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16p11.2 Deletion Syndrome Mice Display Sensory and Ultrasonic Vocalization Deficits During Social Interactions

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Abstract

Recurrent deletions and duplications at chromosomal region 16p11.2 are variably associated with speech delay, autism spectrum disorder, developmental delay, schizophrenia, and cognitive impairments. Social communication deficits are a primary diagnostic symptom of autism. Here we investigated ultrasonic vocalizations (USVs) in young adult male 16p11.2 deletion mice during a novel three-phase male–female social interaction test that detects vocalizations emitted by a male in the presence of an estrous female, how the male changes its calling when the female is suddenly absent, and the extent to which calls resume when the female returns. Strikingly fewer vocalizations were detected in two independent cohorts of 16p11.2 heterozygous deletion males (+/−) during the first exposure to an unfamiliar estrous female, as compared to wildtype littermates (+/+). When the female was removed, +/+ emitted calls, but at a much lower level, whereas +/− males called minimally. Sensory and motor abnormalities were detected in +/−, including higher nociceptive thresholds, a complete absence of acoustic startle responses, and hearing loss in all +/− as confirmed by lack of auditory brainstem responses to frequencies between 8 and 100 kHz. Stereotyped circling and backflipping appeared in a small percentage of individuals, as previously reported. However, these sensory and motor phenotypes could not directly explain the low vocalizations in 16p11.2 deletion mice, since (a) +/− males displayed normal abilities to emit vocalizations when the female was subsequently reintroduced, and (b) +/− vocalized less than +/+ to social odor cues delivered on an inanimate cotton swab. Our findings support the concept that mouse USVs in social settings represent a response to social cues, and that 16p11.2 deletion mice are deficient in their initial USVs responses to novel social cues.

Keywords

mouse model of autism; 16p11.2 deletion; ultrasonic vocalization; social Interaction; autism

Introduction

Recurrent heterozygous deletions and duplications of a ~600kb segment on human chromosomal region 16p11.2 appear in up to 1% of individuals with autism [Christian et al., 2008; Hanson et al., 2010; Kumar et al., 2008; Marshall et al., 2008; McCarthy et al., 2009; Levy et al., 2011; Pinto et al., 2010; Rosenfeld, Coe, Eichler, Cuckle, & Shaffer, 2013; Rosenfeld et al., 2010; Sanders et al., 2011; Sebat et al., 2007; Shen et al., 2010; Weiss et al., 2008]. 16p11.2 deletions and duplications are additionally associated with speech delay, intellectual impairment, and developmental delays [Bassuk et al., 2013; Bijlsma et al., 2009; Cooper et al., 2011; Fernandez et al., 2010; Guilmatre et al., 2009; Hanson et al., 2010; Owen et al., 2014; Rosenfeld et al., 2010; Shinawi et al., 2010; Weiss et al., 2008; Zufferey et al., 2012], schizophrenia [Guilmatre et al., 2009; McCarthy et al., 2009; Rosenfeld et al., 2010; Steinberg et al., 2014; Szatkiewicz et al., 2014], bipolar disorder [Fernandez et al., 2010; McCarthy et al., 2009], depression [Degenhardt et al., 2012; Rosenfeld et al., 2010], and anxiety [Fernandez et al., 2010; Pinto et al., 2010; Rosenfeld et al., 2010]. Physiological abnormalities associated with 16p11.2 copy number variants are diverse, including motor hypotonia, seizures, feeding difficulty, obesity (deletion), low body weight (duplication), immune deficiency, syringomyelia, hearing loss, and cardiac defects [Bassuk et al., 2013; Bijlsma et al., 2009; Ciuladaite et al., 2011; Fernandez et al., 2010; Ghebranious, Giampietro, Wesbrook, & Rezkalla, 2007; Hanson et al., 2010; Jacquemont et al., 2011; Puvabanditsin et al., 2010; Rosenberg et al., 2006; Reinthaler et al. 2014; Sanders et al., 2011; Schaaf et al., 2011; Shinawi et al., 2010; Shiow et al., 2009; Steinberg et al., 2014; Walters et al., 2010]. 16p11.2 deletions and duplications are not fully penetrant and some individuals are largely asymptomatic, though 16p11.2 deletions appear to be more deleterious than duplications [Bijlsma et al., 2009; Cooper et al., 2011; Fernandez et al., 2010; Glessner et al., 2009; Hanson et al., 2010; Kumar et al., 2008; Rosenfeld et al., 2010; Rosenfeld et al., 2013]. Several genes in the 16p11.2 region have been implicated in autism, including *SEZ6L, MAPK3*, and *KCTD13* [Blumenthal et al., 2014; Golzio et al., 2012; Konyukh et al., 2011; Kumar et al., 2009; Schaaf et al., 2011].

Two mouse models of 16p11.2 heterozygous deletions (+/ −) were independently generated [Horev et al., 2011; Tian et al., 2015; Portmann et al., 2014].+/− of both lines exhibited low body weight, perinatal mortality, increased spontaneous locomotor activity in a novel home cage environment, and sporadic motor stereotypies. In addition, we observed that juvenile and adult +/− males and females exhibited normal general health, neurological reflexes, responses to social and nonsocial odors, motor learning, normal social approach, and normal juvenile reciprocal social interaction [Portmann et al., 2014]. We further detected an inability to swim, hypoactivity during the first 5 min in an open field, and a deficit in novel object recognition in multiple cohorts across two testing sites [Portmann et al., 2014].

Mice emit vocalizations in the ultrasonic range in response to olfactory and other sensory cues from a social partner [Whitney & Nyby, 1979; Byatt & Nyby, 1986; Malkesman et al., 2010; Roullet, Wohr & Crawley, 2011]. Here, we investigated another phenotype of potential relevance to autism, ultrasonic vocalizations (USVs) in social settings, in 16p11.2 deletion mice, as well as sensory phenotypes and anxiety-related behaviors, to better

understand the consequences of 16p11.2 deletions in mice. We recorded USVs from male +/
− and their +/+ littermates when exposed to a novel estrous female, when the female was
removed, and subsequently when the same female returned, to evaluate responses to the
presence and absence of a social partner [Hanson & Hurley, 2012; Yang, Loureiro,
Kalikhman, & Crawley, 2013]. Considerable evidence indicates that the USVs detected
during male–female interaction are emitted by the male rather than the female [Wang, Liang,
Burgdorf, Wess, & Yeomans, 2008; White, Prasad, Barfield, & Nyby, 1998; Whitney, Coble,
Stockton, & Tilson, 1973; Sugimoto et al., 2011]. A dramatic reduction in vocalizations by
young adult +/− males first presented with a female, and fewer vocalizations in response to
social pheromones in female urine on an inanimate cotton swab may indicate deficits in
perceiving the presence of, or responding to, salient social cues.

