Dosage-dependent phenotypes in models of 16p11.2 lesions found in autism

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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 dp/+ 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 dp/+ 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/+ and df/dp mice were weaned at the same time as +/+ controls, as expected (Table S1).

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE32012).

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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/+; three +/+; and three dp/+ male mice. 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 difference between dp/+ and +/+ vs. the difference between df/+ and +/+ indicated that genes within 16p11.2 displayed a large difference between dp/+ and +/+ brain, and a much smaller difference between dp/+ and +/+ 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 more 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 using as quantifiable and unbiased approaches as possible. We used HomeCageScan, a system previously used to assess behavioral alterations caused by neurodegenerative disease, neurotoxic agents, and pain (29, 34–36). We investigated behavior of a cohort of 50 male and female mice. The mice were progeny from df/+ × dp/+ crosses, and therefore included +/+ and df/dp diploid controls (n = 15 and n = 9, respectively), df/+ (n = 13), and dp/+ (n = 13). The parents in these crosses came from two chimeras. Thirty-nine of these were later used for MRI (as detailed later). Recording was done in cages that were significantly larger, with a ceiling that was much higher, than a standard mouse cage. The reason for using large cages is that, by minimizing the physical constraints on the animals, a rich spectrum of behaviors evolves (37) and the dynamics of the change in behavior varies between genotypes (38). In this experimental paradigm, the recording cages posed a new environment to the mice being analyzed. In particular, mice had to adapt their climbing abilities to this new environment. In each session, the behavior of four individual mice was recorded simultaneously. Multiple sessions were performed so that the behavior of each of the 50 mice was analyzed. Mice were transferred into the recording cages before the last 2 h of the light period. The recording started immediately and continued for 60 h after the onset of the first dark period (i.e., over three 12-h dark periods and two 12-h light periods). We also tested social behavior and grip strength (Fig. S4). These analyses did not show significant differences and therefore we do not discuss those data.

However, six of eight distinct behavioral measures revealed significant genotype differences. The changes were evident immediately after the mice were introduced into the new cages, as well as throughout the entire period of the test (Fig. 2A and Dataset S3). Five of these six differences were particularly interesting, as these behaviors were affected in opposite directions in df/+ and dp/+ mice relative to diploid (+/+ and df/dp) controls. As was the case for gene expression profiles, the effect of the deletion on behavior was more severe than that of the duplication. As 16p11.2 CNV-associated syndromes sometimes have a gender bias, we asked whether the changes in behavior were sex-specific. Two-way ANOVA did not reveal significant interactions between 16p11.2 dosage and sex for any of the behavioral measures (Dataset S3). Later we describe in detail the behaviors affected by 16p11.2 dosage.

**Response to change in environment.** The 16p11.2 CNV models responded uniquely to environmental change: within the 2-h period after being transferred to the test cage, the distance traveled, as well as the time spent walking, lingering, and resting, depended on genotype (Pdistance = 0.0016, Pwalking = 0.003, Plingering = 0.021, Presting = 0.025; Dataset S3). Tukey’s confi...
The stages of activity that did not occur in other groups. The response to the environment (Fig. 2) during the first 2 h of the test (Fig. 2B). However, the df/+ cohort had a second burst of activity that did not occur in other groups. The response to being transferred to the test cage occurred in three sequential stages defined by the actions of the mice (Fig. 2C). For each group, the first stage was characterized by elevated walking and grooming. The third stage was characterized by resting, which was significantly decreased and increased in df/+ and dp/+ 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.

Fig. 3. Behavioral diurnal rhythms are affected in 16p11.2 CNV mice. Comparison of the distance traveled in cohorts of distinct genotypes (detailed in the text) during 60 h of alternating 12-h dark and 12-h light cycles (i.e., spanning three dark periods plus two light periods). (A) The distance traveled over successive dark (black bars) and light (gray bars) periods is shown in bins of 12 h. Five successive dark or light periods are shown. (B) The average distance traveled during light vs. the average distance traveled during dark. (C) The ratio of light-to-dark activity indicates that df/+ mice are unusual in that their activity is not as restricted to the dark periods as the controls are. Average and SEM are presented in all panels.
genotypes (Fig. 3C). These findings indicate that 16p11.2 CNVs affect diurnal behaviors.

