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Comprehensive behavioral phenotyping of a 16p11.2 del mouse model for neurodevelopmental disorders

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Abstract

The microdeletion of copy number variant 16p11.2 is one of the most common genetic mutations associated with neurodevelopmental disorders, such as Autism Spectrum Disorders (ASDs). Here, we describe our comprehensive behavioral phenotyping of the 16p11.2 deletion line developed by Alea Mills on a C57BL/6J and 129S1/SvImJ F1 background (Del^m). Male and female Del^m mice were tested in developmental milestones as preweanlings (PND2-PND12), and were tested in open field activity, elevated zero maze, rotarod, novel object recognition, fear conditioning, social approach, and other measures during post-weaning (PND21), adolescence (PND42), and adulthood (>PND70). Developmentally, Del^m mice show distinct weight reduction that persists into adulthood. Del^m males also have reduced grasp reflexes and limb strength during development, but no other reflexive deficits whereas Del^m females show limb strength deficits and decreased sensitivity to heat. In a modified version of a rotarod task that measures balance and coordinated motor activity, Del^m males, but not females, show improved performance at high speeds. Del^m males and females also show age-specific reductions in anxiety-like behavior compared to WTs, but neither sex show deficits in a social preference task. When assessing learning and memory, Del^m males and females show age-specific impairments in novel object or

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Conflicts of Interest

The authors report no conflicts of interest.

spatial object recognition, but no deficits in contextual fear memory. This work extends the understanding of behavior phenotypes seen with 16p11.2 deletion emphasizing age and sex-specific deficits; important variables to consider when studying mouse models for neurodevelopmental disorders.

Lay Summary:

Autism spectrum disorder is a common neurodevelopmental disorder that causes repetitive behavior and impairments in social interaction and communication. Here, we assess the effects of one of the most common genetic alterations in ASDs, a deletion of one copy of 29 genes, using a mouse model. These animals show differences in behavior between males and females and across ages compared to control animals, including changes in development, cognition, and motor coordination.

Keywords

16p11.2; neurodevelopmental disorders; behavior; autism spectrum disorders; mouse model; phenotype; copy number variant

Introduction

Copy number variants (CNVs) are alterations of the genome through deletions, duplications, insertions, or translocations that span at least 1 kB. Many of these CNVs can increase susceptibility to neurodevelopmental disorders (Girirajan et al., 2012; Maillard et al., 2015; Migliavacca et al., 2015; Rosenfeld et al., 2010). CNVs have a higher odds ratio than single nucleotide polymorphisms, are rarer, and can be accurately modeled in mice, given the similarities between the human and murine genome, making them an important candidate for study.

One of the most common CNVs associated with Autism Spectrum Disorders (ASDs) is the 16p11.2 microdeletion (Weiss et al., 2008), which spans approximately 600 kilobases and ~29 genes (Arbogast et al., 2016). The genes within this region are involved in a wide range of cellular processes and some are individually implicated in ASDs (Fasano & Brambilla, 2011; Kalkman, 2012). ASDs are a set of neurodevelopmental disorders that cover a wide range of symptomatology with varying degrees of severity. The core symptoms include difficulty with communication and social interaction, restricted interests, and repetitive behaviors (American Psychiatric Association, 2013; Rivet & Matson, 2011).

Estimates suggest the microdeletion of the 16p11.2 region is found in 0.6% of individuals with ASDs, making it one of the most common genetic anomalies associated with ASDs (Hanson et al., 2010; Weiss et al., 2008). The strength of the association between the 16p11.2 microdeletion and neurodevelopmental disorders in patients makes it a compelling focus for further exploration. The syntenic 7qF3 region within the mouse genome is the highly conserved analog to the 16p11.2 region within humans, allowing for modeling of this CNV in mice. To date, 3 distinct mouse lines of 16p11.2 microdeletion CNV have been generated. These models have been analyzed for many behavioral and physiological

abnormalities (Arbogast et al., 2016; Horev et al., 2011; Yang, Mahrt, et al., 2015). Each model displays a distinct phenotypic profile, which may be attributed to differences in the deletion region, background strain, or specific methodology. One of the first mouse models generated, the Mills model (Del^m), carries a deletion of the Slx1b-Sept1 region, which includes 4 genes outside the human BP4-BP5 interval of the CNV on a C57BL/6J:129S1/SvImJ F1 background (Horev et al., 2011). A number of behavioral phenotypes have been described for these mice, by our lab and others (Angelakos et al., 2017; Arbogast et al., 2016; Grissom et al., 2017; Horev et al., 2011; V. Kumar et al., 2018; Nickl-Jockschat et al., 2015; Portmann et al., 2014; Pucilowska et al., 2018; Pucilowska et al., 2015; Tian et al., 2015; Yang, Lewis, Foley, & Crawley, 2015; Yang, Lewis, Sarvi, Foley, & Crawley, 2015; Yang, Mahrt, et al., 2015). However, the present study is the first to provide a comprehensive understanding of different behavioral phenotypes at different ages, with a major focus on preweanling (PND2–12), post-weaning (PND21), adolescent (PND42), and adulthood (PND70). Exploring these different developmental time points is of great import because much of the literature exploring animal models test adults and ASDs are neurodevelopmental disorders; the symptomology of ASDs can differ across development (Chow et al., 2012; Esbensen, Seltzer, Lam, & Bodfish, 2009; Ramsey et al., 2013; Schulte-Rüther et al., 2014; Shattuck et al., 2007). In addition to understanding the neural mechanisms underlying ASDs, research is also aimed at elucidating biological underpinnings of the robust prevalence of ASDs in males relative to females (American Psychiatric Association, 2013; Loomes, Hull, & Mandy, 2017). The reason for such a large sex-specific discrepancy remains unknown and requires more thorough investigation (Baron-Cohen et al., 2011; Begeer et al., 2013). The goal of the current set of behavioral experiments is to assess females and males to determine genotype differences for each sex.

