

1 **Sex-specific stress-related behavioral phenotypes and central amygdala dysfunction in a**
2 **mouse model of 16p11.2 microdeletion**

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14

15 **Abstract**

16 Substantial evidence indicates that a microdeletion on human chromosome 16p11.2 is linked to
17 neurodevelopmental disorders including autism spectrum disorders (ASD). Carriers of this
18 deletion show divergent symptoms besides the core features of ASD, such as anxiety and
19 emotional symptoms. The neural mechanisms underlying these symptoms are poorly understood.
20 Here we report mice heterozygous for a deletion allele of the genomic region corresponding to
21 the human 16p11.2 microdeletion locus (i.e., the ‘*16p11.2 del/+* mice’) have sex-specific
22 anxiety-related behavioral and neural circuit changes. We found that female, but not male
23 *16p11.2 del/+* mice showed enhanced fear generalization – a hallmark of anxiety disorders –
24 after auditory fear conditioning, and displayed increased anxiety-like behaviors after physical
25 restraint stress. Notably, such sex-specific behavioral changes were paralleled by an increase in
26 activity in central amygdala neurons projecting to the globus pallidus in female, but not male
27 *16p11.2 del/+* mice. Together, these results reveal female-specific anxiety phenotypes related to
28 16p11.2 microdeletion syndrome and a potential underlying neural circuit mechanism. Our study
29 therefore identifies previously underappreciated sex-specific behavioral and neural changes in a
30 genetic model of 16p11.2 microdeletion syndrome, and highlights the importance of
31 investigating female-specific aspects of this syndrome for targeted treatment strategies.

32

33 **Introduction**

34 As of 2018, the Autism and Developmental Disabilities Monitoring (ADDM) Network of the
35 Center for Disease Control (CDC) estimated that approximately one in 59 children age eight and
36 younger are currently diagnosed with autism spectrum disorders (ASD) (Baio et al., 2018). ASD
37 is a spectrum of neurodevelopmental conditions defined by two major diagnostic criteria:

38 “persistent deficits in social communication and social interaction across multiple contexts” and
39 “restricted, repetitive patterns of behavior, interests, or activities” (*Diagnostic and Statistical*
40 *Manual of Mental Disorders, DSM-5*, 2013). Diagnoses of ASD often include supplemental
41 association with intellectual disability, catatonia, other defined neurodevelopmental, mental,
42 behavioral disorders, and/or a known medical, genetic, or environmental factor. Furthermore,
43 patients with ASD are commonly diagnosed with one or more comorbid conditions including
44 intellectual disability (Howlin, 2000; Schwartz & Neri, 2012; Tonnsen et al., 2016), attention
45 deficit-hyperactivity disorder (Antshel et al., 2014, 2016; Antshel & Russo, 2019; Jang et al.,
46 2013), obsessive compulsive disorder (Leyfer et al., 2006; Postorino et al., 2017), anxiety
47 (Brookman-Fraze et al., 2018; Gotham et al., 2013; White et al., 2009), and depression
48 (Andersen et al., 2015; Davidsson et al., 2017; Gotham et al., 2013; Matson & Cervantes, 2014),
49 and are at increased risk for suicidality, particularly among females (T Hirvikoski et al., 2019;
50 Tatja Hirvikoski et al., 2016; Kirby et al., 2019).

51
52 Despite the heterogeneity in ASD features, one major consistency is its sex bias in diagnoses. It
53 is well documented that ASD is about 4 times more common in males than in females with an
54 exception for x-linked syndromes, such as Rett Syndrome which is more common in females
55 (Fombonne, 2002). There is significant evidence of divergence among core symptoms of ASD
56 based on sex. Specifically, many studies have found reduced severity of repetitive and or
57 stereotyped behaviors in females than in males (Baron-Cohen, 2009; Beggiato et al., 2017;
58 Knickmeyer et al., 2008; Kopp et al., 2010; Szatmari et al., 2012). In contrast, females show
59 different social impairments compared with males (Beggiato et al., 2017; Dean et al., 2017; Head
60 et al., 2014; Hiller et al., 2014; Werling & Geschwind, 2013). These tend toward more

61 internalizing symptoms and emotional disturbance (Horiuchi et al., 2014; Kreiser & White,
62 2014; Rynkiewicz et al., 2016; Rynkiewicz & Łucka, 2018; Solomon et al., 2012). Females with
63 ASD also show increased risk of eating disorders (Kalyva, 2009), sensory impairments (Lai et
64 al., 2014), sleep disturbances (Hartley & Sikora, 2009), epilepsy and learning disorders (Giarelli
65 et al., 2010). It has been suggested that females may “camouflage” their autism phenotypes
66 better than males owing to fewer social impairments and better executive functioning (Bölte et
67 al., 2011), as well as reduced externalizing symptoms (Werling & Geschwind, 2013). One way
68 that emotional phenotypes often manifest, is as anxiety disorders. In the general population,
69 females have an increased prevalence of stress-related disorders such as anxiety, depression, and
70 PTSD (Breslau, 2002; Kessler et al., 1995; Olf, 2017; Tolin & Foa, 2006). Therefore, it is
71 possible that anxiety-like phenotypes may present differently in males and females with ASD.

72
73 A major limitation of much of the research in ASD has been its emphasis on males. This is not
74 exclusive to ASD research as most research is done in males (Hughes, 2007). Among
75 neuroscience studies in general, the sex bias of human subjects is approximately 5.5 males for
76 every female and with a ratio much higher among animal studies (Beery & Zucker, 2011). This
77 bias precludes our understanding of autism in females and limits our development of effective
78 treatment strategies. Therefore, we sought to examine whether sex differences exist in stress-
79 related behaviors in a mouse model of ASD. To this end, we utilized a model that mimics
80 a microdeletion on human chromosome 16p11.2. Notably, this deletion is one of the most
81 common genetic variations found in ASD, accounting for ~1% of ASD cases (Chen et al., 2017;
82 Kumar et al., 2007; Marshall et al., 2008; Sanders et al., 2011; Sebat et al., 2007; Weiss et al.,
83 2008). Patients with this deletion show repetitive behaviors, hyperactivity, intellectual disability,

84 motor and speech/language delay, and anxiety symptoms (Al-Kateb et al., 2014; Bijlsma et al.,
85 2009; Fernandez et al., 2010; Shinawi et al., 2010; Steinman et al., 2016). Of note, individuals
86 carrying the 16p11.2 deletion, including those non-ASD carriers, are often diagnosed as having
87 anxiety disorders or other mood disorders (Zufferey et al., 2012).

88

89 The mouse model we used was generated by Horev et al. (Horev et al., 2011), and is one of three
90 independently generated mouse genetic models that mimic the 16p11.2 microdeletion (Arbogast
91 et al., 2016; Horev et al., 2011; Portmann et al., 2014). These models, which were created by
92 deleting largely similar genomic intervals in mouse chromosome 7 corresponding to the syntenic
93 16p11.2 microdeletion region in humans, exhibit overlapping phenotypes (Arbogast et al., 2016;
94 Horev et al., 2011; Portmann et al., 2014). In particular, heterozygous deletion mice – hereafter
95 referred to as *16p11.2 del/+* mice – in each of these lines share basic phenotypes such as low
96 body weight and perinatal mortality, and, importantly, also show behavioral phenotypes related
97 to the symptoms of human 16p11.2 microdeletion carriers. These phenotypes include increased
98 locomotor activity, stereotyped and repetitive behaviors, sleep deficits, recognition memory
99 deficits, reward learning deficits, and social deficits (Angelakos et al., 2017; Arbogast et al.,
100 2016; Grissom et al., 2017; Horev et al., 2011; Portmann et al., 2014; Rein & Yan, 2020; Walsh
101 et al., 2018; Yang, Lewis, et al., 2015; Yang, Mahrt, et al., 2015).

102

103 A few studies examined the *16p11.2 del/+* mice for anxiety or fear-related behaviors, but with
104 mixed results. When tested in the open field (OPF) test and elevated plus maze (EPM) test,
105 assays conventionally used to assess ‘anxiety’ in rodents, these mice appear not different from
106 wildtype (WT) mice (Arbogast et al., 2016; Lynch et al., 2020; Yang, Lewis, et al., 2015),

107 (however, see (Pucilowska et al., 2015)). The *16p11.2 del/+* mice were also examined in fear
108 conditioning paradigms. One study shows that the *16p11.2 del/+* mice have impaired contextual
109 fear conditioning (Tian et al., 2015), whereas other studies show that the *16p11.2 del/+* mice
110 have normal contextual fear conditioning and normal visually cued fear conditioning (Lynch et
111 al., 2020; Yang, Lewis, et al., 2015).

