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Abstract
Recurrent copy number variations (CNVs) of human 16p11.2 have been associated with a variety of developmental/neurocognitive syndromes. In particular, deletion of 16p11.2 is found in patients with autism, developmental delay, and obesity. Patients with deletions or duplications have a wide range of clinical features, and siblings carrying the same deletion often have diverse symptoms. To study the consequence of 16p11.2 CNVs in a systematic manner, we used chromosome engineering to generate mice harboring deletion of the chromosomal region corresponding to 16p11.2, as well as mice harboring the reciprocal duplication. These 16p11.2 CNV models have dosage-dependent changes in gene expression, viability, brain architecture, and behavior. For each phenotype, the consequence of the deletion is more severe than that of the duplication. Of particular note is that half of the 16p11.2 deletion mice die postnatally; those that survive to adulthood are healthy and fertile, but have alterations in the hypothalamus and exhibit a “behavior trap” phenotype—a specific behavior characteristic of rodents with lateral hypothalamic and nigrostriatal lesions. These findings indicate that 16p11.2 CNVs cause brain and behavioral anomalies, providing insight into human neurodevelopmental disorders.

Keywords
home-cage, stereotypic behavior, structural variation, brain MRI

Disciplines
Applied Statistics | Biostatistics | Neuroscience and Neurobiology | Statistics and Probability

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Recurrent copy number variations (CNVs) of human 16p11.2 have been associated with a variety of developmental/neurocognitive syndromes. In particular, deletion of 16p11.2 is found in patients with autism, developmental delay, and obesity. Patients with deletions or duplications have a wide range of clinical features, and siblings carrying the same deletion often have diverse symptoms. To study the consequence of 16p11.2 CNVs in a systematic manner, we used chromosome engineering to generate mice harboring deletion of the chromosomal region corresponding to 16p11.2, as well as mice harboring the reciprocal duplication. These 16p11.2 CNV models have dosage-dependent changes in gene expression, viability, brain architecture, and behavior. For each phenotype, the consequence of the deletion is more severe than that of the duplication. Of particular note is that half of the 16p11.2 deletion mice die postnatally; those that survive to adulthood are healthy and fertile, but have alterations in the hypothalamus and exhibit a “behavior trap” phenotype—a specific behavior characteristic of rodents with lateral hypothalamic and nigrostriatal lesions. These findings indicate that 16p11.2 CNVs cause brain and behavioral anomalies, providing insight into human neurodevelopmental disorders.

Accumulating evidence suggests the importance of copy number variations (CNVs) in the etiology of neuropsychiatric disorders, including autism (1), schizophrenia (2–4), developmental delay (5), and other complex traits (6). The 16p11.2 region is particularly intriguing. Whereas deletion of 16p11.2 has been associated with autism (7–9), duplication of 16p11.2 has been associated with autism (9, 10) as well as schizophrenia (11). 16p11.2 CNVs have also been reported in patients with developmental delay, mental retardation, repetitive behaviors (12–16), and a highly penetrant form of obesity (17). A reciprocal effect of 16p11.2 dosage on head size has been noted, as deletions are associated with large head size or macrocephaly, whereas duplications are associated with microcephaly (16). These studies reveal the variability of symptoms in patients carrying the same 16p11.2 CNV, an extreme example being a family with three affected members with symptoms so heterogeneous that they were barely overlapping (18).

Mouse models allow direct assessment of CNVs while reducing variability caused by genetic and environmental factors. We and others have previously used chromosome engineering (19) to model genetic alterations found in complex human diseases including cancer (20) and genomic disorders (21–24), allowing identification of the causative gene and elucidation of the mechanism involved (20, 25–27). Here we used a similar approach to generate mouse models with deletion and duplication corresponding to those found in patients with 16p11.2 CNVs. Because of the evidence for clinical heterogeneity, we screened these models for multiple changes in brain anatomy and behavior by using a combination of high-resolution MRI (28) and a monitoring system that assesses multiple behaviors (29). We found that the deletion and the duplication affect behavior and brain anatomy in opposing ways, with deletion mice exhibiting behaviors that resemble sensorimotor deficits in rats with lateral hypothalamic and nigrostriatal lesions (30, 31).