Methods and Materials

Animals

Male 16p11.2 Del^m mice were procured from The Jackson Laboratory (Stock #013128) and bred with B6129SF1/J females (Stock#101043) from The Jackson Laboratory to generate experimental cohorts of littermates. The colony rooms were maintained between 18–24°C. Animals were maintained on a 12 hour light/dark cycle with light onset at 7:00 am. All animals had *ad lib* access to Purina 5001 rodent chow and tap water. All animals were cared for in accordance with the guidelines of the National Institutes of Health and procedures were approved by the University of Pennsylvania or the University of Iowa Institutional Animal Care and Use Committees and every attempt to minimize pain or discomfort in the animals was made. Experimenters were blind to genotype. Separate cohorts of littermates were used to investigate each early developmental milestone. All testing occurred during the first four hours of the light cycle, except for the social choice procedure, which occurred during the last four hours of the light cycle to spur locomotor activity. Animals were handled prior to procedures that can be affected by handling, such as anxiety-like behavior and fear behavior, to remove the fear of handling as a potential variable.

Behavioral data was collected at specific stages of development, including PND2–12 (pre-weanling) PND21 (pre-pubescence; +/-2 days), PND42 (pubescent; +/-4 days), and PND70

(young adult; ± 7 days). Upon weaning at PND21, same-sex, mixed-genotype littermates were housed 2–4 per cage on corn cob bedding with nestlet material. For each experiment, sample sizes are included in the figure legend, within the bar of each group, or in the x-axis label. For developmental milestone assays, sample sizes reflect number of *litters* rather than number of mice in an effort to minimize error when comparing multiple litters on developmental milestones (Festing, 2006), and each litter was assessed in 2 day increments between PND2–12 (See Supplementary Table S6–S11 for raw data for individual pups). See Supplemental Methods for more details.

Prewaning Developmental Milestones—For all pre-weaning milestone observations, *n* values indicate litter number (Festing, 2006). Tables with individual raw data across litters are included within the supplemental methods (Supplementary Table S6–11). Naturally occurring developmental milestones were assessed in preweanling pups including weight, length, fur development, pinnae detachment, eyelid opening, incisor eruption based on Bignami, Musi, Dellomo, Laviola, and Alleva (1994). Pups measured in developmental milestones were euthanized after the last measurement on postnatal day (PND) 12.

Neuromuscular Reflex Development

Pups were assessed for surface righting responses, negative geotaxis, cliff avoidance, grasp reflex, bar suspension, and screen suspension between PND2–12 (Fig 1).

Post weaning Behavior Assessments—Pups were assessed for various behavioral phenotypes starting at PND21 (Fig 2).

Motor and Sensory Function

Motor Functioning: Animals were assessed for grip strength, activity within an open field, high speed rotarod performance, and temperature sensitivity at PND21 (immediately post-weaning),

Arousal and Anxiety—Animals were assessed for acoustic startle response, habituation, and pre-pulse inhibition. Other cohorts were tested for anxiety-like behavior in the elevated zero maze, and time spent in the center of an open field.

Social Behavior and Cognition—A subset animals were assessed for social preference (Sankoorikal, Kaercher, Boon, Lee, & Brodtkin, 2006), novel and spatial object recognition, and fear conditioning as previously described (Hawk et al., 2012; Vecsey et al., 2007). For social preference behavior, mice were tested at PND30. This age was chosen as a midpoint between PND21 and PND42 because at PND21, mice do not ambulate at high levels or explore novelty robustly whereas PND42 mice may have been undergoing hormonal fluctuations during the maturation process.