112
113 Recent studies indicate that environmental factors can exacerbate ASD symptomatology and
114 impairments in cognitive and adaptive behaviors in *16p11.2* deletion carriers (Hudac et al.,
115 2020), and *16p11.2 del/+* mice show altered coping in response to stress compared with wildtype
116 littermates (Panzini et al., 2017; Yang, Lewis, et al., 2015). In light of these findings and studies
117 showing males and females can exhibit very different behavioral responses to threats or stress
118 (Dalla & Shors, 2009; Gruene et al., 2015), we reasoned that under a stressful situation *16p11.2*
119 *del/+* mice may exhibit sex-specific behavioral changes. However, a potential sex-specific effect
120 of the *16p11.2* deletion on anxiety or fear-related behaviors in mice has not been considered until
121 recently (Lynch et al., 2020). Furthermore, only simple assays, such as OPF and EPM tests, have
122 been used to assess “baseline anxiety” in *16p11.2 del/+* mice, which may not be sufficient to
123 reveal potential changes in anxiety or fear processing in response to stress in these mice.

124
125 To address these issues, in the current study we examined anxiety-related behaviors under
126 different stress conditions in both male and female *16p11.2 del/+* mice and their wild type
127 littermates. We found that female, but not male *16p11.2 del/+* mice showed enhanced fear
128 generalization, a hallmark of anxiety disorders (Dunsmoor & Paz, 2015), after auditory fear
129 conditioning. Furthermore, although at baseline *16p11.2 del/+* mice were not different from

130 their wildtype littermates in the EPM test, consistent with previous studies (Arbogast et al., 2016;
131 Lynch et al., 2020; Yang, Lewis, et al., 2015), we found that female, but not male *16p11.2 del/+*
132 mice showed enhanced anxiety in the EPM after acute restraint stress. Lastly, we found that
133 these sex-specific behavioral changes were paralleled by an increase in activity in the central
134 amygdala – a major limbic structure that regulates anxiety in rodents and primates (Ahrens et al.,
135 2018; Fox et al., 2012; Shackman & Fox, 2016) – of female, but not male *16p11.2 del/+* mice.
136 Together, our work suggests that *16p11.2* microdeletion differentially affects males and females
137 and may disproportionately disrupt stress-regulation brain functions in females. These findings
138 provide insight into understanding how ASD may present differently in females at behavioral
139 and neuronal levels, and raise the question of whether changes to treatment and diagnostic
140 strategies based on sex should be considered.

141

142 **Methods**

143 **Animals**

144 To breed *16p11.2 del/+* mice, we used *16p11.2 del/+* male mice (Stock Number: 013128) and
145 C57/B6 female mice purchased from the Jackson Laboratory, or similar breeders obtained from
146 Pavel Osten's lab at Cold Spring Harbor Laboratory (CSHL). Breeders were housed with a
147 cardboard bio-hut under a 12-hour light/dark cycle (7 am to 7 pm light) with food and water
148 available *ad libitum*. As *16p11.2 del/+* mice exhibit postnatal lethality (Horev et al., 2011), in
149 breeding cages only, standard rodent chow (LabDiet) was supplemented with DietGel® Boost
150 (ClearH2O), a high calorie liquid diet that increased survival of *16p11.2 del/+* pups. Pups were
151 weaned at 3 weeks of age and group housed with wildtype littermates. Mice were genotyped for
152 *16p11.2* microdeletion between 4-8 weeks of age with primers provided by Alea Mills' lab at

153 CSHL.

154

155 Mice of 2-4 months old were used for all behavioral experiments. Mice of 6-10 weeks old were
156 used for all electrophysiology experiments. All experimental mice were housed under a 12-h
157 light/dark cycle (7 a.m. to 7 p.m. light) in groups of 2-5 animals, containing both *16p11.2 del/+*
158 mice and their wildtype littermates. Food and water were available *ad libitum*. All behavioral
159 experiments were performed during the light cycle. Littermates were randomly assigned to
160 different groups prior to experiments. All experimental procedures were approved by the
161 Institutional Animal Care and Use Committee of CSHL and performed in accordance to the US
162 National Institutes of Health guidelines.

163

164 **Behavioral tasks**

165 *Auditory fear conditioning*

166 We followed standard procedures for classical auditory fear conditioning (Li et al., 2013; Penzo
167 et al., 2014, 2015; Yu et al., 2017). Briefly, mice were initially handled and habituated to a
168 conditioning cage, which was a Mouse Test Cage (18 cm x 18 cm x 30 cm) with an electrifiable
169 floor connected to a H13-15 shock generator (Coulbourn Instruments, Whitehall, PA). The Test
170 Cage was placed inside a sound attenuated cabinet (H10-24A; Coulbourn Instruments). Before
171 each habituation and conditioning session, the Test Cage was wiped with 70% ethanol. The
172 cabinet was illuminated with white light during habituation and conditioning sessions.

173

174 During habituation, two 4-kHz 75-dB tones and two 12-kHz 75-dB tones, each of which was 30s
175 in duration, were delivered at variable intervals within an 8-minute session. During conditioning,

176 mice received three presentations of the 4-kHz tone (conditioned stimulus; CS+), each of which
177 co-terminated with a 2-s 0.7-mA foot shock (unless otherwise stated), and three presentations of
178 the 12-kHz tone (CS-), which were not paired with foot shocks. The CS+ and CS- were
179 interleaved pseudo-randomly, with variable intervals between 30 and 90 s within a 10-minute
180 session. The test for fear memory (retrieval) was performed 24 h following conditioning in a
181 novel context, where mice were exposed to two presentations of CS+ and two presentations of
182 CS- (>120 s inter-CS interval). The novel context was a cage with a different shape (22 cm x 22
183 cm x 21 cm) and floor texture compared with the conditioning cage, and was illuminated with
184 infrared light. Prior to each use the floor and walls of the cage were wiped clean with 0.5% acetic
185 acid to make the scent distinct from that of the conditioning cage.

186

187 Animal behavior was videotaped with a monochrome CCD-camera (Panasonic WV-BP334) at
188 3.7 Hz and stored on a personal computer. The FreezeFrame software (Coulbourn Instruments)
189 was used to control the delivery of both tones and foot shocks. Freezing behavior was analyzed
190 with FreezeFrame software (Coulbourn Instruments). Baseline freezing levels were calculated as
191 the average freezing during the first 100 s of the session before any stimuli were presented, and
192 freezing to the auditory stimuli was calculated as the average freezing during the tone
193 presentation. The average of the freezing responses to two CS+ or CS- presentations during
194 retrieval was used as an index of fear. Discrimination Index was calculated as the difference
195 between freezing to the CS+ and CS-, normalized by the sum of freezing to both tones.

196

197 *Shock sensitivity test*

198 Animals were placed in a conditioning Test Cage in a lit, sound attenuated box, as in the fear

199 conditioning experiments, and received two presentations each of 0.2, 0.4, 0.6, 0.8, and 1.0 mA
200 shocks with an inter-shock interval of 30 seconds. Animals were monitored with a monochrome
201 CCD camera (Panasonic WV-BP334) at 4 Hz, and tracked and analyzed using Ethovision XT 5.1
202 (Noldus Information Technologies) to extract distance traveled and movement velocity during
203 the 2s time window of each shock presentation.

204

205 *Acute physical restraint stress*

206 For stress susceptibility experiments, animals underwent a standard protocol of acute physical
207 restraint as described previously (K. Kim & Han, 2006). Mice were immobilized in a well-
208 ventilated 50 mL conical tube for two hours in a dark, sound attenuated chamber. Males and
209 females were kept in separate chambers. Animals were then tested on the EPM 24 hours after the
210 end of the restraint session.

211

212 *Elevated plus maze test*

213 The elevated plus maze (EPM) test apparatus was constructed from white Plexiglas and
214 consisted of two open arms without walls (30 cm long and 5 cm wide) and two arms enclosed by
215 15.25 cm high non-transparent walls. The arms were extended from a central platform (5 cm x 5
216 cm), and were arranged such that the identical arms were opposite to each other. The maze was
217 raised to a height of 50 cm above the floor with an overhead light. At the start of the session,
218 animals were placed in the center zone and allowed to explore the maze for 10 minutes in the
219 absence of the experimenter, while their behavior was videotaped using a monochrome CCD
220 camera (Panasonic WV-BP334) at 4 Hz. The resulting data was analyzed using Ethovision XT
221 5.1 (Noldus Information Technologies). Parameters assessed were total distance travelled,

222 velocity, time spent in the open arms, number of entries to the open arms, and latency to the first
223 entry into an open arm. The maze was thoroughly cleaned with 70% ethanol in between subjects.