Materials and Methods

All procedures were approved by the Institutional Animal Care and Use Committees of the
National Institute of Mental Health Intramural Research Program, University of California
Davis, and Washington State University Vancouver. Generation of the 16p11.2 deletion mice
(contributed to The Jackson Laboratory, stock number 025100) was previously described
[Portmann et al., 2014]. Breeding pairs were imported from Stanford University to NIMH to
generate Cohort 1, and subsequently rederived at UC Davis to generate Cohorts 2 and 3.
16p11.2 deletion and wildtype littermate control mice were tested on a battery of behavioral
assays. Behavioral findings in the +/− mice, described in a recent publication [Portmann et
al., 2014], included low pup survival, low body weights, absence of acoustic startle response,
and impaired novel object recognition, along with normal scores on juvenile reciprocal
social interactions, three-chambered social approach, self-grooming, grip strength, general
health and neurological reflexes, rotarod motor learning, novel home cage activity, and
olfactory habituation/dishabituation. Here, we evaluate USVs emitted by 16p11.2 mice in
response to social cues in three paradigms: (1) our novel three-phase male-female social
interaction task, (2) the female urine sniff test, and (3) separation-induced USVs in pups. To
evaluate sensory, motor, and anxiety-like phenotypes that could contribute to low
vocalization responses to social cues, we evaluated anxiety-like behaviors, 60-min open field
activity, nociceptive responses, olfactory habituation/dishabituation, functional hearing, and
conducted an in-depth analysis of motor stereotypies including circling and back-flipping
behaviors. Only male subjects were tested for adult USVs in response to female cues,
because adult mice rarely vocalize during same-sex interaction without a prolonged period
of isolation (3–7 days) before the experiment [Nakagawa, Matsunaga, & Okanoya, 2012;
Scattoni, Ricceri, & Crawley, 2011], and our subjects were group-housed by sex. All other
tests used animals of both sexes. Supporting Information describes detailed methods on
breeding strategy, genotyping methods, testing procedures for each behavioral assay, and
statistical analyses. Behavioral methods used in this study were comprehensively described
in previous publications [Chadman et al., 2008; Yang & Crawley, 2009; Silverman et al.,
2011; Yang, Silverman & Crawley, 2011; Brielmaier *et al.*, 2012; Ey et al., 2012; Yang et al.,
2012, 2013; Portmann et al., 2014]. The auditory brainstem response (ABR) assay to
measure hearing sensitivity to pure tones across a wide frequency range was conducted in a
similar manner as previously described [Mahrt, Perkel, Tong, Rubel, & Portfors, 2013].

Results

USV call numbers and behaviors in the three-phase male– female social interaction test

The present experiments used male subjects for adult USVs in response to female cues, a well-established standard paradigm to elicit large numbers of calls [Ey et al., 2012; Holy & Guo, 2005; Scattoni et al., 2011].

We examined USVs in +/− mice and control +/+ littermates to determine if the deletion alters this form of response to social cues. Our novel three-phase male– female interaction paradigm consists of a 5-min Phase 1 in which the male subject freely interacted with an unfamiliar female, a 3-min Phase 2 in which the female was absent, and a 3-min Phase 3 in which the same female returned [Yang et al., 2013]. Figure 1 illustrates genotype differences in call numbers in the three-phase male-female social interaction test. Cohort 1 +/− males emitted significantly fewer calls than +/+ males in Phase 1 (Mann–Whitney U test, U=135, P < 0.01) and Phase 2 (U=168, P< 0.05), but not in Phase 3 (U=236, NS) (Fig. 1A). Similar results were seen in Cohort 2 (Fig. 1B). +/− emitted significantly fewer calls than +/+ in Phase 1 (U=10, P< 0.01) and Phase 3 (U=2, P< 0.01). A similar but nonsignificant trend was detected in Phase 2 (U=44, P< 0.075). Figure 1C illustrates the time course of call emission in Cohort 1. +/− males produced fewer USVs than +/− males in min 1 (U=98, P< 0.01), min 2 (U=140, P< 0.01), min 3 (U=143, P< 0.01), min 4 (U=165, P< 0.05), and min 5 (U=167, P< 0.05). A significant effect of time was detected in +/+ (Repeated Measures ANOVA on ranks, P< 0.01), but not in +/− (NS). The observed reduction in call rate across minutes 3–5 justified the use of minutes 1–3 for subsequent scoring of call categories. Figure 1D illustrated a robust genotype difference in number of USVs during the female urine sniff test in which the male subject was presented with a cotton swab saturated with fresh female urine. +/+ emitted high numbers of calls when exposed to a cotton swab containing fresh urine from a +/+ female in estrus, whereas +/− emitted significantly fewer USVs than +/+ ($F_{1,20}$=7.38, P< 0.01). Almost no calls were emitted by either genotype in the 3-min pretest when the male was alone in the cage, indicating that environmental novelty is not a stronger trigger of vocalizations. These results from the female urine sniff test support an interpretation that impaired responsivity to social cues in +/− males is not limited to their response to live moving mice. See Figure 1 legend for statistical results.

Figure 2 illustrates genotype differences in social behaviors in the three-phase male–female social interaction test. In Cohort 1, +/− males exhibited less anogenital sniffing and less following than +/+ males during Phase 1 (Fig. 2A). When the female was returned after a 3-min absence (Phase 3), no genotype differences were found on any behavioral parameter (Fig. 2C). Similarly, Cohort 2 +/− males exhibited less anogenital sniffing and following than +/+ males in Phase 1 (Fig. 2B). In Phase 3, +/− males exhibited less following than +/+ males (Fig. 2D). Mounting attempts rarely occurred in this short test, and were not statistically analyzed. See Supporting Information, Table S1 for statistical results of Figure 2. Correlation analyses between behavioral and USV data were conducted in both cohorts (Cohort 1: 10 +/+ and 12 +/−; Cohort 2: 11 +/+ and 13 +/−). In two cohorts of +/+ and +/− tested, no significant correlations (data not shown) were found between number of USVs

and any behavioral parameter, indicating that social sniff and vocalizations are probably independent measures of response to social cues.

USV call categories

Figure 3 illustrates USV call categories and peak call frequencies during the initial male–female social interaction (Phase 1), after the female was removed (Phase 2), and when the same female was reintroduced (Phase 3), respectively. No significant genotype differences were found for peak call frequency in Phase 1 (Fig. 3A inset, $F_{1,21}=0.07$, NS), Phase 2 (Fig. 3C inset, $F_{1,11}=0.55$, NS), or Phase 3 (Fig. 3E inset, $F_{1,23}=2.37$, NS). Although +/− differed from +/+ in call numbers on some categories, males of the two genotypes displayed mostly similar call repertoires on relative percentages of calls in each category in the presence of the female, as illustrated by relative percentages (Fig. 3A, C, E). Relative percentages provide a more relevant quantification of call categories, since low numbers of total calls by +/− was responsible for the low of number of calls in each category. Physical ability of +/− to emit USVs of normal quantities and qualities is confirmed by the absence of genotype differences in call numbers in Phase 3. See Supporting Information, Table S2 for statistical results of call categories illustrated in Figure 3.