Climbing deficits. The most significant genotype effect reported by HomeCageScan was that df/+ mice remained on the ceiling of the cage for extended periods (F hang = 0.00012; Fig. 2A). 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).

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/dp 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. 4A 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 “behavioral 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 demonstrate that altered dosage of 16p11.2 causes changes in the size of several brain structures, and that deletion and duplication have opposing effects.

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 symptoms 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 macroanatomy in human subjects with 16p11.2 deletion and duplication, respectively, indicating that our animal models recapitulate the human genomic disorders.

The finding that mice with the same CNVs present in humans have neuroanatomical and behavioral phenotypes indicates that 16p11.2 genes are important for brain function in mammals other than humans. For some human CNV-associated syndromes such as 7q11.23 deletion (i.e., Williams–Beuren syndrome) and the reciprocal duplication (42), loss and gain are associated with opposing clinical features. Indeed, this is the case for head size alterations associated with 16p11.2 CNVs (16), but certainly not for behavioral symptoms of these patients (10, 13, 16). In mice, we see that loss and gain of 16p11.2 cause distinct and opposing behavioral phenotypes. Similarly, mouse chromosome engineered models of human 17p11.2 deletion/duplication-associated syndromes had opposing phenotypes for some, but not all, clinical phenotypes studied (22).

The side-by-side comparison of mice with deletion and duplication of the region corresponding to human 16p11.2 reveals that expression of most genes within the engineered interval correlates directly with dosage, and that a number of neuroanatomical and behavioral phenotypes are affected in opposite directions by loss and gain. Deletion has a more severe effect than duplication on each phenotype—viability, gene expression, brain structure, and behavior—in keeping with the severity of deletion vs. duplication of 16p11.2 in humans. For examples, duplications are occasionally seen in asymptomatic carriers, but carriers of the deletion are
responds to the new cage with heightened activity, but only changes survive strict statistical analyses (37, 43). Each genotype duplication have opposite consequences. These highly significant volumetric changes in 16p11.2 CNV mice, revealing that deletion and duplications (10, 13, 16); however, to our knowledge, there is no evidence that loss and gain of 16p11.2 affect edge, 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 functional. 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 mobility gradient that recapitulates the ontogeny of movement (44). In contrast, df/+ mice do not show the mobility gradient: their ceiling-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-induced 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 indicator for early intervention, as these features appear before the core symptoms that define autism (51).

16p11.2 CNVs Affect Many Brain Regions. Changes in head circumference and abnormal brain structure have been reported in patients with 16p11.2 CNVs (14, 16). By using MRI, we find significant volumetric changes in eight different brain regions. Brains of df/+ (but not dp/+ or dp/-) mice, have significant volumetric changes relative to controls, but the most extensive difference is between df/+ and dp/+ mice, emphasizing the opposing effects that 16p11.2 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 knowledge, 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 functional. 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 mobility gradient that recapitulates the ontogeny of movement (44). In contrast, df/+ mice do not show the mobility gradient: their ceiling-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-induced 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 indicator for early intervention, as these features appear before the core symptoms that define autism (51).

Fig. 4. MRI identifies structural changes in brains of 16p11.2 CNV mice. The relative volume (percentage of total brain volume) of eight brain regions is increased in df/+ mice. (A) Three-dimensional representation of the mouse brain highlights eight regions (colored as in legend) affected by 16p11.2 dosage. (B) Relative volumes (shown as percentage of total brain volume) are dependent on dosage. Mean and SEM are shown. Statistically significant pairwise differences to the df/+ group (determined by t test followed by Bonferroni-Holm procedure) are depicted as follows: cyan indicates that df/+ differs from at least one other cohort, magenta indicates cohorts that differ significantly from df/+, and black indicates groups that do not differ significantly from df/+ . Full pair-wise comparisons are shown in Dataset S4.