224

225 *Auditory discrimination test*

226 Mice were first trained in an auditory two-alternative choice (2-AC) procedure as previously
227 described (Ahrens et al., 2015). Briefly, mice initiated each trial by poking their nose into the
228 center port of a three-port operant chamber. After a silent delay of random duration (200–300
229 ms, uniformly distributed), a frequency-modulated target sound was presented. The carrier
230 frequency of the target indicated to the animal which of the two side ports would provide 10 μ l
231 of water reward. For a target carrier frequency of 4kHz, reward was available only at the left
232 port. For a target of 12 kHz, reward was provided at the right port. Mice were only rewarded in
233 trials in which they chose the correct port as their first choice. Sound intensity was set at 60 dB-
234 SPL, and sound duration was 100 ms. The modulation frequency was set to 15 Hz. Incorrect
235 choices were punished by a 4s timeout and a white noise presentation.

236

237 After mice reached a performance level of 70% in the 2-AC task, they were tested for auditory
238 discrimination. Mice initiated a trial by a nose poke into the center port. After a silent delay of
239 random duration (200-300 ms), a frequency modulated sound was presented for 100 ms. The
240 frequency of the sound was randomly selected from a group of eight frequencies (4, 4.68, 5.48,
241 6.4, 7.49, 8.77, 10.26 and 12 kHz). These frequencies were chosen such that they were
242 equidistant from each other on the logarithmic (Log_2) scale. All frequencies less than 6.9 kHz
243 (the geometric mean of 4 and 12 kHz) were rewarded if the mouse chose the left water port, and
244 those greater than 6.9 kHz were rewarded with water in the right water port. The volume of the

245 water reward was 5 μ l to ensure that the mice performed sufficient number of trials for each of
246 the frequencies. Data from five consecutive sessions were collected (250-350 trials per session).
247 Responses of each mouse to the eight sound frequencies was transformed into the percentage of
248 ‘proportion right choice’, which is the percentage of the trials in which the mouse chose the
249 water port on the right side. These data were fitted using the following logistic function(Ahrens
250 et al., 2015; Gilchrist et al., 2005):

$$251 \quad y = \frac{A1 - A2}{1 + \left(\frac{X_0}{X}\right)^p} + A2$$

252 where X_0 represents the median threshold and p determines the slope of the curve; $A1$ and $A2$ are
253 the upper and lower bounds of the equation, respectively. A sigmoidal psychometric curve was
254 thus generated. The median threshold X_0 and parameter p of this curve were then obtained for
255 each animal, and the data were pooled for each group.

256

257 **Stereotaxic Surgery**

258 Standard surgical procedures were followed for stereotaxic injection (Li et al., 2013; Penzo et al.,
259 2015; Yu et al., 2016, 2017). Briefly, mice were anesthetized with isoflurane (3% at the
260 beginning and 1% for the rest of the surgical procedure), and positioned in a stereotaxic injection
261 frame (myNeuroLab.com). A digital mouse brain atlas was linked to the injection frame to guide
262 the identification and targeting (Angle Two Stereotaxic System, myNeuroLab.com). The
263 injection was performed at the following stereotaxic coordinates for GPe: -0.46 mm from
264 Bregma, 1.85 mm lateral from the midline, and 3.79 mm vertical from skull surface.

265

266 For injection of the retrograde tracer, we made a small cranial window (1–2 mm²), through
267 which the tracer (~0.3 μ l) was delivered via a glass micropipette (tip diameter, ~5 μ m) by

268 pressure application (5–20 psi, 5–20 ms at 0.5 Hz) controlled by a Picospritzer III (General
269 Valve) and a pulse generator (Agilent). During the surgical procedure, mice were kept on a
270 heating pad maintained at 35°C and were brought back to their home-cage for post-surgery
271 recovery and monitoring. Subcutaneous Metacam (1-2 mg kg⁻¹ meloxicam; Boehringer
272 Ingelheim Vetmedica, Inc.) was given post-operatively for analgesia and anti-inflammatory
273 purposes.

274

275 The retrograde tracer cholera toxin subunit B (CTB) conjugated with Alexa Fluor™ 555 (CTB-
276 555) was purchased from Invitrogen, Thermo Fisher Scientific (Waltham, Massachusetts, USA).
277 CTB was used at a concentration of 1mg/ml in phosphate-buffered saline and kept at -20°C.

278

279 ***In vitro* electrophysiology**

280 For the *in vitro* electrophysiology experiments, mice were anaesthetized with isoflurane and
281 perfused intracardially with 20 mL ice-cold artificial cerebrospinal fluid (ACSF) (118 mM NaCl,
282 2.5 mM KCl, 26.2 mM NaHCO₃, 1 mM NaH₂PO₄, 20 mM glucose, 2 mM MgCl₂ and 2 mM
283 CaCl₂, pH 7.4, gassed with 95% O₂ and 5% CO₂). Mice were then decapitated and their brains
284 quickly removed and submerged in ice-cold dissection buffer (110.0 mM choline chloride, 25.0
285 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM KCl, 0.5 mM CaCl₂, 7.0 mM MgCl₂, 25.0 mM
286 glucose, 11.6 mM ascorbic acid and 3.1mM pyruvic acid, gassed with 95% O₂ and 5% CO₂).
287 Coronal sections of 300 μm containing the central amygdala (CeA) were cut in dissection buffer
288 using a HM650 Vibrating-blade Microtome (Thermo Fisher Scientific). Slices were immediately
289 transferred to a storage chamber containing ACSF at 34 °C. After 40 min recovery time, slices
290 were transferred to room temperature (20–24°C) and perfused with gassed ACSF constantly

291 throughout recording.

292

293 Whole-cell patch clamp recording was performed as previously described (Li et al., 2013).

294 Briefly, recording from CeA neurons was obtained with Multiclamp 700B amplifiers and

295 pCLAMP 10 software (Molecular Devices, Sunnyvale, California, USA), and was visually

296 guided using an Olympus BX51 microscope equipped with both transmitted and epifluorescence

297 light sources (Olympus Corporation, Shinjuku, Tokyo, Japan). The external solution was ACSF.

298 The internal solution contained 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM

299 HEPES, 2.5 mM MgCl₂, 4 mM Na₂ATP, 0.4 mM Na₃GTP, 10 mM sodium phosphocreatine and

300 0.6 mM EGTA (pH 7.2). Miniature excitatory post-synaptic currents (mEPSCs) were recorded at

301 -70 mV with bath application of 100 μM GABA antagonist, picrotoxin (PTX), and 1 μM sodium

302 channel blocker, tetrodotoxin (TTX). The internal solution contained 115 mM cesium

303 methanesulphonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM MgCl₂, 4 mM Na₂-ATP, 0.4 mM

304 Na₃GTP, 10 mM Na-phosphocreatine and 0.6 mM EGTA (pH 7.2, 290 mOsm). Data was

305 collected in gap-free mode in pClamp 10 (Molecular Devices) for 5 minutes at room temperature

306 and analyzed using Mini Analysis Program (Synaptosoft). For recordings on CeA neurons

307 projecting to the GPe, CTB-555 was injected into the GPe 3 days prior to the recording. Slices of

308 the GPe were examined for accuracy in the injection location. Animals with incorrect injection

309 locations were not used for recording.

310

311 **Data analysis and statistics**

312 All statistics are indicated where used. Statistical analyses were performed with GraphPad Prism

313 Software (GraphPad Software, Inc., La Jolla, CA). Normality was tested by D'Agostino-Pearson

314 or Shapiro-Wilk normality tests. Non-normal datasets were log-transformed for normality before
315 statistical testing. All behavioral experiments were controlled by computer systems, and data
316 were collected and analyzed in an automated and unbiased way. Virus-injected animals in which
317 the injection site was incorrect were excluded. No other animals were excluded.

318

319 **Results**

320 **Female-specific increase in fear generalization in *16p11.2 del/+* mice**

321 One hallmark of anxiety disorders is fear generalization (Dunsmoor & Paz, 2015). Fear
322 generalization can be assessed in mice using a fear conditioning paradigm with a discrimination
323 component (see Methods), in which mice are trained to associate one auditory stimulus
324 (conditioned stimulus, CS) (CS+) with a foot shock (unconditioned stimulus, US), while a
325 different auditory stimulus (CS-) is presented without the shock. In a fear retrieval test 24 hours
326 following the conditioning, both freezing in response to the CS+ and that to the CS- are
327 measured and used to calculate a discrimination index, which is the difference between an
328 animal's average freezing to the CS+ and that to the CS-, normalized to the sum of the two
329 measurements.