Pup USVs and body weights

Separation calls by mouse pups represent a different type of vocalization in a social setting. Pup calls may be more analogous to human infant crying than to adult social communication. We evaluated numbers and categories of vocalizations in +/+ and +/− pups separated from the nest for the 3-min test. To determine whether body weight affects calling, we weighed each animal after each separation session. In Cohort 1, no significant genotype differences were found on number of USVs emitted by pups tested on postnatal day 6 (P6) (Fig. 4A). A trend was seen for +/− pups to emit fewer USVs than +/+ littermates on P8 (Fig. 4B). In this cohort, +/− pups had significantly lower body weights than +/+ pups on P6, as previously reported [Portmann et al., 2014] and on P8 (data not shown). In Cohort 2, a significant main effect of genotype was detected across days, with +/− pups emitting significantly fewer USVs than +/+ pups on P6. A significant main effect of genotype was found for body weight (Fig. 4E), with +/− pups having lower body weights than +/+ pups on P6, 8, 10, and 12. Similarly, in Cohort 3, a significant main effect of genotype was found for number of USVs across days (Fig. 4D). Posthoc comparisons indicated that +/− pups emitted fewer calls than +/+ pups on P6. A significant main effect of genotype was again found for body weight (Fig. 4F). +/− pups weighed less than +/+ pups on P6, 8, 10, and 12. These results indicate that the 16p11.2 deletion affected number of calls in separated mouse pups on P6 in two of three cohorts. As previously reported [Portmann et al., 2014], +/− pups displayed reduced body weights. Figure G and H illustrate call repertoires and peak call frequencies in 8-day old pups. Peak call frequency was significantly higher in +/− pups than in +/+ pups (Fig. 4G inset, $F_{1,21}=15.4$, $P<0.01$). Genotype differences were found in three call categories: Downward, Short, and Frequency steps. Call category differences in pups, at a developmental time point when the pinna of the ear remains closed and presumably mouse pups are not yet able to hear [Ehret 2005; Grimsley, Monaghan, & Wenstrup, 2011], will be interesting to explore further. See Supporting Information, Table S3 for statistical results of Figure 4.

Acoustic startle and prepulse inhibition

The ability to hear sounds in the human hearing range was assayed in the acoustic startle response test that measures flinching responses following a loud sound. As in our previous study [Portmann et al., 2014], +/− exhibited essentially no startle response at any decibel level, while +/+ responded to acoustic startle tones normally (Fig. 5A, $F_{1,22}$=22.06, P< 0.01). Results of prepulse inhibition indicated normal sensory gating in +/+ mice (Fig. 5B). Because +/− mice are deaf (see below), prepulse inhibition data of +/− do not reflect sensorimotor gating ability and are included for illustrative purposes only.

16p11.2 +/− have no ABRs across a wide range of frequencies

The absence of acoustic startle responses in +/− mice indicated the possibility of deafness, and prompted us to conduct comprehensive analysis using more broad test of hearing sensitivity based on auditory brain stem responses (ABR) to pure tones across a wide frequency range. Figure 6 illustrates ABR thresholds to pure tones between 8 and 100 kHz in +/+ and +/− tested at 6–7 weeks (young) and 27–32 weeks (middle-aged). In young +/+, the minimum threshold was 12 kHz (21 ± 6 dB SPL) and the maximum threshold was 32 kHz (71 ± 18 dB SPL). These mice did not have any evoked brainstem responses above 32 kHz. Similar results were found in the middle-aged +/+ mice with the exception that the thresholds were significantly higher in the middle-aged mice than in the young mice (12 kHz; 27 ± 2 dB SPL and 32 kHz; 93 ± 2 dB SPL, Kruskal–Wallis, P< 0.01). These higher thresholds are consistent with documented age-related hearing loss in C57BL/6N mice [Kane et al., 2012], the predominant genetic background in the 16p11.2 line. The ABR thresholds of +/+ were similar to previously reported ABR thresholds of C57B6/6J and other normal hearing strains [Kane et al., 2012; Zheng, Johnson, & Erway 1999]. None of the young or middle-aged +/− displayed any evoked ABR to any frequencies presented at the maximum intensity, indicating complete deafness.

Pain sensitivity and olfaction

In the hot plate test, latency to respond to a 55°C heat stimulus was significantly longer in +/− than +/+, indicating reduced sensitivity to thermal pain. Consistent results were found in Cohort 1 ($F_{1,27}$=9.4, P< 0.01) and Cohort 2 ($F_{1,23}$=5.5, P< 0.05) (Fig. 7A,B). No genotype differences were found on response latency in the tail flick test in Cohort 1 ($F_{1,25}$=0.41, NS) or Cohort 2 ($F_{1,21}$=0.36, NS) (Fig. 7C,D). Since tail flick is primarily a spinal reflex, while hot plate responses involve cortical regions [Pastoriza, Morrow, & Casey, 1996; South & Smith, 1998], the 16p11.2 deletion may affect central processing of pain perception, of relevance to unusual sensory responsiveness in some cases of autism [Elwin, Ek, Schroder, & Kjellin, 2012; Moore 2014]. As in our previous study [Portmann et al., 2014], olfactory habituation/dishabituation responses to sequentially presented nonsocial (water, banana, and vanilla) and social odors (from two cages of sex-matched novel mice) were normal in 16p11.2 deletion mice (Fig. 7E). See Supporting Information, Table S5 and Figure 7 legend statistical results.

Home cage behaviors

No unusual sleeping, huddling, nesting, feeding, or aggressive behaviors were seen during observations of home cages in the vivarium room at three different times of the day (data not shown), indicating that the 16p11.2 deletion did not affect easily observable home cage behaviors.

Anxiety-like behaviors

Figure 8 illustrates results of the elevated plus-maze and light ↔ dark exploration tests of anxiety-like behaviors in two cohorts of 16p11.2 mice. In the 5-min elevated plus-maze test (Fig. 8A–F), +/− exhibited low levels of overall exploratory activity and normal or lower levels of anxiety-like behaviors. In the 10-min light ↔ dark exploration test (Fig. 8G–L), +/− exhibited normal exploratory activity and normal or lower levels of anxiety-like behaviors. See Supporting Information, Table S6 for statistical results of Figure 8.

Open field exploration

Two cohorts were tested for locomotor activity in a novel open field across a 60-min session. As shown in Figure 9, In Cohort 1, one out of 15 +/− exhibited high levels of repetitive circling. None of the +/+ exhibited any circling or other stereotypic behaviors. With data from the +/− circler included, there were no genotype differences on any measures across 60 min. Similarly, in Cohort 2, one out of 15 +/− exhibited circling. See Supporting Information, Table S7 for statistical results of Figure 9. Unlike in cohort 1, +/− in Cohort 2 had increased center time as compared to +/+. The combined results of the two cohorts indicate normal open field exploratory activity across 60 min in +/−, although in both cohorts there was a trend for +/− to travel less during the first 5 min, similar to our previous findings [Portmann et al., 2014].

Motor stereotypies

In agreement with our previous report [Portmann et al., 2014], when placed in a novel empty cage for 60 min, a small number of +/− displayed stereotypic circling and backflipping (Table 1).

The same individual +/− consistently displayed the same stereotypies across ages in most but not all cases. No +/+ exhibited circling or backflipping at any age (data not shown). These quantitative results indicate that motor stereotypies are a consequence of the 16p11.2 deletion, but represent a trait with low penetrance.

Adult body weights

+/− weighed significantly less than +/+ littermates at 6 weeks (+/+, 22.1 ± 0.70 g; +/−, 16.4 ± 0.61 g; $F_{1,62}=36.7$, $P < 0.01$), 8 weeks (+/+, 24.3 ± 0.76 g; +/−, 17.1 ± 0.86 g; $F_{1,58}=35.7$, $P < 0.01$), and 10–12 weeks (+/+, 26.9 ± 0.91 g; +/−, 19.4 ± 0.94 g; $F_{1,45}=31.1$, $P < 0.01$). Low body weights in 16p11.2 deletion mice were seen in previous cohorts [Portmann et al., 2014], consistently from birth throughout development. No significant correlations were detected between body weight and USV numbers in +/− ($r=0.24$, NS), supporting an

interpretation that decreased vocalization number was not a direct result of lower body weight.