These findings provide evidence that brain anatomy and behavior depend on dosage of the region corresponding to 16p11.2.

Results

Generation of Mouse Models for Human 16p11.2 CNVs. We asked whether altered dosage of the region corresponding to 16p11.2 causes abnormalities in mice. Genes mapping to the 0.52-Mb 16p11.2 CNV in humans cluster within a 0.44-Mb region of mouse chromosome 7 (Fig. 1A). Using chromosome engineering (19) as we have previously (20, 27, 32), we generated mice with one copy [heterozygous for a deletion or deficiency (df) allele], as well as mice with three copies [heterozygous for a duplication (dp) allele] of the region corresponding to 16p11.2 (Fig. 1B and Fig. S1). Endpoints for the rearrangement were selected based on human data (1), with each gene in the interval being conserved in mouse (Dataset S1). Gene targeting constructs were generated using MICER (33), and sequential targeting in mouse ES cells resulted in integration of loxP sites and selection cassettes at each endpoint (Fig. 1B and Fig. S1). Cre-mediated recombination and drug selection within eight independent doubly targeted clones revealed that three clones had been targeted in cis and five clones had been targeted in trans, which generated df/+ and df/dp ES cells, respectively (Fig. 1B and Figs. S1 and S2). Five independent df/dp clones were used for blastocyst injection, producing 40 different male chimeras that were crossed to +/+ females. Ten of these chimeras (representing two independent ES cell clones) produced df/+ and df/+ mice that were identified by PCR (Fig. 1C). This approach provides mouse models for directly assessing the consequences of both the 16p11.2 CNV losses (i.e., deletion) and gains (i.e., duplication) found in humans.

We established both df/+ and df/+ mice, but at weaning we noticed that df/+ mice were underrepresented and litter sizes were smaller than expected (Table S1). Before weaning, df/+ mice were sometimes small (Fig. 1D), but as adults, they were essentially the same size as their siblings and appeared healthy (SI Experimental Procedures). To determine whether df/+ mice were dying during embryogenesis, we crossed df/+ males to +/+ females, and harvested embryos at day 13.5 of development [i.e., embryonic day (E) 13.5] as well as just before birth (E17.5–E18.5); progeny from similar crosses using the same studs as well as their male siblings were also genotyped at weaning (Table S1). Whereas litter sizes during embryogenesis averaged 9.4 embryos and the ratio of df/+ embryos was Mendelian, litter sizes at weaning averaged only 5.0 mice and the ratio of df/+ mice was half that expected. In addition, litter sizes were normal and df/+
mice were present in expected ratios immediately after birth, whereas dead pups lacking a milk pouch were sometimes found later on. Therefore, some df/+ mice die after birth, indicating that 16p11.2 loss can compromise survival.

**Gene Expression in Multiple Brain Regions Corresponds to 16p11.2 Dosage.** To validate the models, we analyzed gene expression profiles in the brain and determined whether expression corresponded with dosage. We measured mRNA intensities in 37 microarray hybridizations representing four brain regions (olfactory bulbs, cortex, cerebellum, and brainstem; five samples were hybridized twice for estimation of technical errors) in two df/+ and three df/+ animals. All mice were F1 C57BL/6N:129Sv hybrids; therefore, other than the engineered CNV, their genomes were identical. A scatter plot of the gene expression intensity differences between dp/+ and df/+ vs. the difference between df/+ and df/+ indicated that genes within 16p11.2 displayed a large difference between df/+ and df/+ brain, and a much smaller difference between df/+ and df/+ brain (Fig. S3A). Two-way ANOVA with brain region and dosage as main factors indicated that, of 33 genes in the engineered region, expression of 26 was affected directly by dosage (Dataset S2). Gdpd3, mapping within the engineered region, showed extreme up- and down-regulation; this reflected differences in Gdpd3 expression in C57BL/6N vs. 129Sv strains. Further analysis indicated that expression of genes in the region was significantly altered in each of the four brain regions analyzed, and that expression was affected by deletion than by duplication (Fig. S3B). These findings indicate that copy number dictates gene expression levels in multiple brain regions, and that loss has the largest effect.