330

331 Interestingly, we found that during a habituation session before the conditioning, female *16p11.2*
332 *del/+* mice showed small (10-20%) but robust increase in freezing to the auditory stimuli
333 compared with their wildtype (WT) littermates (Figure 1A, left). Male *16p11.2* mice did not
334 show such change (Figure 1A, right). However, we did not observe a significant difference in
335 freezing during the first tone presentation in the subsequent conditioning session (i.e., before
336 mice received any shocks) between genotypes for either the female or the male mice (Figure 1B,

337 D), suggesting that the enhanced freezing in *16p11.2 del/+* female mice during habituation may
338 be related to the fact that the auditory stimuli were novel to the animals.

339

340 After fear conditioning and upon memory retrieval, both female and male *16p11.2 del/+* mice
341 showed levels of freezing similar to those of their WT littermates in response to the CS+ (Figure
342 B, D), consistent with previous findings that *16p11.2 del/+* mice have intact fear conditioning
343 (Lynch et al., 2020; Yang, Lewis, et al., 2015). Surprisingly, however, female, but not male
344 *16p11.2 del/+* mice showed increased freezing to the CS- compared with WT littermates (Figure
345 1B, D), resulting in reduced levels of fear discrimination in female, but not male *16p11.2 del/+*
346 animals (Figure 1C, E). In addition, we found that female, but not male *16p11.2 del/+* mice
347 showed enhanced reactions to foot shocks compared with WT mice, as measured by enhanced
348 movement velocity and distance immediately following shocks of varying intensities (Figure 2).
349 These results suggest that female *16p11.2 del/+* mice have enhanced fear generalization
350 following fear conditioning, which could result from heightened alertness (as indicated by
351 increased freezing during habituation) or an increase in sensitivity to aversive stimuli (as
352 indicated by increase reactivity to foot shocks), or both.

353

354 ***16p11.2 del/+* mice have normal auditory perception**

355 An alternative explanation for the enhanced fear generalization in female *16p11.2 del/+* mice is
356 that these mice have an impairment in auditory processing, such that they cannot effectively
357 discriminate between a 4-kHz tone and a 12-kHz tone, which were used as CS+ and CS-,
358 respectively, during fear conditioning. To test this possibility, we trained a new cohort of mice,
359 including *16p11.2 del/+* mice and their WT littermates, in an auditory two-alternative choice (2-

360 AC) task that depended on discriminating between a 4-kHz tone and a 12-kHz tone (Figure 3A;
361 see Methods) (Ahrens et al., 2015). Both female and male *16p11.2 del/+* mice learned the 2-AC
362 task at a rate similar to that of their WT littermates (Figure 3B, C). In fact, male *16p11.2 del/+*
363 mice tended to be faster than WT mice in learning the task (Figure 3C), though this difference
364 did not reach significance. In addition, the performance of female and male *16p11.2 del/+* mice
365 in discriminating a series of sounds with frequencies ranging from 4 to 12 kHz (Figure 3D-F and
366 H-J), or with different intensities (Figure 3G, K), was indistinguishable from their WT
367 littermates. These results indicate that *16p11.2* microdeletion does not affect auditory perception
368 in mice, ruling out the possibility that the enhanced fear generalization in female *16p11.2 del/+*
369 mice is confounded by an impairment in auditory processing in these mice.

370

371 **Stress induces an increase in anxiety in female *16p11.2 del/+* mice**

372 In fear conditioning, mice receive electric shocks as the aversive US, which is a significant
373 stressor to animals. Therefore, the enhanced fear generalization in female *16p11.2 del/+* mice
374 after fear conditioning points to a possibility that these animals are prone to stress-induced
375 anxiety. To further test this possibility, we sought to examine anxiety-like behaviors in mice
376 subjected to a different stressor. For this purpose, we used physical restraint (Methods), a well
377 characterized stress-induction procedure in rodents which has been shown to affect the function
378 of the central amygdala (Varodayan et al., 2018, 2019). As described previously (Zimprich et al.,
379 2014), animals are physically restrained in a well-ventilated 50 mL conical tube for 2 hours in a
380 dark, sound attenuated box. 24 hours later, animals were tested on the EPM (Methods). We
381 found a significant interaction between sex and genotype in the time spent in the open arms
382 (Figure 4A) and significant effects of sex on movement velocity (Figure 4B) and distance

383 traveled (Figure 4C). Post-hoc analysis revealed that the stressed female *16p11.2 del/+* mice
384 spent significantly less time in the open arms of the EPM compared to their female WT
385 littermates (Figure 4A). We did not find any change in time spent in the open arms in male
386 *16p11.2 del/+* mice.

387

388 We also examined anxiety levels in naïve mice using the EPM test. Compared with naïve female
389 or male WT littermates, naïve female or male *16p11.2 del/+* mice, respectively, did not show
390 any change in the time spent in the open arms (Figure 4D), movement velocity (Figure 4E) and
391 distance traveled (Figure 4F). This result is consistent with previous findings (Arbogast et al.,
392 2016; Lynch et al., 2020; Yang, Lewis, et al., 2015). Together, our results indicate that female
393 *16p11.2 del/+* mice have increased susceptibility to stress-induced anxiety.

394

395 ***16p11.2 del/+* mice have central amygdala dysfunction**

396 Previous studies have revealed that the central amygdala (CeA) is particularly responsive to
397 stress and is a major contributor to anxiety-related behaviors (Ahrens et al., 2018; Fox et al.,
398 2012; Shackman & Fox, 2016). Therefore, we examined whether the *16p11.2* microdeletion
399 affects CeA neuronal function in a sex-specific manner. We recorded miniature excitatory
400 postsynaptic currents (mEPSCs) – a measurement of total excitatory synaptic drive onto the
401 recorded neurons – from CeA neurons in acute brain slices prepared from female or male
402 *16p11.2 del/+* mice, as well as their respective WT littermates (Figure 5A). We found significant
403 effects of sex and genotype on mEPSC frequency in randomly recorded central amygdala
404 neurons (Figure 5B-D). Post-hoc analysis revealed that females with *16p11.2* microdeletion
405 specifically had increased mEPSC frequency compared with female wildtype littermates. There

406 was no difference in mEPSC amplitude between genotypes or sexes (Figure 5E). These results
407 indicate that female, but not male *16p11.2 del/+* mice have enhanced excitatory synaptic drive
408 onto CeA neurons.

409

410 We recently identified a pathway from the CeA to the globus pallidus externa (GPe), which
411 conveys information of the US and is critical for learning in fear conditioning (Giovanniello et
412 al., 2020). Importantly, optogenetic activation of the CeA-GPe pathway increases fear
413 generalization whereby animals increase their freezing to CS-. Therefore, we sought to
414 determine whether the GPe-projecting CeA neurons are affected by the *16p11.2* microdeletion.
415 To this end, we used a retrograde labeling strategy whereby fluorescently conjugated CTB was
416 injected in the GPe to label the GPe-projecting CeA neurons (Figure 6A; Methods). Three days
417 after the CTB injection, we recorded mEPSCs selectively from the CTB-labeled GPe-projecting
418 CeA neurons in acute brain slices prepared from female or male *16p11.2 del/+* mice, as well as
419 their respective WT littermates (Figure 6A, B). Again, we found a significant interaction
420 between sex and genotype whereby females with *16p11.2* microdeletion exhibited increased
421 mEPSC frequency compared with wildtype littermates (Figure 6D, E). Thus, our results indicate
422 that the *16p11.2* microdeletion caused a female-specific enhancement of excitatory synaptic
423 drive onto CeA neurons, and moreover suggests dysfunction in the CeA-GPe pathway as a
424 potential mechanism for the increased stress susceptibility and fear generalization identified in
425 female *16p11.2 del/+* mice.

426

427 **Discussion**

428 Our results indicate that female, but not male, *16p11.2 del/+* mice have increased susceptibility

429 to anxiety-like phenotypes following stressful life events, revealing a previously
430 underappreciated sex-specific effect in the modulation of behavior by 16p11.2 microdeletion.
431 Furthermore, we identify that CeA dysfunction, in particular that in the CeA-GPe circuit, may
432 underlie the female-specific behavioral phenotypes caused by the 16p11.2 microdeletion. These
433 findings are consistent with the vast literature that females affected with ASD show distinct
434 behavioral symptoms compared with males (Beggiato et al., 2017; Dean et al., 2017; Head et al.,
435 2014; Hiller et al., 2014; Werling & Geschwind, 2013) in particular the more internalizing
436 symptoms and emotional disturbances (Horiuchi et al., 2014; Kreiser & White, 2014;
437 Rynkiewicz et al., 2016; Rynkiewicz & Łucka, 2018; Solomon et al., 2012). Our findings are
438 also consistent with the notion that in the general population, females have an increased
439 prevalence of stress-related disorders such as anxiety, depression, and PTSD (Breslau, 2002;
440 Kessler et al., 1995; Olf, 2017; Tolin & Foa, 2006). Our study thus urges a careful examination
441 of anxiety and other emotional symptoms, as well as functional changes in the amygdala-basal
442 ganglia circuits in 16p11.2 microdeletion carriers, in particular in female carriers. In general, our
443 study also urges sex-specific diagnostic and treatment strategies for ASD.