Discussion

Individuals with 16p11.2 deletions have a high incidence of language impairment, commonly manifested as delayed onset of expressive language, complete lack of spoken language, low verbal skills, and difficulties in articulation [Bijlsma et al., 2009; Ciuladaite et al., 2011; Hanson et al., 2010; Raca et al., 2013; Schaaf et al., 2011; Shinawi et al., 2010; Weiss et al., 2008]. We investigated vocalizations in 16p11.2 heterozygous deletion mice, to test the hypothesis that vocal emissions would similarly be unusual in the mouse model of 16p11.2 deletion syndrome. Employing a novel paradigm of social interaction, separation, and reintroduction [Yang et al., 2013], we discovered that young adult male heterozygous 16p11.2 deletion mice displayed moderately reduced social interactions and strikingly reduced USVs during the initial interaction session with an unfamiliar estrous female. These results suggest that 16p11.2 deletion males are less responsive to social cues when first encountering female conspecifics. Other major findings are sensory deficits (deafness and increased pain threshold) in +/− mice. Anxiety-like behaviors and open field exploration appear largely normal in +/− mice.

One possible explanation for the low number of emitted USVs in adult +/− males is their inability to hear the female. However, existing literature and current data do not support this interpretation. First, females generally do not vocalize during male–female interactions [Sugimoto et al., 2011; Wang et al., 2008; White et al., 1998; Whitney et al., 1973]. Second, genotype differences in call numbers (+/− males emitted significantly fewer USVs than +/+ males) were found during both male–female interaction and the female urine sniff test, suggesting that social vocalization deficits in +/− males are not solely due to their inability to hear USVs or movements of a live female partner. Males of both genotypes emitted markedly more calls when interacting with a live female than when sniffing female urine alone, suggesting that motion, visual, and tactile cues could contribute to the salience of social cues in mice. Further, low USV numbers in +/− males are not due to an inability to vocalize, because both genotypes in Cohort 1 emitted similar numbers of USVs when the female returned in Phase 3, indicating that the mutation did not disable the vocal apparatus, nor directly impair other physical abilities required to emit USVs. Normal levels of vocalizations during Phase 3 may, therefore, reflect improved social cue detection, interest, and/or responsiveness after the olfactory and visual cues of the female mouse became familiar. Alternatively, the lack of genotype difference in Phase 3 may be due to a lack of habituation to familiar social cues in +/−, which could indicate a social memory deficit.

A genotype difference in call number was detected in Cohort 2 during Phase 3. The slight discrepancy between Phase 3 data of the two cohorts may be attributable to the fact that Cohort 2 males had a brief 5-min pretest experience with females, whereas Cohort 1 did not. No correlations were detected between social sniffing and call numbers, in either genotype, indicating that USV emission and social sniffing are independent response traits evoked by social stimuli. Our previous study [Portmann et al., 2014] presents findings indicating that +/− have normal olfactory habituation/dishabituation to nonsocial and social odors and have

normal vision, suggesting that social vocalization deficits in +/− males are not attributable to impaired olfaction or vision. Body weight was lower in +/−, but there were no correlations between body weights and call numbers in either genotype, indicating that low body weight does not impair the ability to emit USVs.

Analysis of qualitatively different call types revealed few category differences between genotypes. Focusing on percentages of call categories, a more accurate representation than number of calls when the totals are low, we discovered that the deaf +/− and hearing +/+ males differed in only one category, Short, in Phase 1, only in the Upward category in Phase 2, and none in Phase 3. These results indicate that the deaf +/− and hearing +/+ had similar call repertoires and lend support to the suggestion that auditory experience may not have a crucial role in regulating USV in mice [Mahrt et al., 2013]. In pups tested at an age before hearing onset [Ehret 2005; Sonntag, Englitz, Kopp- Scheinpflug, & Rubsamen, 2009], genotype differences were found in both percentage and absolute number of Downward, Short, and Frequency Steps calls, an intriguing finding that may be worth further investigation.

One of the most striking findings in this study is the complete deafness in our line of +/− mice. We replicated our previous finding that adult +/− are deaf to low frequency sounds [Portmann et al., 2014] and further demonstrated that the +/− mice are deaf to high frequency tones as well. In contrast, the Mills line of 16p11.2 deletion mice [Horev et al., 2011] appear to display normal hearing in an operant task with an auditory contingency (personal communication, Anne Churchland, Cold Spring Harbor Laboratory, Ted Abel, University of Pennsylvania). Differences between the present Portmann/Dolmetsch line and the Mills line may be related to differences in background strain and to the precise length of the deletion.

To fully understand the extent of hearing loss, and because the B6 background strain is known to display an age-related hearing loss, we evaluated 16p11.2 mice at two ages. At both young and middle ages, +/− mice failed to display an ABR to stimulus tones in the 8−100 kHz frequency range, consistent with their lack of acoustic startle responses. In contrast, young +/+ mice displayed ABR thresholds up to 32 kHz at levels comparable to young B6 mice [Kane et al., 2012; Zheng et al., 1999]. It may prove fruitful to explore interactions between genes in the 16p11.2 deletion sequence and B6 background genes that may regulate hearing at young ages in our line, and in comparison to the genetic background of the Mills 16p11.2 deletion line. Since deafness is rare in human 16p11.2 deletion syndrome, with only six cases of hearing loss reported [Schaaf et al., 2011; Shinawi et al., 2010; Rosenfeld et al., 2010], species differences are likely to affect the consequences of the 16p11.2 deletion on hearing.

Absence of ABR responses to high frequency tones in +/+ mice does not imply that they cannot detect USVs. Minimal or no ABR responses in +/+ mice to frequencies within the range of social USVs is comparable to single neuron electrophysiological results in the auditory midbrain [Portfors & Roberts, 2014]. Very few neurons in the mouse auditory system respond to pure tones greater than 60 kHz, yet neurons that respond to lower frequency tones do respond to the USVs that contain energy in the 60 kHz and higher

frequency range [Holmstrom Eeuwes, Roberts, & Portfors, 2010 ; Portfors, Roberts, & Jonson, 2009; Portfors & Roberts, 2014]. Electrophysiological and modeling experiments suggest that the low frequency neurons respond to combinations of frequencies in the USVs that create cochlear distortions [Portfors & Roberts, 2014; Portfors et al., 2009]. Thus, the finding that the mice in this study did not have ABRs to frequencies greater than 32 kHz was expected, but it does not imply that the +/+ mice cannot detect the USVs emitted during social interactions. In addition, it has been shown that normal hearing CBA/CaJ mice can discriminate different USVs [Neilans, Holfoth, Radziwon, Portfors, & Dent, 2014].

Multiple behavioral phenotypes were evaluated in this study, to identify potential artifacts that could conceivably influence social behaviors and USVs. Motor stereotypies were observed in a small number of adult +/− mice, consistent with previous reports on our Portmann–Dolmetsch line and in the Mills line of 16p11.2 deletion mice [Horev et al., 2011; Portmann et al., 2014]. A higher threshold for a thermal nociceptive response in +/− mice may reflect unusual responsiveness to pain. Motor stereotypies and unusual pain response are additional phenotypes with potential face validity to symptoms of 16p11.2 deletion syndrome, particularly in cases diagnosed with autism [Lord et al., 2006]. No consistent genotype differences were detected across parameters on two anxiety-like tasks or on summed open field parameters.