**df/+ and dp/+ Mice Have Distinctive Behavioral Phenotypes. General survey of behavior.** The clinical evidence that patients with 16p11.2 CNVs have highly heterogeneous symptoms suggested that if the corresponding genomic alteration did in fact cause behavioral alterations in mice, the phenotypes might also be highly variable. Therefore, we believed it imperative to monitor the 16p11.2 CNV models for multiple behaviors by using as quantifiers the distance walked, grip strength, and a much smaller difference between df/+ and df/+ brain, and a much smaller difference between df/+ and df/+ brain (Fig. S3A). Two-way ANOVA with brain region and dosage as main factors indicated that, of 33 genes in the engineered region, expression of 26 was affected directly by dosage (Dataset S2). Gdpd3, mapping within the engineered region, showed extreme up- and down-regulation; this reflected differences in Gdpd3 expression in C57BL/6N vs. 129Sv strains. Further analysis indicated that expression of genes in the region was significantly altered in each of the four brain regions analyzed, and that expression was affected by deletion than by duplication (Fig. S3B). These findings indicate that copy number dictates gene expression levels in multiple brain regions, and that loss has the largest effect.

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**df/+ and dp/+ Mice Have Distinctive Behavioral Phenotypes.**

*Fig. 1.* Generation of 16p11.2 models. (A) Genes mapping to human 16p11.2 CNVs are conserved in mouse. (B) Schematic of the chromosome engineering strategy used to generate mouse models of 16p11.2 CNVs. Step 1 is gene targeting at the 135k15 locus; step 2 is gene targeting at the 276k12 locus in the 135k15-targeted ES cells; and step 3 is Cre-mediated recombination. Cis and trans indicate that loxp sites (yellow triangles) had integrated on the same or different chromosome homologues, respectively. (C) Molecular validation. PCR products using primers specific for the positive control (β-actin), targeting at the first and second endpoints (135k15 and 216k12 loci, respectively), the df allele, and the dp allele are shown. (D) Before weaning, df/+ mice (Right) tend to be smaller than their +/- siblings (Left; 88 and 15.4 g, respectively, for the females shown). Note the light-colored tail and ears of the df/+ mice, which is a result of the presence of the Agouti transgene. In A and B, chromosome positions are shown in megabases. Further information is provided in Figs. S1 and S2, Dataset S1, and SI Experimental Procedures.
multiple behaviors depend on 16p11.2 dosage. (B) Median distance traveled vs. time during the first 2 h after cage transfer. (C) Detailed behaviors during the first 2 h after cage transfer. Medians of the cumulative time of four distinct behaviors (rearing, walking, grooming, resting) vs. time elapsed from the beginning of the trial are shown for each genotype. During this period, df/+ mice do not rest; instead, they have a second peak of activity compared with the other genotypes. Distance traveled and time spent walking were significantly decreased and increased in df/+ and dp/+ mice, respectively, relative to +/-; indeed, df/+ mice had a burst of walking and rearing, whereas resting was absent during this stage. Thus, the rate of certain behaviors is affected reciprocally by loss and gain of 16p11.2 dosage in response to environmental challenge. In addition, the sequence of these behaviors is disrupted in df/+ mice. Thus, 16p11.2 CNVs affect both the rate and the timing of specific behaviors.