Statistical Analyses

Behavior was analyzed using factorial analysis of variance (ANOVA) or independent t-test analyses when appropriate (Graphpad Prism 8). For repeated measures ANOVAs, the Geisser-Greenhouse epsilon was calculated to determine sphericity and the correction was

applied to the analysis. For two-way ANOVAs, the Spearman's test was conducted. If homoscedasticity was violated, as demonstrated by a significant χ^2 , data was log transformed and reanalyzed (noted in results where applicable). If transformed, analyses reported reflect those conducted on transformed data. However, graphical representations utilize raw values for easier interpretation of findings. All statistical tests are stated in the results. Importantly, our *a priori* intention was to assess the impact of genotype within each sex at different ages. Two-tailed Sidak *post hoc* analyses were conducted where appropriate. Statistical significance was set at $p < 0.05$.

See Supplemental Information for more detailed methodology.

Results

I. Pre-Weanling Developmental Milestones

To determine the physical well-being of the mice, we examined physical health, including body weight and other developmental milestones.

Physical Development

Body Weight: Del^m males had significantly lower body weight compared to WT^s ($p < 0.01$) with weight differences at PND 8 and PND10 ($p < 0.05$; Fig 3A). Del^m females do not have lower weight compared to WT females at any specific age although genotype was significantly different overall (Fig 3C). Body weight was also assessed after weaning through adulthood. Due to a significant Spearman's test ($R_s = 0.18$, $p < 0.01$), data were log transformed. Del^m males and females had lower weight compared to WT^s ($p < 0.001$) with differences at PND 21, 42, and 70 ($p < 0.001$; Fig 3B, 3D; Supplementary Table S1).

Body length, eye opening, and fur, ear, and incisor development: Pups were observed for developmental milestones from PND 2–12. Only male mutants displayed significantly reduced body length at PND10 ($p < 0.05$). No other genotype differences in body length were found in either sex across development (not significant [ns]; Supplementary Fig S1A; S1B), in the age when eyes open (ns; Supplementary Fig S2A; S2E), fur develops (ns; Supplementary Fig S2B; S2F), the pinnae develop (ns; Supplementary Fig S2C; S2G), or incisors erupt (ns; Supplementary Fig S2D; S2H; Supplementary Table S1).

Neuromuscular Reflex Development

Surface Righting Response: No differences were found in the latency of male (Supplementary Fig S3A) or female (Supplementary Fig S3E) Del^m mice to right themselves after being laid on their backs at any age tested. (Supplementary Table S2).

Negative Geotaxis: Male (Supplementary Fig S3B) and female (Supplementary Fig S3F) Del^m pups did not differ from WT^s at any age in the latency to orient themselves with head upward (Supplementary Table S2).

Cliff Avoidance: For cliff avoidance, Del^m and WT mice were tested for the time it takes to withdraw from the edge of a surface. Del^m males had significantly less cliff avoidance

($p < 0.05$), taking more time to retreat from the edge (Fig. 4A). Females had no genotypic differences in cliff avoidance (Fig. 4D; Supplementary Table S2).

Grasp Reflex: Grasp reflex was defined as the curling of toes around a paperclip when mildly pressed onto the plantar surface of the forepaws. Del^m pups had no delay in the development of grasp reflex (Fig 4B, 4E; Supplementary Table S2).

Bar Suspension: Del^m pups were also measured for time they could remain suspended from a T-bar. Analyses revealed no overall genotype differences in the ability to perform the task (data not shown). When analyzing the latency to fall, genotype had a significant effect of Genotype in males ($p < 0.05$; Supplementary Fig S3C), but not females (Supplementary Fig S3J). However, Sidak multiple comparisons showed no significant differences between genotype at any age, suggesting no differences in the amount of time spent holding on between Del^m and WT mice at any specific age (Supplementary Table S2).

Screen Suspension: For horizontal suspension, two female adolescent data points were removed as statistical outliers ($> 2 \text{ SD} \pm \text{mean}$). Del^m mice showed significantly reduced holding times compared to WT at PND4 ($p < 0.05$) and PND6 ($p < 0.01$; Fig 4C), but not at other ages, with no genotype differences between females (Fig 4F; Supplementary Table S2). For vertical screen suspension, ANOVAs showed no significant effect in males (Supplementary Fig S3D) or females (Supplementary Fig S3H; Supplementary Table S2).

II. Post weaning Behavior Assessment

Motor Functioning

Grip Strength: We measured the forepaw and hindpaw grip strength of Del^m and WT mice. Due to a significant Spearman's test ($R_s = 0.25$, $p < 0.01$), data were log transformed and a two-way ANOVA was conducted on the transformed data. Del^m males had significantly lower grip strength at all ages tested ($p < 0.05$; Fig 5A) and Del^m females had lower grip strength at PND21 and PND42, but not PND70 (Fig 5E; Supplementary Table S3).

Investigation of hindpaw grip strength (Fig. 5B) revealed significantly lower grip strength in Del^m males at PND21 and PND42 ($p < 0.001$), but not PND70 whereas Del^m females (Fig 5F) had significantly lower grip strength at PND21 and PND70, but not PND42, demonstrating significant impairments of grip strength in male and female mutant mice (Supplementary Table S3).