Generally normal scores on anxiety-related, exploration and olfactory tests reported here, and previously [Portmann et al., 2014] indicate that responses to novelty, conflict, and sensory detection of social and nonsocial odors are similar across genotypes, and therefore, unlikely to be responsible for the vocalization differences during social interactions. Moderately reduced adult reciprocal social interactions in the present two cohorts of adult 16p11.2 mice, in contrast to the lack of genotype differences we previously reported for juvenile reciprocal social interactions and 3-chambered social approach in adult 16p11.2 mice [Portmann et al., 2014], suggest that the 16p11.2 deletion may have selective effects on responsiveness to highly salient social cues such as female pheromones. Reduced vocalization responses to social cues in 16p11.2 deletion mice, along with their sensory abnormalities, well-replicated sporadic motor stereotypies, and a previously reported cognitive deficit [Portmann et al., 2014], thus recapitulate features of several symptoms of the 16p11.2 deletion syndrome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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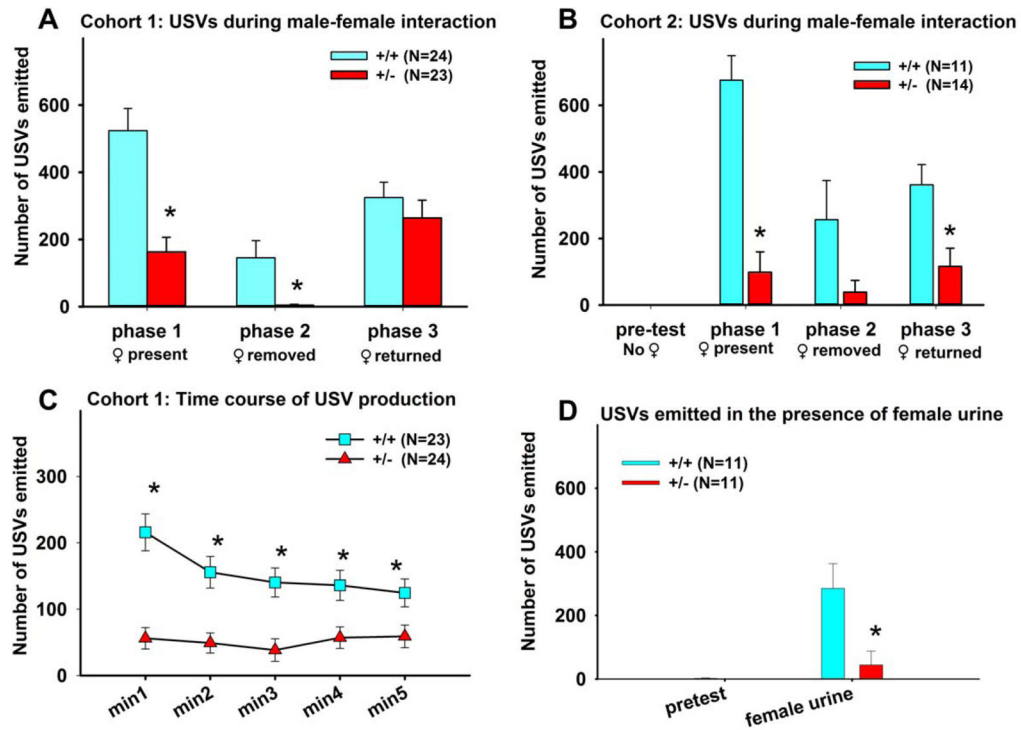
Figure 1.
Reduced numbers of ultrasonic vocalizations in +/− males in the three-phase male–female social interaction test and the female urine sniff test. (A) In Cohort 1, male heterozygotes (+/−) emitted fewer USVs than wildtype littermates (+/+) during the initial male–female social interaction session (Phase 1) and after the female was removed (Phase 2). USV numbers in +/− and +/+ were similar during the second interaction session when the female was reintroduced (Phase 3). (B) In Cohort 2, no calls were emitted by any mice of either genotype during a 3-min pretest period when the subject was alone in the test cage, confirming the specificity of USVs to the presence of a social partner in our paradigm. +/− males again emitted fewer USVs in Phase 1, with a nonsignificant trend for fewer calls in Phase 2, and fewer calls in Phase 3. (C) Time course of call emission in Cohort 1. +/− produced fewer USVs than +/+ in minutes 1–5. A significant effect of time was detected in +/+, but not in +/−. Data are presented as mean ± standard error of the mean. *, $P < 0.05$ +/+ vs. +/−. (D) +/− males emitted fewer USVs than +/+ males in the presence of fresh urine from a +/+ non-littermate female in estrus, provided on a cotton swab, in a 3-min test session. *, $P < 0.05$ +/+ vs. +/−.
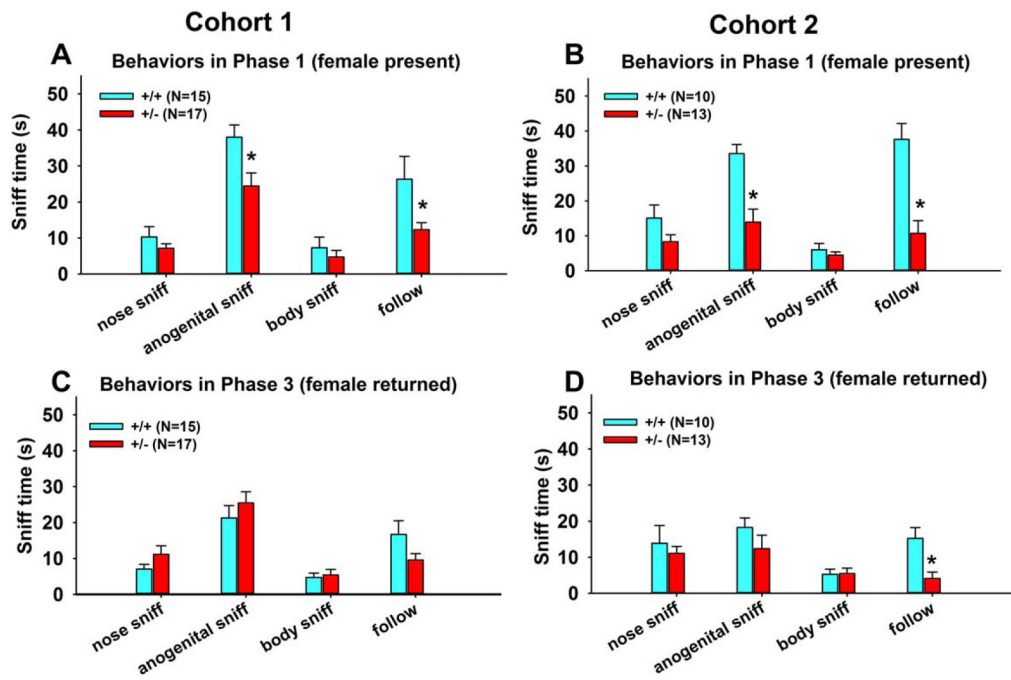
Figure 2.
Reduced social sniffing in 16p11.2 +/− males in the three-phase male-female social interaction test. (A) Cohort 1 +/− males exhibited less anogenital sniffing and following, as compared to +/+ males, during the first male-female interaction session (Phase 1). (C) No genotype differences were detected during the second exposure to the female (Phase 3). (B and D) Cohort 2 +/− males exhibited less anogenital sniffing and less following than +/+ males in Phase 1, and less following in Phase 3. None of the individual social interaction parameter scores was significantly correlated with total numbers of USVs, indicating that social sniffing and vocalization are probably independent measures of response to social cues. Data are presented as mean ± standard error of the mean. *, $P < 0.05$ +/+ vs. +/−. See Supporting Information, Table S1 for statistical results of Figure 2.
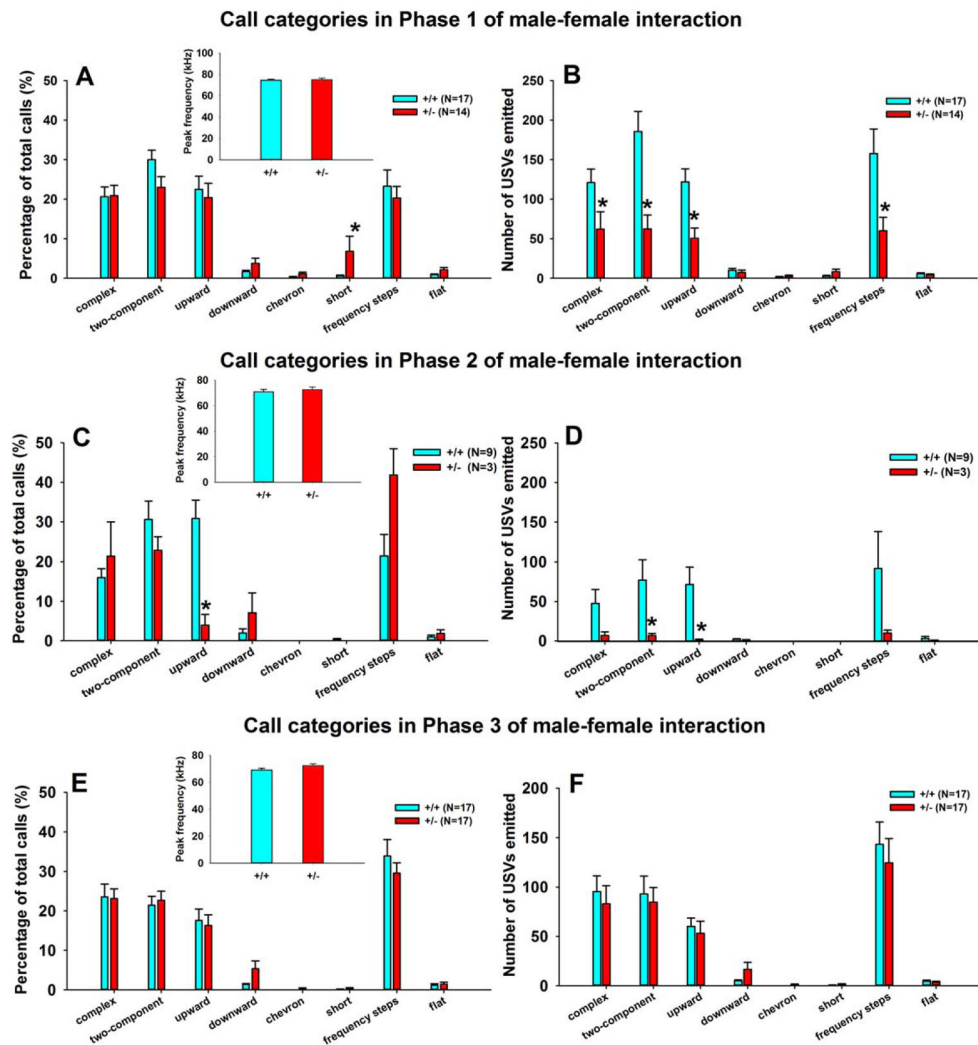
Figure 3.