444

445 Three lines of evidence suggest that heightened alertness or an increase in sensitivity to aversive
446 stimuli, or to the stimuli signaling potential threat, may underlie the increased susceptibility to
447 anxiety-like phenotypes in female *16p11.2 del/+* mice following stressful experiences. First,
448 *16p11.2 del/+* mice, especially females, show increased freezing when they are exposed to an
449 unfamiliar sound, which is a sign of uncertainty or potential danger. Second, female *16p11.2*
450 *del/+* mice have enhanced reactivity to foot shocks. Third, CeA neurons in female *16p11.2 del/+*
451 mice have enhanced sensitivity to excitatory inputs. This enhanced sensitivity may lead to

452 heightened alertness or attention, as the CeA has been implicated in selective processing of
453 salient information (Calu et al., 2010; Roesch et al., 2012).

454

455 The CeA has central roles in the generation of fear and anxiety states (Ahrens et al., 2018;
456 Andreatta et al., 2015; Calhoun & Tye, 2015; Davis et al., 2010; Etkin & Wager, 2007; Fox et
457 al., 2012, 2015; Gungor & Paré, 2016; Jennings et al., 2013; S.-Y. Kim et al., 2013; Li et al.,
458 2013; Marcinkiewicz et al., 2016; Mobbs et al., 2010; Penzo et al., 2015; Shackman & Fox, 2016;
459 Tovote et al., 2015; Wager et al., 2008; Walker & Davis, 2008; Yu et al., 2017). In parallel,
460 amygdala dysfunction has been implicated in the pathogenesis of ASD. Abnormal developmental
461 trajectory of the amygdala has been observed in ASD (Amaral et al., 2008). Brain imaging
462 studies indicate that the amygdala is enlarged precociously in children with autism (Schumann et
463 al., 2004; Sparks et al., 2002), and that amygdala enlargement in autistic children is associated
464 with anxiety symptoms (Juranek et al., 2006). In addition, cellular changes in the amygdala have
465 been reported in ASD (Amaral et al., 2008). In a recent study (Giovanniello et al., 2020), we
466 found that a subpopulation of neurons in the CeA send direct projections to the GPe, and the
467 CeA-GPe pathway conveys US information and controls learning during fear conditioning. In
468 the current study, we found that an enhanced excitatory drive onto GPe-projecting CeA neurons
469 parallels the anxiety phenotypes of female *16p11.2 del/+* mice. These findings together strongly
470 suggest a role of CeA-GPe circuit dysfunction in susceptibility to anxiety after stress, and
471 warrant future studies to elucidate how this circuit is involved in 16p11.2 microdeletion
472 syndrome.

473

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482

483 **Author contributions**

484 J.G. and B.L. conceived and designed the study. J.G. conducted the experiments and analyzed
485 data. S.A. conducted the experiments with the auditory discrimination task and assisted with
486 other electrophysiology experiments. K.Y. identified the CeA-GPe projections and assisted with
487 experiments. J.G. and B.L. wrote the paper with input from all authors.

488

489 **Competing interests**

490 The authors declare no competing financial interests.

491

492 **References**

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- 892

1 **Sex-specific stress-related behavioral phenotypes and central amygdala dysfunction in a**
2 **mouse model of 16p11.2 microdeletion**

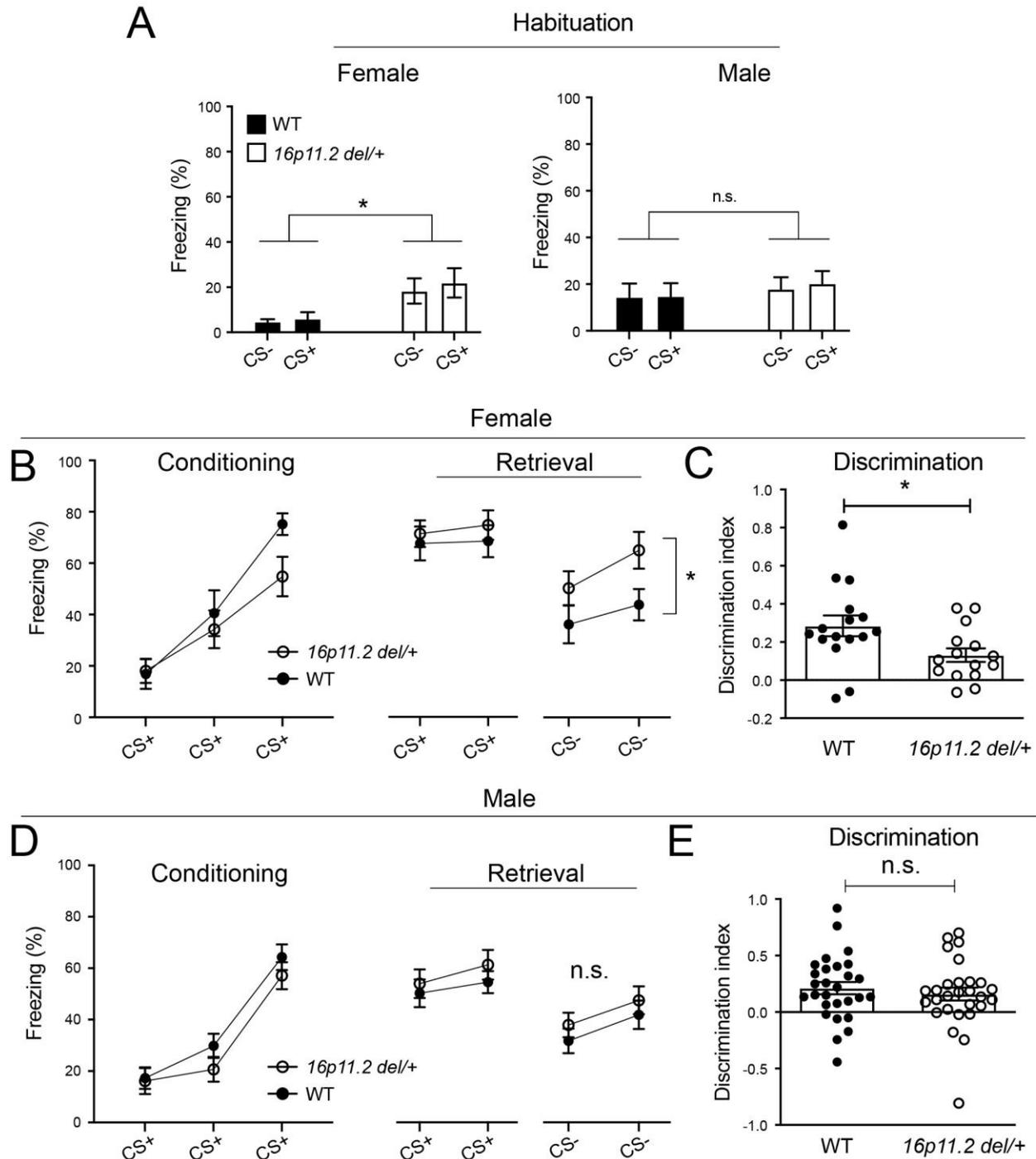
3 Jacqueline Giovanniello^{1,2,3}, Sandra Ahrens^{2,4}, Kai Yu², Bo Li^{1,2}#