Spontaneous Activity in an Open Field: Animals were tested in the open field for 10-min and the distance travelled during the test was recorded and analyzed. At any age of testing, male and female Del^m mice did not differ from WT. (Supplementary Fig S4A–S4F; Supplementary Table S3). Overall, the distance travelled did not differ between genotypes for males or females at any age tested (PND70 data adapted from Angelakos et al., 2017).

High Speed Rotarod: Mice were analyzed on a rotarod that was modified to attain high speeds to detect repetitive motor activity and motor coordination. The low- and high-speed phases of the test were analyzed separately. At low speeds, male and female mutants were

not different than WT during any trial and each genotype improved equivalently across trials (Fig. 6A; 6C). However, during high speed trials, Del^m had significantly longer latencies than WT at Trial 2–5 ($p < 0.05$) in males (Fig 6B), but not in females (Fig 6D; Supplementary Table S3). Thus, male Del^m mice demonstrate enhanced performance at higher speeds compared to WT.

Sensory Functioning

Heat and Cold Sensitivity: For males, a Genotype x Age RM two-way ANOVA of the response of males to decreasing temperature (Cold Sensitivity; Fig. 5C) revealed a significant interaction ($p < 0.05$), but no differences between the groups. For increasing temperatures, A significant Spearman's test ($R_s = 0.25$, $p < 0.01$), resulted in doing a log transformation on the heat sensitivity data. A two-way ANOVA was conducted on the transformed data. No differences were seen in males (Heat Sensitivity; Fig. 5D). Females had no differences between genotype for Cold Sensitivity (Fig 5G) but a significant Genotype x Age interaction ($p < 0.01$) for Heat Sensitivity with Del^m females showing reduced sensitivity to heat compared to female WT mice at PND21 (Fig 5H; Supplementary Table S3).

III. Arousal and Anxiety

Del^m at different ages were tested for acoustic startle response (ASR) with specific intensities (dB) of white noise stimuli to generate an input/output response curve in addition to measures of prepulse inhibition (PPI) and habituation.

Acoustic Startle Response and Habituation—Habituation was investigated by comparing the responses to an initial block of 6, 120 dB stimuli to a subsequent block of 6, 120 dB stimuli delivered at the end of the procedure session. Independent t-tests revealed a significant effect of genotype at PND21 ($p < 0.05$) but no other ages in males (Supplementary Fig S6A–C) whereas females have no habituation differences between genotype (Supplementary Fig S6D–F).

Discrete stimuli intensities were used to test reactivity and verify that the neural circuitry required for the response was intact, as another model of the 16p11.2 deletion are deaf and cannot perform this task (Portmann et al., 2014). When analyzing input/output data, Del^m males showed ASR reactivity like their WT littermates, demonstrating intact hearing and neural circuitry (Fig 7A–C). For females, differences were only seen at PND70 ($p < 0.05$) although Sidak multiple comparisons revealed no significant Vmax responses in female Del^m mice across intensities (Fig 7D–F; Supplementary Table S4).

Prepulse Inhibition—No differences were seen in the no-prestimulation trials in any group ($p > 0.05$; data not shown). Del^m males had intact PPI (Fig 8A–C) whereas Del^m females had impaired PPI at PND21 compared to WT ($p < 0.05$; Fig 8D–F; Supplementary Table S4).

Anxiety-Related Behavior: Elevated Zero Maze and Open Field Center Activity

Elevated Zero Maze: In males, independent t-test analyses of the time spent in the open areas of the EZM across Genotype showed a trend towards increased time in the open areas at PND21 ($p=0.058$), but the effect was not significant at any age (PND70 data adapted from Angelakos et al., 2017; Supplementary Fig S5A–C). For females (Supplementary Fig S5G–I), independent t-test analyses across Genotype showed no effect of gene on time in the open area of the maze at any age for time spent in the open areas of the maze (Supplementary Table S5). No differences were seen in distance travelled in the EZM between genotypes for any age in either sex, ($p>0.05$; data not shown).

Open Field Center Activity: When tested in the open field, independent t-test analyses across Genotypes showed that males (Supplementary Fig S5D–F) did not differ in center activity at any age whereas Del^m females (Supplementary Fig S5J–L) spent significantly more time in the center than WT^s at PND70 ($p<0.01$), suggesting reduced anxiety-like behavior (Supplementary Table S5).

Social Behavior and Cognition

Social Approach: Cohorts of mice were tested for social approach at PND30 (juvenile). No genotype differences in cylinder exploration time during the choice phase were found in juvenile male mice (Supplementary Fig S7A) or female mice (Supplementary Fig S7B; Supplementary Table S5). All mice demonstrated a sniffing preference for the social stimulus versus a novel object.