Call categories of USVs emitted during Phase 1 (the first exposure to a novel estrous female), Phase 2 (after the female was removed from the interaction cage), and Phase 3 (when the female returned). In all three phases, +/+ and +/− differed in call numbers but displayed generally similar repertoires. (A, C, E) Analysis of percentages of call types indicated that +/− had a higher percentage of Short than +/+ in Phase 1, a lower percentage of upward in Phase 2, and did not differ from +/+ in Phase 3. (B, D, E) Analysis of number of calls in each category indicated that +/− emitted fewer Complex, Two-component, Upward, and Frequency steps as compared to +/+ in Phase 1, fewer Two-component and Upward in Phase 2, and did not differ from +/+ in Phase 3. Only Cohort 1 male subjects that emitted USVs were included in call category analysis. Out of a total of 23 +/− in Cohort 1, 14 called in Phase 1, 3 in Phase 2, and 17 in Phase 3. Out of a total of 24 +/+ in Cohort 1, 17 called in Phase 1, 9 in Phase 2, and 17 in Phase 3 (see Supporting Information, Table S8). No significant genotype differences were found for peak call frequency in Phase 1 (A inset), Phase 2 (C inset), or Phase 3 (E inset). Data are presented as mean ± standard error of the

mean. *, $P<.05$ +/ + vs. +/−. See Supporting Information, Table S2 for statistical results of Figure 3.
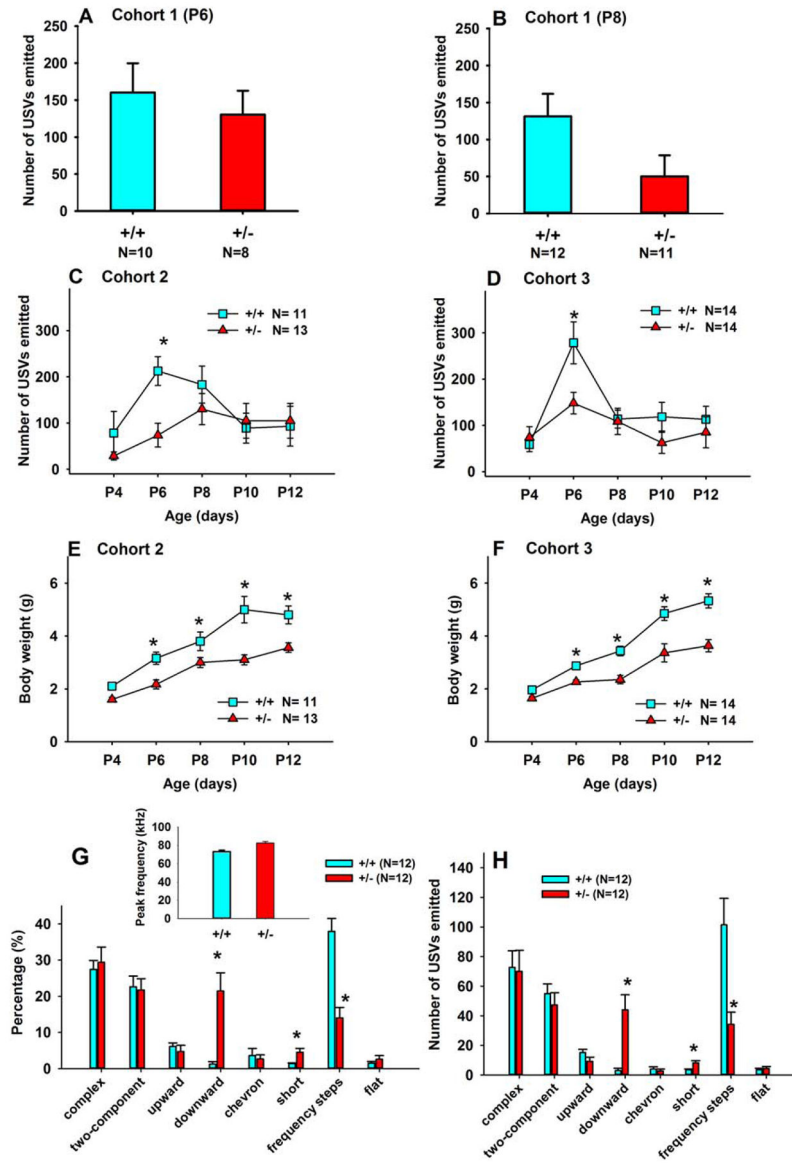
Figure 4.
Pup vocalizations and body weights. (A, B) Pups from Cohort 1 were tested on postnatal days 6 and 8. A trend was detected for +/− pups to emit fewer USVs as compared to +/+ littermates on P8. (C) In Cohort 2, a significant genotype effect was found across P4, 6, 8, 10, and 12, with +/− emitting fewer USVs than +/+ littermates on P6. (D) Similarly, in Cohort 3, a significant genotype effect was found across days, with +/− emitting fewer USVs than +/+ on P6. (E–F) Consistent results from multiple cohorts indicated that +/− weighed less than +/+ across days, with genotype differences being significant on P6, 8, 10, and 12. (G, H) +/− and +/+ pups differed in three call categories: Downward, Short, and Frequency steps. Peak call frequency was higher in +/− than +/+ (Panel G inset). Data are presented as mean ± standard error of the mean. *, $P < 0.05$ +/+ vs. +/−. See Supporting Information, Tables S3 and S4 for statistical results of Figure 4.
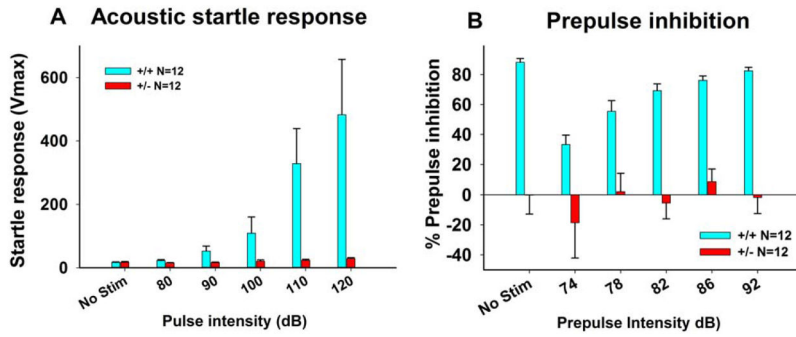
Figure 5.