Diurnal deficits. Sleeping disorders are frequently reported in many psychiatric disorders, including autism (40). Because we recorded behavior over sequential dark/light intervals, we could assess the effect of 16p11.2 CNVs on light and dark cycling by using previously established methods (29). Mice are nocturnal, and indeed, each genotype was most active during the dark periods (Fig. 3A). The +/-, df/+ , and dp/+ mice were most active during the initial dark period, with activity decreasing in successive dark periods. Although activity of df/+ mice was highest in the first dark period, during subsequent dark cycles, the mice adapted and had activity levels similar to controls. The activity of df/dp and +/- mice was indistinguishable in light and dark periods; dp/+ mice were notably less active in the dark (but not light) period (Fig. 3B). In striking contrast, df/+ mice were significantly more active than mice of other genotypes in both light and dark periods. Furthermore, df/+ mice were unique, as they had a higher ratio of light to dark activity compared with the other genotypes. Distance traveled during light vs. the average distance traveled during dark.
The most significant genotypic effect reported by HomeCageScan was that df/+ mice remained on the ceiling of the cage for extended periods ($F_{hang} = 0.00021$; Fig. 2 A). Therefore, we further investigated the climbing patterns of the mice. The ceiling-climbing behavior of controls was dynamic and changed over the course of the session. Shortly after being introduced into the test cage, diploid controls climbed up to the lower part of the V-shaped ceiling, remained there briefly, and then returned to the floor. During this early phase of testing, control mice returned to the floor with the rear part of their bodies leading, i.e., they hung on the ceiling with their forelimbs, touched the floor with their hindlimbs, and then left the ceiling (Movie S1). In subsequent climbing episodes, control mice traveled to higher and more distant locations on the ceiling, gradually progressing to the highest point of the cage. The climbing behavior of controls developed in two dimensions: first, they left the ceiling from different locations and returned to different places on the floor; second, they could climb down from the ceiling with their head and forepaws leading, i.e., they hung on the ceiling by their hindlimbs and then touched down on the floor with their forelimbs.

In contrast to the adaptability of controls, the ceiling-climbing behavior of df/+ mice was extremely stereotypic throughout the test period. Like control mice during the early phase of being introduced into the test cage, df/+ mice returned to the floor with their hindlimbs leading. However, in contrast to control mice, df/+ mice did not progress to the stage at which they were able to climb down from the ceiling with their head and forelimbs leading. In further contrast to controls, df/+ mice did not climb off the ceiling from different spots; they continued to go up to and down from the ceiling at the same location (Table 1). Some df/+ mice became “trapped” on the ceiling for extensive periods, apparently lacking the ability to return to the floor of the cage (Movie S2). Other df/+ mice developed stereotypic ways of coming down from the ceiling (Movie S3) that they repeated hundreds of times during the course of the session. This repetitive behavior continued throughout the recording period, even after the mice had performed hundreds of climbing episodes. This analysis revealed that 16p11.2 deletion mice show nonprogressive, stereotypic motor behavior that is similar to stereotypic behavior caused by lateral hypothalamic and nigrostriatal lesions (30, 31).

### Discussion

**16p11.2 CNV Models Provide Insight into Human Syndromes.** CNVs affecting 16p11.2 have been associated with autism and other neurodevelopmental/neuropsychiatric syndromes (1, 7, 9, 12–16), yet several issues remain unresolved. Are these conditions unique to humans? Do loss and gain cause the same syndrome? Does dosage of 16p11.2 affect brain architecture? Why are some of patients with the same CNV diverse? To begin to address these issues, we engineered mice heterozygous for deletion and duplication of the interval corresponding to 16p11.2 CNVs found in humans. The striking changes we discovered in gene expression profiles, viability, brain architecture, and most importantly behavior, provide functional evidence that 16p11.2 CNVs cause phenotypes in mice, that loss and gain have opposing effects, and that multiple brain regions and behaviors are affected. Our finding that brain volume size is affected reciprocally in deletion vs. duplication mice is concordant with the macrocephaly phenotype described in human subjects with 16p11.2 deletion. Because “behavior trap” resembles a phenotype described in rats with lesions in the lateral hypothalamus, we performed detailed MRI analysis of the hypothalamus. Most changes between df/+ and dp/+ were located in the posterior region of the hypothalamus, with pronounced changes in the lateral zone (Fig. 5). These findings support the hypothesis that the lateral hypothalamus is affected in df/+ mice. In addition, we found that Mapk3—which maps within the region corresponding to human 16p11.2—is expressed robustly in specific brain regions including the lateral hypothalamus and the nigrostriatal tract (Fig. S6). These findings demonstrated that altered dosage of 16p11.2 causes changes in the size of several brain structures, and that deletion and duplication have opposing effects.