Novel Object Recognition: For analysis, the percentage of time the animal spent sniffing the object to be replaced was recorded during training and compared directly to the time spent sniffing the novel object during testing 24 hours later. This preference index analysis has been used in other studies as a viable indicator of performance (You et al., 2017). No mice displayed a preference for either of the two objects during the first exposure (data not shown). At PND42, males had a trending interaction between Genotype x Trial ($p=0.057$) and Sidak comparisons showed significant learning in WT^s that is not seen in Del^m mice ($p<0.001$; Fig 9A). At PND70, males showed no differences in time spent investigating a novel object ($p<0.05$; Fig 9B). For females at PND42, neither genotype differed in time spent investigating a novel object ($p<0.05$; Fig 9E). However, at PND70, Del^m females did not spend more time sniffing the novel object at test compared to the last training session 24 hours prior, suggesting impaired memory for the object (Fig 9F; Supplementary Table S5).

Spatial Object Recognition: In the spatial object recognition procedure, no mice displayed a preference for any of the three objects during the first training trial, demonstrating no aversive or attractant quality of any object, per se (data not shown). For males at PND42, a Genotype x Trial repeated measures ANOVA revealed a trending interaction ($p=0.066$). Sidak post hoc comparisons show that WT males spent significantly more time sniffing the displaced object at test compared to the last training session, ($p<0.01$), whereas Del^m mice did not (Fig 9C). The same pattern occurred at PND70 (Fig 9D). For PND42 females, one female adolescent data point was removed as a statistical outlier (>2 SD $\pm$ mean; Fig 9G). Each genotype displayed significant time spent sniffing the displaced object at test. At

PND70 however, analyses showed only WT females had significant interactions with the displaced object at test. (Fig 9H; Supplementary Table S5)

Fear Conditioning: At PND70, mutants and WT mice did not have differences in contextual fear memory retention in males (Supplementary Fig S8A) or females (Supplementary Fig S8B; Supplementary Table S5).

Discussion

Microdeletion of the 16p11.2 CNV is one of the most common genetic alterations associated with ASDs and other neurodevelopmental disorders. We and others have characterized mouse models of the 16p11.2 CNV microdeletion to help inform mechanisms of deficits related to neurodevelopmental disorders. In our experiments, we find differences in many phenotypes, including developmental milestones, motor activity, anxiety-like behavior, and cognition (Angelakos et al., 2017; Grissom et al., 2017; Horev et al., 2011; V. Kumar et al., 2018; Nickl-Jockschat et al., 2015; Portmann et al., 2014; Yang, Mahrt, et al., 2015). *Del^m* mice had lower body weight that starts in preweanlings and persists throughout adulthood in comparison to WT littermates (Brunner et al., 2015; Horev et al., 2011). This persistent weight reduction suggests that one or more genes within the 16p11.2 region are required for proper metabolic processing.

In addition to body weight, we also provided a detailed analysis of multiple developmental milestones in the *Del^m* mice. For fur development, body length, and pinnae detachment, *Del^m* mice do not differ from WT mice. Differences in neuromuscular development and reflexes were seen between *Del^m* and WT pups. Notably, only *Del^m* males at early ages showed differences in cliff avoidance compared to WT mice (PND2), reduced grasp reflexes (PND6), and reduced horizontal screen suspension (PND4; PND6). These results suggest transient differences between *Del^m* and WT mice for reflexive development, but only in male mutant mice. This is consistent with children with 16p11.2 deletions who may show muscle symptoms at a very young age (Bauman, 1992; Shetreat-Klein, Shinnar, & Rapin, 2014). Additionally, we report reduced grip strength in *Del^m* mice (Fig 3), which is consistent with ASDs; children with ASDs have a negative correlation between grip strength and severity of the disorder; the lower the grip strength, the more severe symptoms an individual expresses (Kern et al., 2011; Kern et al., 2013). These findings suggest some relation between ASD symptomology and the 16p11.2 deletion in mice, but not for all motor and reflex development parameters.

We saw no differences in spontaneous activity in a short-term open field procedure (Supplementary Fig S4), consistent with other studies (Panzini, Ehlinger, Alchahin, Guo, & Commons, 2017). One issue with the open field measure is that animals are placed into a novel environment, usually during the light part of the cycle. Thus, this procedure may not reflect home cage activity in rodents, nor does it provide any data for activity during the active dark cycle. In a previously published report, we showed that *Del^m* mice display significantly more home cage hyperactivity in the dark phase compared to WT mice when investigated in a 24 hour activity monitoring procedure (Angelakos et al., 2017). This is consistent with other results showing *Del^m* mice being more active than WT mice in

distance travelled and time spent moving during the light and dark cycles (Horev et al., 2011). These findings suggest that the Del^m may display hyperactivity in specific situations.

One intriguing finding in the Del^m male mice was improved performance in the high-speed rotarod task, which is typically used to measure balance and coordinated motor activity (Pritchett & Mulder, 2003). In this study, we utilized a customized rotarod that allowed for acceleration to higher speeds than a standard rotarod. Del^m mice are not different from WTs when tested on an acceleration from 4 to 40 rpm over 5-min. However, when the top speed is increased to 8 to 80 rpm, Del^m male mice display significantly better performance than WTs. Female mutants do not show the same improvement (Fig. 4). This phenotype may be associated with repetitive behaviors, which are a hallmark of ASDs (Richler, Bishop, Kleinke, & Lord, 2007). The reason for the sex difference in performance remains unknown and should be addressed by future research. Taken together with the reflexive data, these findings suggest that models of neurodevelopmental disorders should also be studied from the perspective of understanding potential alterations in motor and sensory development.