4

5

6 **FIGURES AND FIGURE LEGENDS**

7

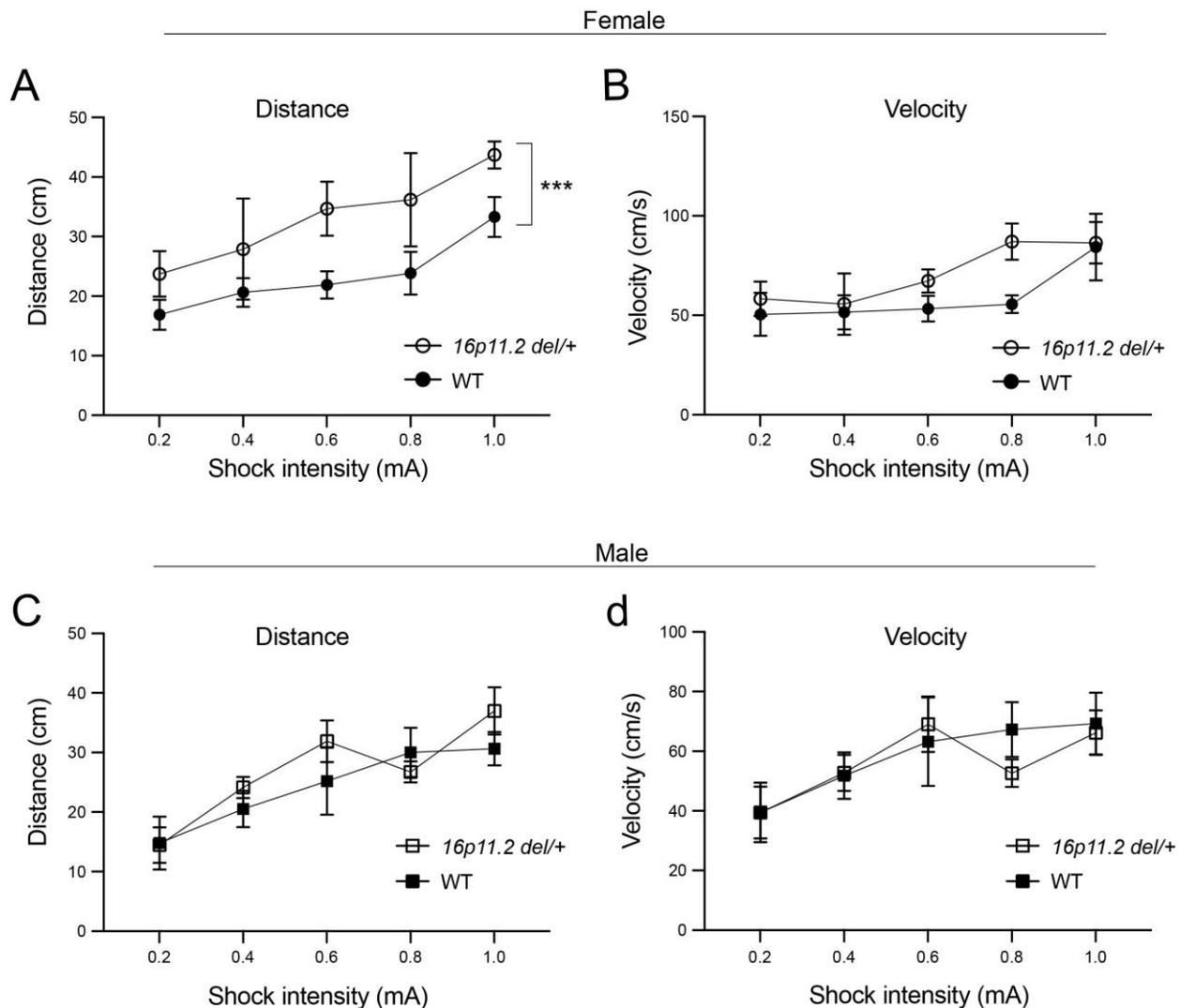


8

9 **Figure 1. Female *16p11.2 del/+* mice exhibit fear generalization following fear conditioning**

10 (A) Freezing behavior of male and female *16p11.2 del/+* mice and their respective wildtype (WT)
 11 littermates in response to CS+ and CS- during habituation (female *16p11.2 del/+*, n = 15
 12 mice, WT, n = 16), $F(1, 29) = 6.023$, $P = 0.0204$; male (*16p11.2 del/+*, n = 28 mice, WT, n =
 13 28), $F(1, 54) = 0.3433$, $P = 0.5604$; * $P < 0.05$, n.s., nonsignificant; two-way ANOVA with
 14 repeated measures).

- 15 (B) Freezing to each stimulus presentation during conditioning and retrieval for female mice
16 (conditioning, $F(1,29) = 1.419$, $P = 0.2432$; CS+ retrieval, $F(1,29) = 0.4314$, $P = 0.5165$; CS-
17 retrieval, $F(1,29) = 5.765$, $P = 0.0230$; * $p < 0.05$; two-way ANOVA with repeated measures
18 and post-hoc Sidak's test).
- 19 (C) Discrimination index $[(CS^+ - CS^-) / (CS^+ + CS^-)]$ for female mice (* $P = 0.0192$, Mann-
20 Whitney t-test,).
- 21 (D) Freezing to each stimulus presentation during conditioning and retrieval for male mice.
22 (conditioning, $F(1,54) = 0.9938$, $P = 0.3233$; CS+ retrieval, $F(1,54) = 0.6327$, $P = 0.4298$; CS-
23 retrieval, $F(1,54) = 0.8779$, $P = 0.3530$; two-way ANOVA with repeated measures).
- 24 (E) Discrimination index for male mice ($P = 0.3742$, n.s., nonsignificant, Mann-Whitney t-test).
25
- 26 Data are presented as mean \pm s.e.m.



27

28 **Figure 2. Female *16p11.2 del/+* mice show enhanced reactivity to foot shock**

29 (A) Distance traveled during 2-s shock presentations for female mice ($F(1,50) = 14.94$, $P =$
30 0.0003 ; $***P < 0.001$; two-way ANOVA; *16p11.2 del/+*, $n = 4$; WT, $n = 8$).

31 (B) Movement velocity during 2-s shock presentations for female mice ($F(1,50) = 2.596$, $P =$
32 0.1135 ; two-way ANOVA).

33 (C) Distance traveled during 2-s shock presentations for male mice ($F(1,50) = 1.410$, $P = 0.2407$;
34 two-way ANOVA; *16p11.2 del/+*, $n = 7$; WT, $n = 5$).

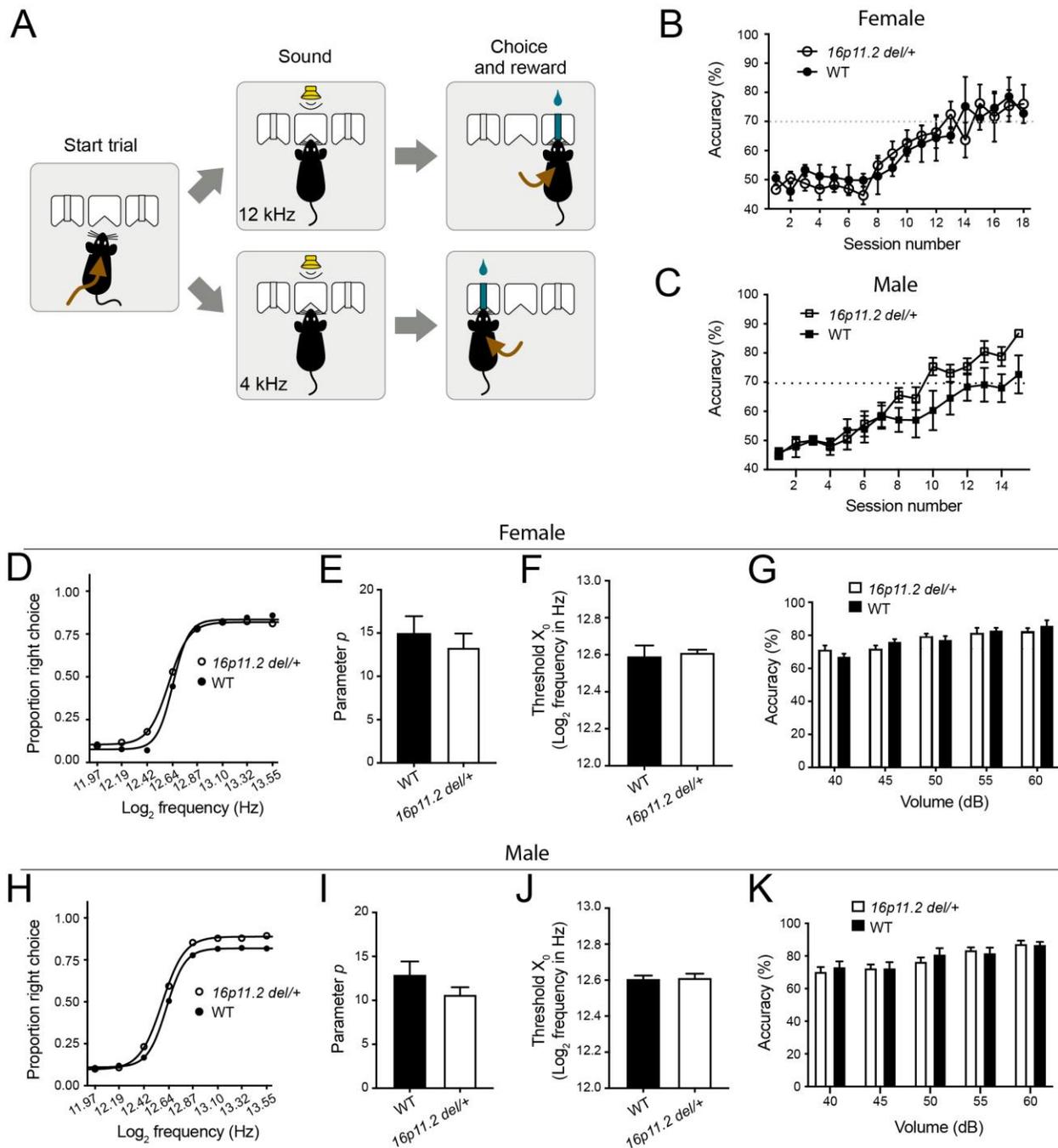
35 (D) Movement velocity during 2-s shock presentations for male mice ($F(1,50) = 0.1467$, $P =$
36 0.7033 ; two-way ANOVA).