16p11.2 CNV Models Have Distinct Changes in Brain Architecture. To identify brain regions altered in 16p11.2 CNV mice, we used MRI to analyze the brains of 39 mice from the cohort that had already been analyzed for behavioral phenotypes (Fig. 4) (28). We included both male and female mice in the cohort, which consisted of +/+ and df/df diploid controls (n = 9 and n = 8, respectively), df/+ (n = 11), and dp/+ (n = 11). Anesthetized mice were perfused and euthanized, and the brain (which remained within the skull) was subjected to MRI. Sixty-two different brain regions (41) were examined, and their volumes were assessed as the percentage of total brain volume averaged for each of the four models (Dataset S4).

Significant changes between brains of df/+ and +/+, as well as between brains of df/+ and dp/+ mice, were noted (Fig. 4 and Fig. S5). Although brains of +/+ and dp/+ mice were not significantly different, a clear trend was found for some regions. Brain structures significantly affected after stringent correction for multiplicity (with the Holm procedure) included the basal forebrain, superior colliculus, fornix, hypothalamus, mammillothalamic tract, medial septum, midbrain, and periaqueductal gray (Fig. 4 A and B). For each structure, the volumetric changes were more extensive between df/+ and dp/+ than between df/+ and +/+, indicating that loss and gain of 16p11.2 dosage affects these regions in opposite ways (Fig. 4 and Fig. S5).

Because the “behavior trap” resembles a phenotype described in rats with lesions in the lateral hypothalamus, we performed detailed MRI analysis of the hypothalamus. Most changes between df/+ and dp/+ were located in the posterior region of the hypothalamus, with pronounced changes in the lateral zone (Fig. 5). These findings support the hypothesis that the lateral hypothalamus is affected in df/+ mice. In addition, we found that Mapk3—which maps within the region corresponding to human 16p11.2—is expressed robustly in specific brain regions including the lateral hypothalamus and the nigrostriatal tract (Fig. S6). These findings demonstrated that altered dosage of 16p11.2 causes changes in the size of several brain structures, and that deletion and duplication have opposing effects.

### Table 1. Number of mice from each cohort that performed different climbing behaviors

<table>
<thead>
<tr>
<th>Group</th>
<th>Climb down from high point</th>
<th>Climb down with head leading</th>
<th>Stereotypic climb down</th>
</tr>
</thead>
<tbody>
<tr>
<td>df/+</td>
<td>13/13</td>
<td>2/13</td>
<td>1/13</td>
</tr>
<tr>
<td>+/-</td>
<td>15/15</td>
<td>15/15</td>
<td>15/15</td>
</tr>
<tr>
<td>df/df</td>
<td>6/9</td>
<td>6/9</td>
<td>6/9</td>
</tr>
<tr>
<td>dp/+</td>
<td>1/13</td>
<td>1/13</td>
<td>1/13</td>
</tr>
</tbody>
</table>

Data shown are from the second night of the test, as shown in Movies S1, S2, and S3. Note that most of the dp/+ mice did not climb on the ceiling.
Changes in head cir-

Several human studies and mice. (df/dp diploid controls are not signi-
mice do not show the mobility gradient: their
Holm test followed by Bonferroni |
and cohorts, with brightness
and dp/+ D (A, most posterior; D, most anterior). Colors indicate
cohorts with an FDR of 0.05. The sections performed along four
models are dosage-dependent.

Details of alterations in the hypothalamus detected by MRI. Three-
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controls, providing genetic evidence that the structural
delictions and duplications (10, 13, 16); however, to our knowl-
dosage has on brain architecture. Importantly,
brains of df/dp diploid controls are not significantly different
from +/- controls, providing genetic evidence that the structural
changes in df/+ and dp/+ models are dosage-dependent.

