSC amplitude for CeA neurons in female mice ($p = .7262$; Mann-Whitney *t* test). Data are from the same cells as in panel **(D)**. **(F)** Quantification of mEPSC frequency for CeA neurons in male mice ($p = .2596$; Mann-Whitney *t* test; male *16p11.2 del/+*, $n = 21$ cells from 4 mice, male WT, $n = 14$ cells from 3 mice). **(G)** Quantification of mEPSC amplitude for CeA neurons in male mice ($p > .9999$; Mann-Whitney *t* test). Data are from the same cells as in panel **(F)**. Data are presented as mean ± SEM. BA, basolateral amygdala; CeA, central amygdala; CeL, lateral division of the CeA; CeM, medial division of the CeA; LA, lateral division of the BA; mEPSC, miniature excitatory postsynaptic current; WT, wild-type.

that an enhanced excitatory drive onto GPe-projecting CeA neurons parallels the anxiety phenotypes of female *16p11.2 del/+* mice. Of note, the electrophysiology experiments in the CeA were performed on mice that were not subjected to the behavioral procedures. In addition, these mice were younger than the mice used for the behavioral experiments (see Supplemental Methods). However, because the 16p11.2 microdeletion was constitutively present from birth in these mice, it likely affected the development of the CeA, leading to functional changes in CeA circuits even at earlier stages. We propose that early developmental and functional changes caused by the 16p11.2 microdeletion, such as those that we report here, predispose the affected individuals to anxiety-related symptoms. Our findings together strongly suggest a role of CeA-GPe circuit dysfunction in susceptibility to anxiety after stress and warrant future studies to elucidate how this circuit is involved in 16p11.2 microdeletion syndrome.

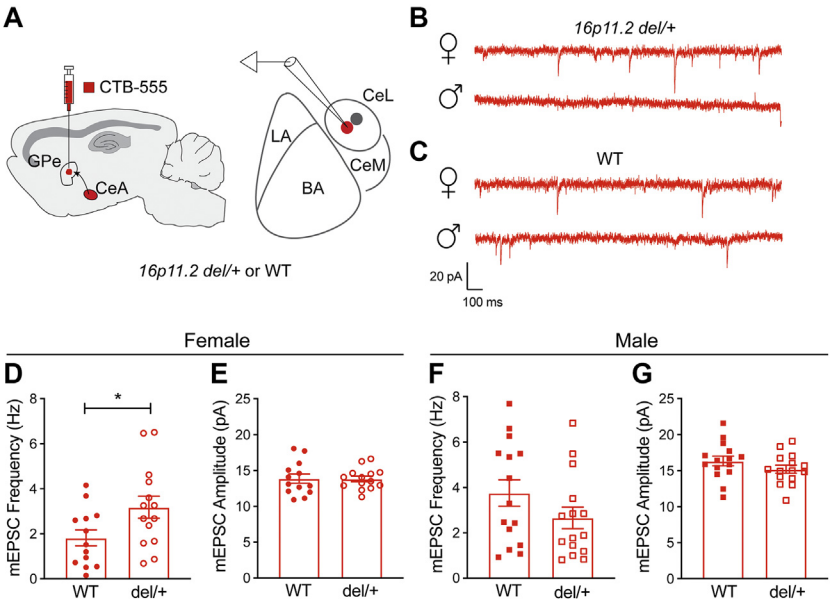

Figure 6. Female *16p11.2 del/+* mice have increased excitatory synaptic transmission onto GPe-projecting CeA neurons. **(A)** Schematic of the experimental design. CTB-555 was used to retrogradely label GPe-projecting CeA neurons. **(B, C)** Representative mEPSC traces from GPe-projecting CeA neurons recorded from male and female *16p11.2 del/+* **(B)** and WT **(C)** mice. **(D)** Quantification of mEPSC frequency for GPe-projecting CeA neurons in female mice ($p = .0482$; $^{*}p < .05$; Mann-Whitney *t* test; female *16p11.2 del/+*, $n = 14$ cells from 5 mice, female WT, $n = 13$ cells from 7 mice). **(E)** Quantification of mEPSC amplitude for GPe-projecting CeA neurons in female mice ($p = .7564$; Mann-Whitney *t* test). Data are from the same cells as in panel **(D)**. **(F)** Quantification of mEPSC frequency for GPe-projecting CeA neurons in male mice ($p = .2017$; Mann-Whitney *t* test; male *16p11.2 del/+*, $n = 15$ cells from 3 mice, male WT, $n = 15$ cells from 5 mice). **(G)** Quantification of mEPSC amplitude for GPe-projecting CeA neurons in male mice ($p = .1770$; Mann-Whitney *t* test). Data are from the same cells as in panel **(F)**. Data are presented as mean ± SEM. BA, basolateral amygdala; CeA, central amygdala; CeL, lateral division of the CeA; CeM, medial division of the CeA; CTB-555, cholera toxin subunit B conjugated to Alexa Fluor 555; GPe, globus pallidus externa; LA, lateral division of the BA; mEPSC, miniature excitatory postsynaptic current; WT, wild-type.

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JG and BL conceived and designed the study. JG conducted the experiments and analyzed data. SA conducted the experiments with the auditory discrimination task and assisted with other electrophysiology experiments. KY identified the CeA-GPe projections and assisted with experiments. JG and BL wrote the paper with input from all authors.

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