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 dimensions 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 dimensions 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 the signiﬁcance of the difference, as speciﬁed by the FDR. AHN, anterior hypothalamic nucleus; DMH, dorsomedial hypothalamic nucleus; FX, columns 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 16p11.2 neonates die after birth, a finding that may have relevance to autism incidence. The precise cause of death in 16p11.2 mice could be related to feeding deficits, but this remains to be investigated. Based on our findings, we suggest that efforts be made to determine whether 16p11.2 deletion is associated with unexplained cases of infant death. If these findings generalize to other genotypes associated with autism, they may explain puzzling aspects of the human condition. The recent increase in autism incidence might be partially attributed 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 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/+; df/dfp, and df/+ mice. Thirty-nine of these mice were also analyzed by MRI. Hypothesis testing was followed by correction for multiplicity (51 Experimental Procedures provides additional details).

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19. Sebat J, et al. (2006) TheDeranged system: Mice carrying rearrangements corresponding to the human CNVs 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/+; df/dfp, and df/+ mice. Thirty-nine of these mice were also analyzed by MRI. Hypothesis testing was followed by correction for multiplicity (51 Experimental Procedures provides additional details).

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Horev et al. 10.1073/pnas.1114042108

SI Experimental Procedures

Chromosome Engineering. Gene targeting. Plasmids specific for each endpoint of the rearrangement were converted to gene targeting vectors pTV-MHPN216k12A Δ.92kb AflIII and pTV-MHPP135k15Δ.47kb AflI by removing 1.92 kb AflIII and 0.47 kb AflI fragments, respectively. The 135k15 locus was targeted by electroporating linearized pTV-MHPP135k15Δ.47kb AflI plasmid into AB2.2 ES cells (129Sv genetic background), selecting in puromycin, and identifying homologous recombinants by Southern analysis and PCR (as detailed later). The 216k12 locus was targeted by electroporating linearized pTV-MHPN216k12A Δ.92kb AflIII plasmid into 135k15-targeted cells, selecting in G418, and identifying homologous recombinants by Southern analysis and PCR (as detailed later).

Cre-mediated recombination. Eight independent doubly targeted clones were electroporated with approximately 25 μg Cre-expressing pOG231 plasmid, cells were selected in HAT, and six to 12 individual subclones were analyzed for resistance to HAT, G418, and puromycin by sib-selection.

Generation of mice. Multiple df/dp subclones were used to establish mouse strains by injection into C57BL/6J (Jackson Laboratory) host blastocysts. Male chimeras were mated to C57BL/6N females (Taconic) to establish df/+ and dp/+ mice.

Genotyping. Southern blot. To analyze the 135k15 locus via Southern analysis, genomic DNA was digested with XhoI and hybridized with the 0.3-kb probe generated with the following primers: 135k15flankFWD no. 956 (5′-CTCTCCTCACCCATGTACAGAG-3′); 135k15flankREV no. 957 (5′-ACCTACGGGGGCCTATCTTAG-3′); this probe detects endogenous 11.3-kb and targeted 10.3-kb fragments. To analyze the 216k12 locus via Southern blot, genomic DNA was digested with EcoRI and hybridized with the 1.9-kb probe generated with the following primers: 216k12gapFWD no. 1089 (5′-CTCTTACACAGCTGGAGGA-3′); 216k12gapREV. no. 1090 (5′-TCTGGTACCTCTGACCAAGG-3′); this probe detects endogenous 15.1-kb and targeted 11.6-kb fragments. Recombined df and dp alleles were detected by unique patterns of Southern blots using the same endpoint probes as earlier (Figs. S1 and S2 show details).

PCR. Targeted 135k15 and 216k12 loci can also be detected by PCR, generating 2.8-kb and 3-kb products, respectively [135k15 primers, 135k15gapREV no. 1674 (5′-GACATGGTGCGACTACCTATATATCC-3′) vector S1. no. 1675 (5′-TCTGGTACCTCTGACCAAGG-3′)]; 216k12 primers, 5′ vector S1. no. 1672 (5′-GGCGCCCAATACTCGAAACCCGC-3′)/216k12gapFWD no. 1673 (5′-CTCTTACACAGCTGGAGGA-3′)]. Recombined df and dp alleles were detected by PCR using the following primers: df forward (5′-GCTCTATGCAATAATTAGCACG-3′)/df reverse (5′-CCGATTTTCACATAATGACACA-3′); dp forward (5′-CGGTGGATTTTCAGCTGAGA-3′)/dp reverse (5′-GCCAACAGCTGATCCGGAAACC-3′). Figs. S1 and S2 provide more details.