Acoustic startle response and sensorimotor gating in 16p11.2 mice. (A) +/+ showed normal acoustic startle responses, whereas a complete absence of startle response to all decibel levels was seen in +/−, replicating our previous findings on deafness in +/− [Portmann et al. 2014]. (B) +/+ exhibited normal prepulse inhibition. Because the +/− mice are deaf, prepulse inhibition data of +/− do not reflect sensory gating ability, and are included for illustrative purposes only. Data are presented as mean ± standard error of the mean.
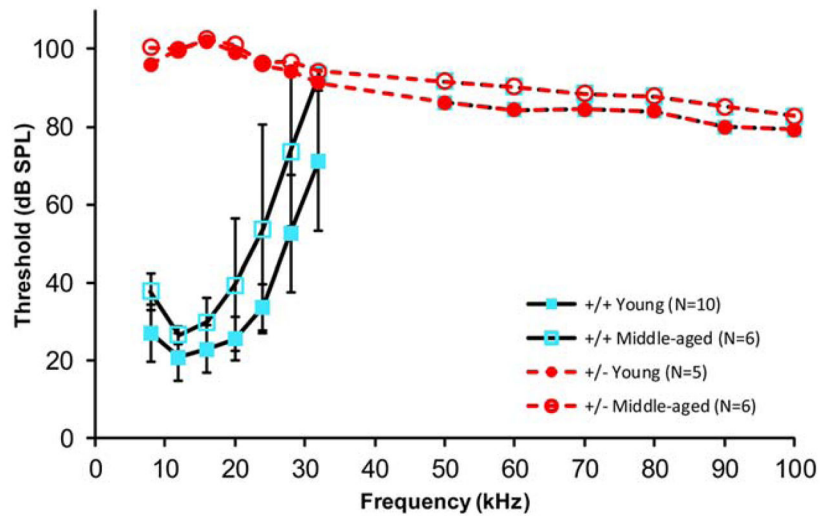
Figure 6.
Deafness was confirmed by auditory brainstem response (ABR) test. ABR thresholds to pure tones between 8 and 100 kHz in +/+ and +/− tested at 6–7 weeks (young) and 27–32 weeks (middle-aged). The dashed lines indicate that no brainstem response was detected at maximum output intensity. Differences between dashed lines are due to differences in maximum output of the two speakers used in experiments with the different aged mice. In both young and middle-aged +/+, the minimum threshold was detected at 12 kHz and the maximum threshold at 32 kHz. Tones 50 kHz and higher did not elicit any response in +/+ of any age, even at the maximum intensity (dashed lines). Middle-aged +/+ had significantly higher thresholds than young +/+ to tones of all frequencies, indicating age-related hearing loss in +/+, consistent with the B6 genetic background. No young or middle-aged +/− displayed evoked responses at any frequencies presented at maximum intensity (dashed line), indicating complete deafness in all +/. Error bars indicate standard deviations.
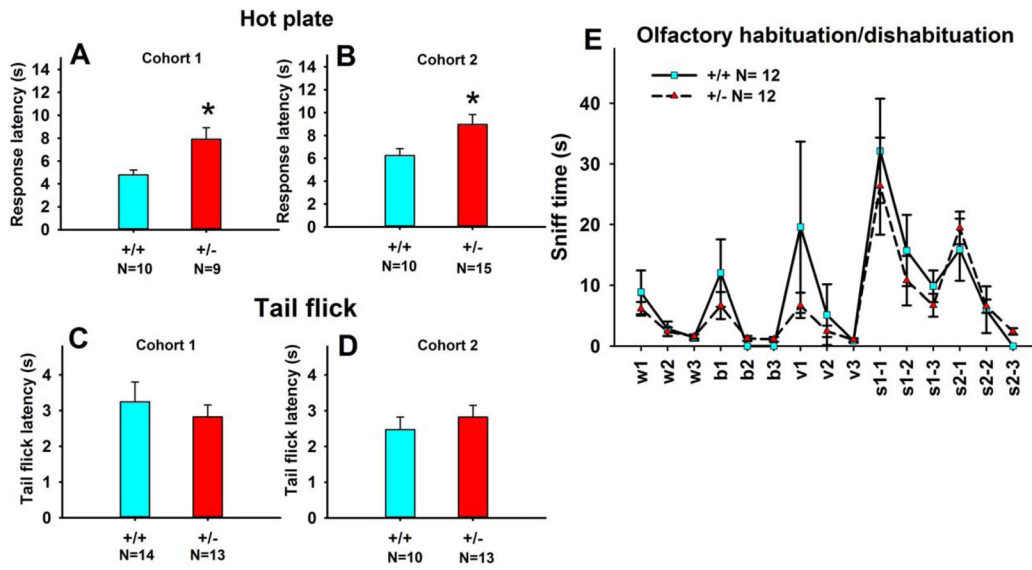
Figure 7.
Reduced pain sensitivity and normal olfaction in +/− mice. (A–D) +/− mice exhibited increased latency to respond in the hot plate test, which is dependent on higher brain function, but not in the tail flick test, which is a spinal reflex, suggesting a small reduction in pain sensitivity. Consistent results were detected in two cohorts. (E) Both +/− and +/+ exhibited significant habituation and dishabituation responses to sequentially presented non-social and social odors, indicating a normal ability to differentiate odors in both genotypes. This result was consistent with our previous finding [Portmann *et al.* 2014]. Data are presented as mean ± standard error of the mean. *, $P < 0.05$ +/+ versus +/−. See Supporting Information, Table S5 for statistical results of Figure 7E.