The acoustic startle response (ASR) procedure was employed to investigate sensorimotor gating (Geyer, Swerdlow, Mansbach, & Braff, 1990). In this procedure, less intense non-startle evoking stimuli precede a loud, startle-evoking stimulus. The prepulse stimuli acts as a cue to attenuate the startle response to the louder stimuli. In this experiment, only female Del^m showed a reduced PPI response at PND21, but not at any other age. Other groups have also shown that Del^m mice do not display deficits in either acoustic startle or PPI (Brunner et al., 2015), suggesting that these mutant mice do not have any hearing deficits, a phenotype often reported in 16p11.2 microdeletion carriers (Berman et al., 2016; Jenkins et al., 2016). Our results suggest minimal deficits in acoustic startle or PPI in Del^m mice compared to WTs although female Del^m mice have increased startle responses with reduced PPI at PND21.

Anxiety-like behavior was assessed in various tasks as individuals with ASDs are at a high risk of developing anxiety disorders (MacNeil, Lopes, & Minnes, 2009; Rodgers, Glod, Connolly, & McConachie, 2012). When assessed in the EZM (Supplementary Fig S5), male mutants trended towards reduced anxiety-like behavior at PND21, but not at any other age whereas females did not differ at any age from WTs. We also analyzed activity in the center portion of the open field arena; the more time a mouse spends in the center area of an open field, the less anxiety-like behavior the mouse is exhibiting (Bailey & Crawley, 2009). Our study found increased center activity (reduced anxiety-like behavior) in PND70 Del^m females, but no differences in male mutants. However, other labs reported that male Del^m mice spend less time in the center of an open field, indicating anxiety-like behavior relative to WTs (Pucilowska et al., 2015). The reason for the differences between studies could be a result of procedural details. Across other measures, others have found that Del^m do not display an anxiety-like phenotype (Brunner et al., 2015). These findings suggest that anxiety-like behaviors in Del^m mice depend on the conditions used to test the behavior and may be age- and sex-specific.

Given the importance of social behavior in ASDs, investigating the impact of 16p11.2 deletion is highly relevant. One common procedure used to test social behavior is the three-

chamber social approach task, which measures a preference for a social stimulus versus a novel object; WT mice demonstrate an innate preference for the social stimulus (Moy et al., 2004). In this procedure, Del^m mice demonstrate no alterations in social behavior compared to WT, with all mice showing a significant preference for the social stimulus (Brunner et al., 2015; S6). These findings are consistent with other studies of social approach in 16p11.2 deletion mouse models. However, another group has demonstrated that 16p11.2 mice exhibit decreased preference to social novelty compared to WT mice (Yang et al., 2015). Future experiments should investigate other forms of sociability. In addition, measures of communication including ultrasonic vocalization (USVs) should be explored. Mice are vocal throughout their lifespan with the earliest vocalizations occurring shortly after birth and peaking by postnatal day 8 (Crawley, 2007). Mice will vocalize as a form of communication in social settings, especially when pups are separated from the mother (Branchi, Santucci, & Alleva, 2001), and when males are courting a female (Holy & Guo, 2005). One report found that Del^m neonatal mice do not display differences in USVs compared to WT, with the peak of vocalizations coming at postnatal day 7 (Brunner et al., 2015). Our unpublished observations suggest that PND5 Del^m males, but not females, display altered call sequences compared to WT littermates (Agarwalla et al., *in preparation*).

In this study, we found sex-specific, age-dependent memory impairments in mice as measured in a novel object recognition task. PND42 Del^m males show memory impairments in novel object recognition at PND42 that resolves at PND70, whereas female Del^m mice display impairments that appear at PND70, which were not seen at PND42. Others find that Del^m mice have impaired novel object performance; they do not spend significantly more time with the novel object compared to the original object (Pucilowska et al., 2015). In other models of the 16p11.2 deletion, mutant mice display impaired novel object performance (Arbogast et al., 2016; Portmann et al., 2014; Yang, Lewis, Sarvi, et al., 2015). Interestingly, in one strain of 16p11.2 deletion mice, environment has an impact on novel object recognition deficits; the deficits are attenuated if animals are housed in same-genotyped cages rather than mixed-genotyped cages (Yang, Lewis, Foley, et al., 2015). This suggests transient cognitive deficits in mutant mice, which may be related to phenotypes seen in 16p11.2 microdeletion carriers (Hanson et al., 2015; Weiss et al., 2008; Zufferey et al., 2012).