37

38 Data are presented as mean \pm s.e.m.

39

40



41
42 **Figure 3. 16p11.2 del/+ mice have normal auditory perception**

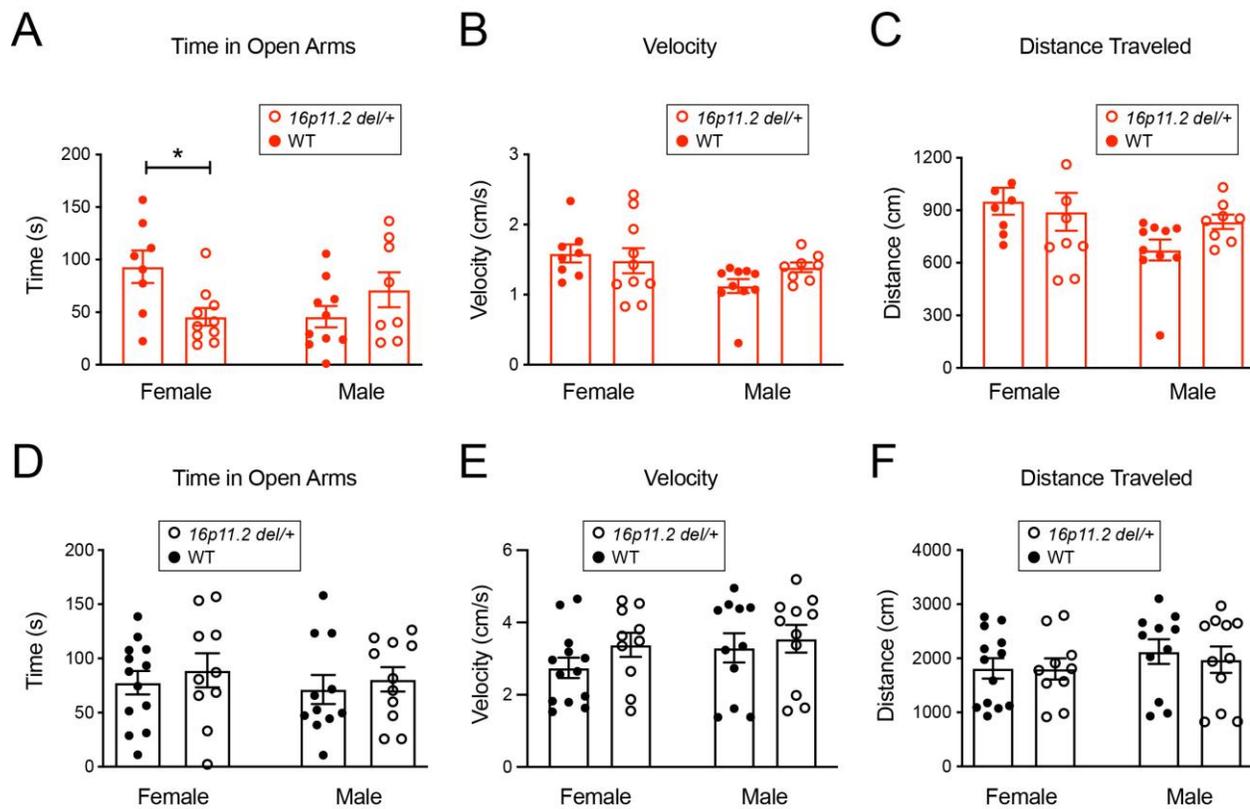
43 (A) A schematic of the behavioral task.

44 (B) Performance levels across training for female mice ($F(1,8) = 0.005112$, $P = 0.9448$; two-way
45 ANOVA; 16p11.2 del/+, $n = 7$, WT, $n = 3$).

46 (C) Performance levels across training for male mice ($F(1,14) = 2.557$, $P = 0.1321$; two-way
47 ANOVA; 16p11.2 del/+, $n = 9$; WT, $n = 7$).

48 (D) Psychometric response curve for frequencies between 4 and 12 kHz (in Log₂ values) for
49 female mice.

- 50 (E) Quantification of the slope of the psychometric curve (parameter p) for female mice ($P =$
51 0.5878 , t-test).
- 52 (F) Quantification of the median threshold, X_0 , from the psychometric function for female mice
53 ($P = 0.6465$, t-test).
- 54 (G) Average performance levels at 4 and 12 kHz for stimuli volume between 40 and 60 dB for
55 female mice ($F(1,8) = 0.04474$, $P = 0.8378$; two-way ANOVA with repeated measures).
- 56 (H) Psychometric response curve for frequencies between 4 and 12 kHz (in Log_2 values) for male
57 mice.
- 58 (I) Quantification of the slope of the psychometric curve (parameter p) for male mice ($P =$
59 0.1713 , t-test).
- 60 (J) Quantification of the median threshold, X_0 , from the psychometric function for male mice (P
61 $= 0.8607$, t-test).
- 62 (K) Average performance levels at 4 and 12 kHz for stimuli volume between 40 and 60 dB for
63 male mice ($F(1,14) = 0.0173$, $P = 0.8972$; two-way ANOVA with repeated measures).
- 64
- 65 All data are presented as mean \pm s.e.m.

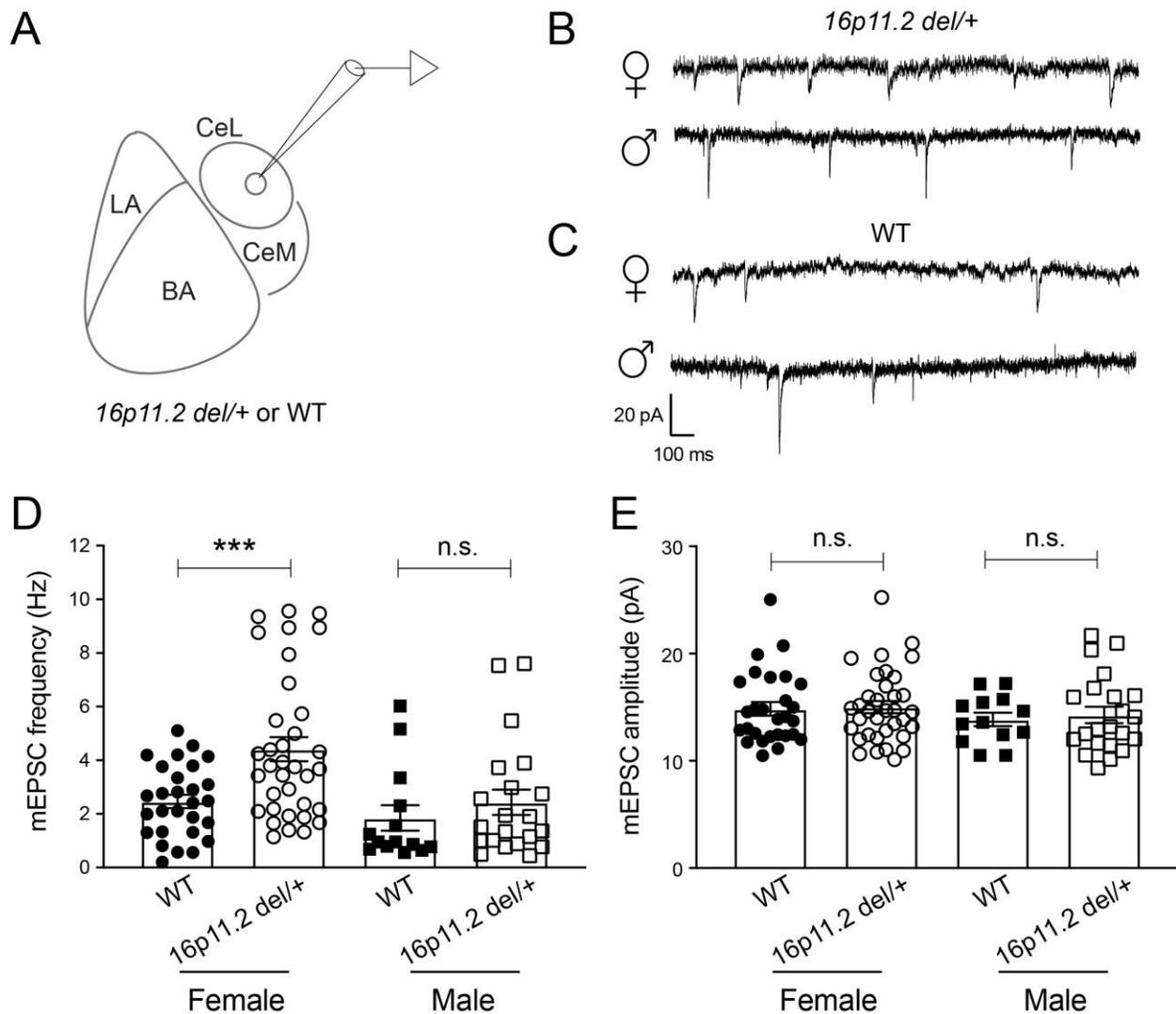


66
67 **Figure 4. Female *16p11.2 del/+* mice exhibit enhanced stress-induced anxiety-like behavior**