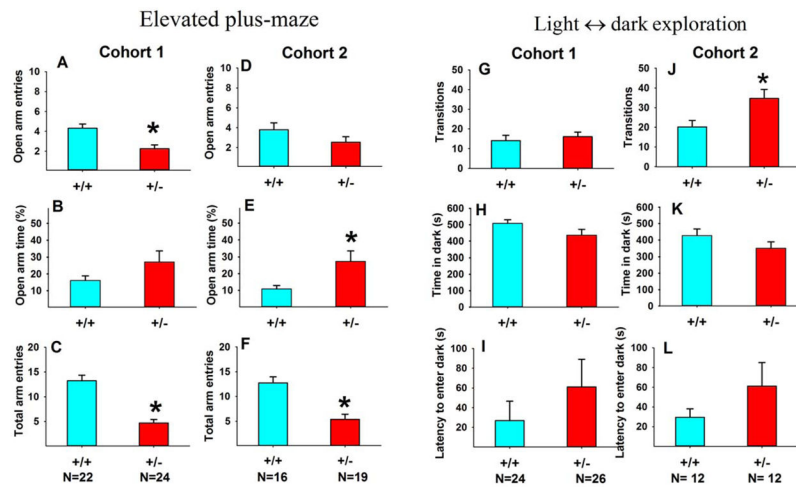
Figure 8.
No consistent anxiety-like phenotypes in 16p11.2 mice. In the elevated plus-maze test, +/− had normal open arm entries (A and D), normal or higher % open arm time (B and E), and markedly lower total arm entries than +/+ (C and F). Lower total arm entries indicated reduced exploratory activity which confounds the interpretation of an anxiety-like phenotype. In the light ↔ dark exploration test, +/− made normal or more transitions between chambers (G and J), and were not different from +/+ on time spent in the dark chamber (H and K) and latency to enter the dark chamber (I and L), indicating normal anxiety-like behaviors. Results on several measures from two anxiety-related tasks indicate a generally normal anxiety profile in 16p11.2 deletion mice. Data are presented as mean ± standard error of the mean. *, $P < 0.05$ +/+ versus +/−. See Supporting Information, Table S6 for statistical results of Figure 8.
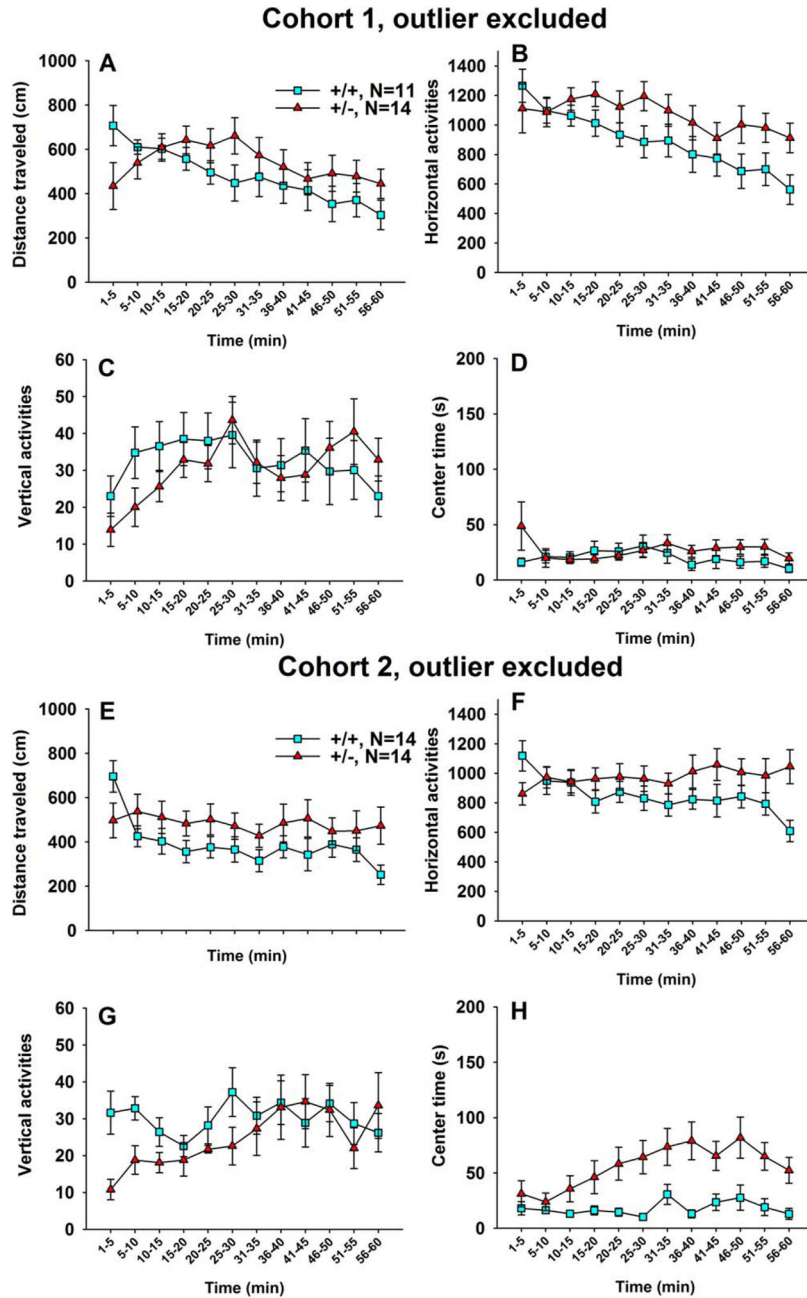
Figure 9.
Open field activity across a 60-min session revealed no genotype differences in locomotor activity. (A–D) In Cohort 1, no significant genotype differences were found overall on total distance traveled, horizontal activity, vertical activity, and center time. A trend was seen for +/− mice to be more active than +/+ mice, but only when the one circling +/− mouse was included (data not shown). (E–H) In Cohort 2, no significant genotype differences were found overall on total distance traveled, horizontal activity, vertical activity. Center time was higher in +/− than in +/+. A trend was found for +/− to move less than +/+ in the first 5 min, consistent with our previous report [Portmann et al. 2014]. Data are presented as mean ±

standard error of the mean. See Supporting Information, Table S7 for statistical results of Figure 9.

Table 1

Repetitive Circling and Backflipping were Detected in a Small Number of +/− at 6, 8, and 10–12 Weeks of Age

Age	Cohort 1		Cohort 2	
	High circling	**High backflipping**	**High circling**	**High backflipping**
6 weeks	0/33	0/33	1/40	1/40
8 weeks	1/33	2/33	0/36	2/36
10–12 weeks	1/30	3/30	0/27	1/27

The same individuals generally exhibited motor stereotypies consistently across ages. No +/+ exhibited repetitive circling and backflipping at any age. Data are presented as the number of +/− exhibiting repetitive circling/backflipping out of total number of +/− tested.