When Del^m mice were assessed in a more hippocampus-dependent task, spatial object recognition, males had persistent deficits whereas only PND70 females had deficits (Fig. 7). These findings suggest a specific cognitive impairment in an age- and sex-dependent manner, but some find that Del^m mice do not display deficits in other spatial tasks such as the Y-maze (Portmann et al., 2014), suggesting further research is needed by assessing animals in various spatial memory tasks to obtain a clear phenotype.

We also investigated Del^m performance on contextual fear conditioning. We found no differences between genotypes for contextual fear memory retention (Supplementary Fig S7), although others do find a deficit in the Del^m with a higher shock level (Tian et al., 2015). Perhaps this stronger shock provided a higher behavioral ceiling allowing differences to emerge between genotypes. Others have found that with certain mutant models, shock intensity may need to be changed to see a difference (Shaban et al., 2006). The previous

study also used a different genetic background that may explain the difference in findings between labs (Tian et al., 2015).

Conclusions

The experiments presented here are the first to explore a multitude of developmental milestones in preweanling pups of a common genetic mutation associated with ASDs and other neurological disorders. Additionally, these experiments provide vital information about sex-specific effects by assessing males and females in all measures. Our results demonstrate stable phenotypic differences in *Del^m* mice as compared with other studies including reduced body weight, deficits in novel object memory, and minimal deficits in social approach. One of the most interesting findings was male-specific improvement in a customized accelerating rotarod task, suggesting improved motor coordination. Overall, the 16p11.2 hemideletion is currently the most stable and reproducible genetic risk factor associated with ASDs and other neurodevelopmental disorders (Fernandez et al., 2009; R. A. Kumar et al., 2009; Weiss et al., 2008). These experiments provide a thorough and robust analysis of the 16p11.2 mutant mouse model, which future studies can build upon by looking at additional phenotypes and the mechanisms underlying phenotypes expressed by *Del^m* mice. Finally, our study is one of the few to assess behavioral phenotypes in males and females (but see Angelakos et al., 2017; Grissom et al., 2017), although no comparisons between sexes were analyzed in the current experiments. Future studies should look to determine the impact of sex and control for certain aspects of development, including estrous cycles. Understanding sex-specific effects of this CNV deletion will provide an opportunity to begin understanding the underlying causes of these disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Preweanling Test Schematic.

Figure of preweanling behavioral procedures and timelines. Animals were assessed in various developmental milestones, starting at PND2 and measured through PND12. Animals were euthanized after their PND12 behavioral assessments.

Figure 2. Post-Weaning Test Schematic.
Figure of post-weaning behavior procedures and timelines. Not all behaviors were performed at all ages and not all mice were tested at all ages. Each number indicates a behavior or group of similar behaviors. No individual animal was subjected to the same behavior twice or two behaviors from the same group. Animals that participated in social approach test at PND 30 were not used at PND 21 or 42, but may have been tested in another assay at PND 70. Acoustic startle/PPI and contextual fear conditioning were considered stressful and were always terminal experiments.

Figure 3. Preweaning Developmental Milestones – Body Weight.

Comparison of preweaning developmental milestones of body weight during preweaning and post weaning. **A)** PND8–12 Del^m males display significantly reduced body weight compared to WT. Subject number located in figure legend and represents number of litters. **B)** Male mutants maintain significantly reduced body weight through adolescence and adulthood (PND21–70). Subject number for each group located inside each bar. **C)** PND10–12 Del^m females display significantly reduced body weight compared to WT. Subject number located in figure legend and represents number of litters. **D)** Female mutants maintain significantly reduced body weight through adolescence and adulthood (PND21–70). Subject number for each group located inside each bar. Males represented in blue; Del^m mutants in dark blue, WT in light blue. Females represented in pink; Del^m mutants in dark pink, WT in light pink. Values are displayed as mean ($\pm$ SEM). Significance values are set at $p < 0.05$. (* = $p < 0.05$, *** = $p < 0.001$).

Figure 4. Prewaning Developmental Milestones – Neuromuscular Reflex Development.

Comparison of neuromuscular reflexes across preweaning development. **A)** Male mutants took longer to avoid a cliff edge at PND2 than WT, but not at any other age. **B)** No significant genotype differences in grasp reflex at any age. **C)** Male WTs displayed significantly better limb strength in the horizontal screen suspension task at PND4 and PND6, but not at any other age. **D)** Female mutants did not take longer to avoid a cliff edge than WT at any age. **E)** Amount of positive responses of the grasping reflex did not differ between mutant and WT females at any age. **F)** Female mutants and WTs show no differences in limb strength in the horizontal screen suspension task. Males represented in blue; Del^m mutants in dark blue, WTs in light blue. Females represented in pink; Del^m mutants in dark pink, WTs in light pink. Subject number located in figure legend and represents number of litters. Values are displayed as mean ($\pm$ SEM). Significance values are set at $p < 0.05$. (* = $p < 0.05$, ** = $p < 0.01$).

Figure 5. Preweaning Developmental Milestones – Motor and Sensory Function.