68 (A) Time spent in the open arms of EPM 24 hours after stress exposure ($F(1,32) = 8.553$, $P =$
69 0.0063 ; * $P < 0.05$; two-way ANOVA with post-hoc Sidak's test; female *16p11.2 del/+*, $n =$
70 10 , female WT, $n = 8$, male *16p11.2 del/+*, $n = 8$, male WT, $n = 10$).
71 (B) Movement velocity on the EPM 24 hours after stress exposure ($F(1,32) = 0.3917$, $P = 0.5358$;
72 two-way ANOVA). Same mice as in A are used.
73 (C) Distance traveled on the EPM 24 hours after stress exposure ($F(1,32) = 0.3918$, $P = 0.5358$;
74 two-way ANOVA). Same mice as in A are used.
75 (D) Time spent in the open arms of EPM in naïve mice ($F(1,41) = 0.6545$, $P = 0.4232$; two-way
76 ANOVA; female *16p11.2 del/+*, $n = 10$, female WT, $n = 13$, male *16p11.2 del/+*, $n = 11$;
77 male WT, $n = 11$).
78 (E) Movement velocity on the EPM in naïve mice ($F(1,41) = 1.587$, $P = 0.2148$; two-way
79 ANOVA). Same mice as in D are used.
80 (F) Distance traveled on the EPM in naïve mice ($F(1,41) = 0.1314$, $P = 0.7189$; two-way
81 ANOVA). Same mice as in D are used.

82
83 Data are presented as mean \pm s.e.m.

84



85
86 **Figure 5. Female *16p11.2 del/+* mice have increased excitatory synaptic transmission onto**
87 **CeA neurons**

88 (A) A schematic of the experimental design.

89 (B, C) Representative mEPSC traces from CeA neurons recorded from male and female *16p11.2*
90 *del/+* (B) and WT (C) mice.

91 (D) Quantification of mEPSC frequency for CeA neurons ($F(1, 94) = 7.759$, $P = 0.0065$; *** $P <$
92 0.001 ; two-way ANOVA with post-hoc Sidak's test; female *16p11.2 del/+*, $n = 35$ cells from 4
93 mice, female WT, $n = 28$ cells from 3 mice, male *16p11.2 del/+*, $n = 21$ cells from 4 mice, male
94 WT, $n = 14$ cells from 3 mice).

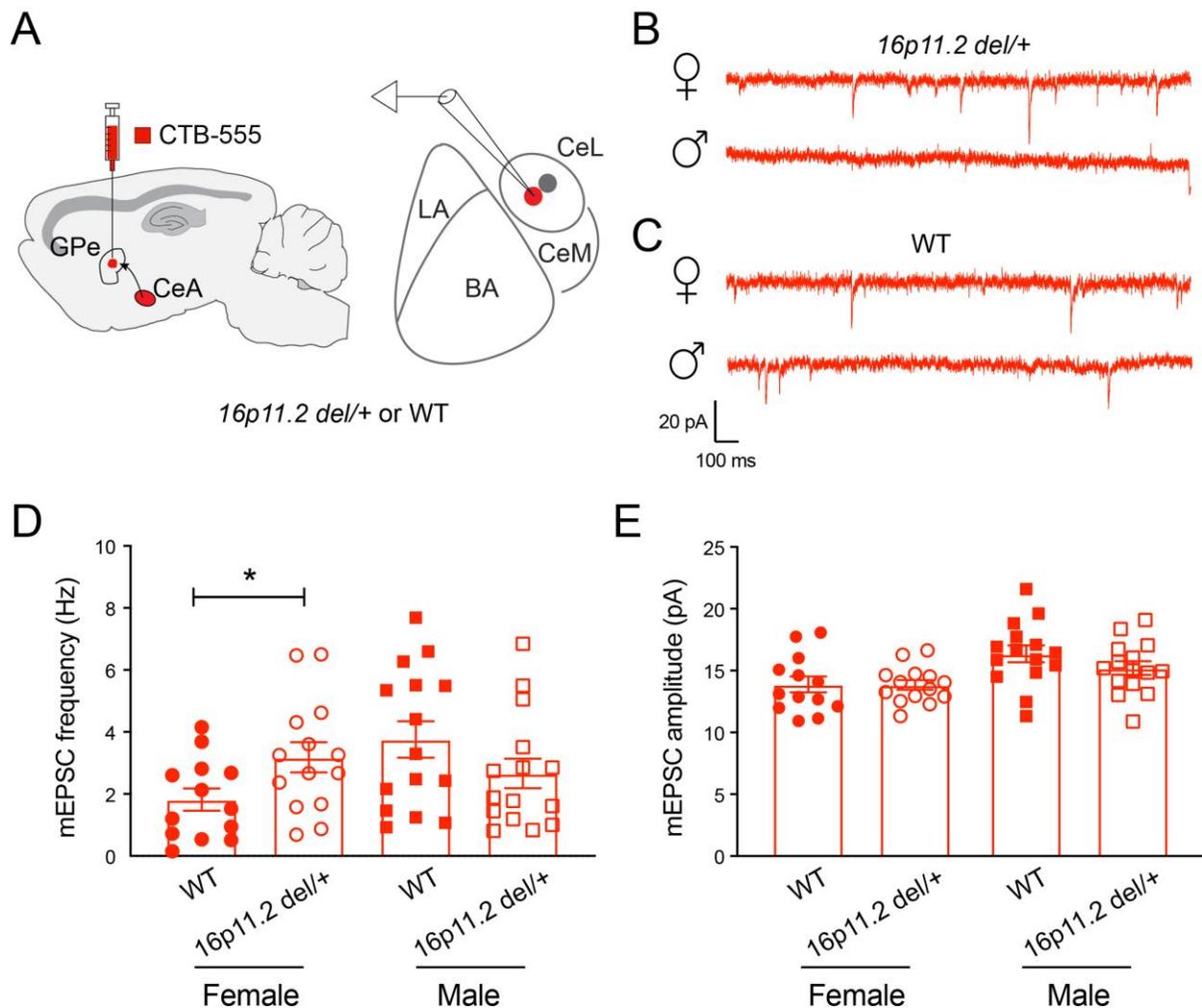
95 (E) Quantification of mEPSC amplitude for CeA neurons ($F(1,94) = 0.1620$, $P = 0.6882$; two-
96 way ANOVA). Data are from the same cells as in D.

97

98 Data are presented as mean \pm s.e.m.

99

100



101
102 **Figure 6. Female *16p11.2 del/+* mice have increased excitatory synaptic transmission onto**
103 **GPe-projecting CeA neurons**
104 (A) A schematic of the experimental design. CTB-555 was used to retrogradely label GPe-
105 projecting CeA neurons.
106 (B, C) Representative mEPSC traces from GPe-projecting CeA neurons recorded from male and
107 female *16p11.2 del/+* (B) and WT (C) mice.
108 (D) Quantification of mEPSC frequency for GPe-projecting CeA neurons ($F(1, 53) = 6.251$, $P =$
109 0.0155 ; $*P < 0.05$; two-way ANOVA with post-hoc Sidak's test; female *16p11.2 del/+*, $n = 14$
110 cells from 5 mice, female WT, $n = 13$ cells from 7 mice, male *16p11.2 del/+*, $n = 15$ cells from 3
111 mice, male WT, $n = 15$ cells from 5 mice).
112 (E) Quantification of mEPSC amplitude for GPe-projecting CeA neurons ($F(1,53) = 1.055$, $P =$
113 0.3090 ; two-way ANOVA). Data are from the same cells as in D.
114
115 Data are presented as mean \pm s.e.m.