16p11.2 CNVs Affect Multiple Behaviors. Several human studies
compared the behavioral symptoms of patients with 16p11.2
deletions and duplications (10, 13, 16); however, to our knowl-
egy, there is no evidence that loss and gain of 16p11.2 affect
behavior in opposing ways. Even with patients harboring the same
16p11.2 lesion, there is a broad spectrum of clinical symptoms,
some patients being severely affected and others highly func-
tional. By simultaneously analyzing multiple behaviors in the
context of a new environment, we identify a number of behaviors
that are altered in 16p11.2 CNV mice, revealing that deletion and
duplication have opposite consequences. These highly significant
changes survive strict statistical analyses (37, 43). Each genotype
responds to the new cage with heightened activity, but only df/+ mice have a second burst of activity at a time when controls are
already resting. When control mice have become accustomed to
their new environment, they have a gradual increase in freedom of
movement on the ceiling over the course of the trial, i.e., a mo-
bility gradient that recapitulates the ontogeny of movement (44).
In contrast, df/+ mice do not show the mobility gradient: their
celling-climbing behavior is restricted to specific locations and
their movements are stereotypic. Interestingly, this ceiling-
climbing behavior is similar to the behavior trap described in rats
with lateral hypothalamic lesions and 6-hydroxydopamine-in-
duced lesions (30, 31), a well characterized model of Parkinson
disease. Other phenotypes of these rats are feeding problems (45,
46), sensory neglect, and abnormal gait (30, 31, 47–49). Indeed,
abnormal gait and motor delay (13, 16, 18, 50), attention deficits
(13), and feeding defects (16) are common in patients with
16p11.2 deletion. Moreover, motor development problems are
common in autism spectrum disorders and may serve as an in-
dicator for early intervention, as these features appear before the
core symptoms that define autism (51).

Fig. 5. Details of alterations in the hypothalamus detected by MRI. Three-
dimensional models of the surface of the hypothalamus (Bottom), coronal
images showing the regions affected (Middle), and magnification focusing on
the hypothalamus (Top). Red indicates voxels that differ significantly between
df/+ and dp/+ cohorts with an FDR of 0.05. The sections performed along four
locations marked A–D (A, most posterior; D, most anterior). Colors indicate
voxels that differ significantly between df/+ and dp/+ cohorts, with brightness
indicating the significance of the difference, as specified by the FDR. AHN, anterior hypothalamic nucleus; DMH, dorsomedial hypothalamus; FX, col-
umns of the fornix; LHA, lateral hypothalamic area; MTT, mammillothalamic
tract; OPT, optic tract; PH, posterior hypothalamic nucleus; ZI, zona incerta.
Deletion of 16p11.2 Causes Lethality in Neonates. A major finding of this work is that approximately half of df/+ neonates die after birth, a finding that may have relevance to autism incidence. The recent increase in autism incidence (52) might be partially attributable to factors that improve pre- and postnatal survival. Human studies are consistent with this idea, as it is much more common for inherited rare copy number polymorphisms that affect coding regions to be duplications than deletions (53).

Closing. This work demonstrates the value of using mice to model CNVs found in human disorders. This approach provides functional evidence that 16p11.2 CNVs affect brain anatomy and behavior in mice, with loss and gain having opposing effects. Multiple brain regions are affected, with deletion of 16p11.2 causing profound behavioral changes such as hyperactivity, difficulty adapting to change, sleeping abnormalities, and repetitive or restricted behaviors. In addition, our findings suggest a potential link between 16p11.2 copy number alterations and infant mortality. Finally, we note a similarity in phenotype between 16p11.2 deletions and rats with lateral hypothalamic lesions. These 16p11.2 CNV models should prove valuable for elucidating the physiological basis of neurodevelopmental syndromes and for evaluating their treatments.

Experimental Procedures
Mice carrying rearrangements corresponding to the human CNVs (Dataset 1) were established by using chromosome engineering as described previously (19, 20, 27, 32). HomeCageScan system (CleverSys) was used to analyze behavior in a cohort of 50 adult df/+ and df/df, and df/+ mice. Thirty-nine of these mice were also analyzed by MRI. Hypothesis testing was followed by correction for multiplicity (5I Experimental Procedures provides additional details).

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