Comparison of grip strength and temperature sensitivity across post weaning development.

A) WT males have significantly better forepaw grip strength compared to mutants at all ages. **B)** WT males also have significantly better hindpaw grip strength at PND21 and PND42, but not PND70. **C)** Male mutant and WT do not differ in sensitivity to cold at any age. **D)** Male mutant and WT do not differ in sensitivity to heat at any age. **E)** WT females have significantly better forepaw grip strength compared to mutants at PND21 and PND42, but not PND70. **F)** WT females also have significantly better hindpaw grip strength at PND21 and PND70, but not PND42. **G)** Female mutant and WT do not differ in sensitivity to cold at any age. **H)** Female mutants have significantly higher tolerance to heat with an increased temperature to evoke a response compared to WT at PND21, but not at any other age. Males represented in blue; Del^m mutants in dark blue, WT in light blue. Females represented in pink; Del^m mutants in dark pink, WT in light pink. Subject number for each group located inside each bar. Values are displayed as mean ($\pm$ SEM). Significance values are set at $p < 0.05$. (* = $p < 0.05$, *** = $p < 0.001$).

Figure 6. Post weaning Behavior Assessment – Motor Functioning.

Comparison of motor coordination in a high-speed rotarod task. **A)** Male mutant and WT's do not differ in latencies to fail in the 4–40 rotarod task across trials. **B)** In the 8–80 rotarod task, male mutants have significantly longer latencies to fail, demonstrating improved performance compared to WT's during trials 2–5, but not 1 and 6. **C)** Female mutant and WT's do not differ in latencies to fail in the 4–40 rotarod task across trials. **D)** Female mutant and WT's do not differ in latencies to fail in the 8–80 rotarod task across trials. Males represented in blue; Del^{m} mutants in dark blue, WT's in light blue. Females represented in pink; Del^{m} mutants in dark pink, WT's in light pink. Subject number located in figure legend. Values are displayed as mean ($\pm$ SEM). Significance values are set at $p < .05$. (* = $p < 0.05$, ** = $p < 0.01$).

Figure 7. Post weaning Behavior Assessment - Arousal – Acoustic Startle.

Comparison of acoustic startle response across ages. **A)** Males do not differ in startle response across stimuli intensities at PND21. **B)** Males do not differ in startle response across stimuli intensities at PND42. **C)** Males do not differ in startle response across stimuli intensities at PND70. **D)** Females do not differ in startle response across stimuli intensities at PND21. **E)** Females do not differ in startle response across stimuli intensities at PND42. **F)** Female mutants do not differ in startle response across stimuli intensities at PND70. Males represented in blue; *Del^m* mutants in dark blue, WTs in light blue. Females represented in pink; *Del^m* mutants in dark pink, WTs in light pink. Subject number located in the figure legend. Values are displayed as mean ($\pm$ SEM). Significance values are set at $p < .05$.

Figure 8. Post weaning Behavior Assessment - Arousal – Prepulse Inhibition (PPI).

Comparison of PPI across ages. **A)** Males do not differ in PPI across stimuli intensities at PND21. **B)** Males do not differ in PPI across stimuli intensities at PND42. **C)** Males do not differ in PPI across stimuli intensities at PND70. **D)** Female WTs have significantly higher PPI to the 81 dB stimulus compared to mutants at PND21. **E)** Females do not differ in PPI across stimuli intensities at PND42. **F)** Females do not differ in PPI across stimuli intensities at PND70. Males represented in blue; Del^m mutants in dark blue, WTs in light blue. Females represented in pink; Del^m mutants in dark pink, WTs in light pink. Subject number located in the figure legend. Values are displayed as mean ($\pm$ SEM). Significance values are set at $p < .05$. (* = $p < 0.05$).

Figure 9. Post weaning Behavior Assessment – Cognition – Object Recognition.

Comparison of learning and memory in novel object and spatial object recognition tasks at different ages. **A)** Male WTs show significant increase in time spent sniffing the novel object when comparing between training and testing, demonstrating preference for the novel object. However, male mutants do sniff the novel object significantly more, indicating memory impairment at PND42. **B)** At PND70, male mutant and WTs show significant preference for sniffing the novel object **C)** In spatial object recognition at PND42, only male WTs show significant increase in time spent sniffing the displaced object when comparing between training and testing. **D)** At PND70, only male WTs show significant preference **E)** In novel object recognition, females do not show significant interaction with the displaced object at test at PND42. **F)** At PND70, female mutants show memory impairment compared to WTs. **G)** In spatial object recognition at PND42, WT and mutants show significant interaction with the displaced object at test. **H)** At PND70, only WTs show significant interaction with the displaced object at test. Males represented in blue; Del^m mutants in dark blue, WTs in light blue. Females represented in pink; Del^m mutants in dark pink, WTs in light pink. Subject number located on the x-axis. Values are displayed as mean ($\pm$ SEM). Significance values are set at $p < .05$. (* = $p < 0.05$, *** = $p < 0.001$).