Microarray. Adult male C57BL/6N:129Sv F1 hybrid df/+; dp/+; and +/+ mice (obtained from 129Sv df/dp and cis pre-Cre/+ chimeras × C57BL/6N crosses) were euthanized at 20 to 24 wk of age, and the brains were surgically removed and immersed in PBS solution on ice. Each brain was cut into four distinct parts: olfactory bulbs, cerebellum, brainstem, and cortex, which were immediately flash-frozen in liquid nitrogen. Tissues were ground under liquid nitrogen by using a mortar and pestle, and RNA was extracted by using TRIzol reagent (Invitrogen) according to manufacturer instructions. We assessed the integrity and quality of the input RNA using the RNA 6000 Nano (Agilent) and the Total RNA assay, and assessed the size distribution of products at the amplified RNA and cDNA stage by using the mRNA assay. The samples were amplified and converted to cDNA using the Whole Transcript Expression kit (Ambion), fragmented and labeled with the Whole Transcript Sense Target Labeling Assay (Affymetrix), and hybridized to Mouse Gene 1.0 ST Affymetrix arrays. The arrays were hybridized and washed with the FS450 fluids station (Affymetrix) and scanned with the GC.S300 7G GeneChip Scanner (Affymetrix). Data were extracted using Expression Console (Affymetrix) with the RMA sketch workflow for gene level analysis. Log2 values of the intensity were further analyzed with Matlab.

Statistical Procedures for Microarray. Descriptive statistics. When indicated, principal component analysis was performed (princomp; Matlab, statistics toolbox).

Hypothesis testing. We performed two-way ANOVA (anovan; Matlab) with 16p11.2 dosage and brain region as main factors, whereby dosage is defined as being 1 (df/+ genome) or greater than 1 (+/+ or dp/+ genotypes) as explained in the main text. The P values of the dosage were corrected for multiplicity using the false discovery rate (FDR) linear step-up procedure (1).

Behavioral Analyses. Mouse health and weight. All mice used for the behavioral analyses were healthy, and all mice could reach the food bin (located on the ceiling of the cage) when they stood on their hindlimbs. Weights were measured for mice at 7 to 9 wk of age as well as after the HomeCageScan behavioral testing (32–35 wk of age). Weight ranges at 7 to 9 wk of age were as follows: df/+; 13 to 22 g; +/+; 16 to 27 g; df/dp, 17 to 28 g; and dp/+; 20 to 31 g. Weight ranges at 32 to 35 wk of age were as follows: df/+; 19 to 32 g; +/+; 22 to 40 g; df/dp, 22 to 39 g; and dp/+; 23 to 45 g. During gross dissection, we noticed that df/+ mice tended to be lean and, in many cases, had very little abdominal fat tissue compared with their littermates. This feature was not quantified systematically.

Description of cohort and behavior monitoring system. We analyzed behavior of cohort of 50 mice; these were progeny of multiple F1 crosses that originated with two chimeras; this cohort included +/+ (n = 15; four male and 11 female), df/dp diploid controls (n = 9; seven male and two female), df/+ (n = 13; six male and seven females), and dp/+ (n = 13; six male and seven female). This cohort was made up of male and female progeny from df/+ × dp/+ crosses; both parents were C57BL/6N:129Sv F1 hybrids. Thirty-nine of these mice were used for MRI following the behavioral analyses (as detailed later). Behavior was analyzed in four mice in parallel by placing individual mice into four polycarbonate cages with dimensions of 48 x 26 x 20 cm (Ancare R20 series) during the light period. Water and food were provided ad libitum on the wire bar of the ceiling of the cage. The behavior of each mouse was recorded and monitored with HomeCageScan (version 3.0; CleverSys) until 60 h after the beginning of the first dark period.

Response to change analysis. Data were exported in bins of 30 s, and the eight behavioral categories were calculated by summing the time or count of the relevant behaviors in each bin. Following Hen et al. (2) the categorized data were then smoothed using robust Lowess (malowess; Matlab, bioinformatic toolbox), and the median of each category was reported for each individual mouse. For hypothesis testing, each behavioral category was transformed
Data were exported in bins of \( \delta = 6 \) h, and eight different behavioral categories were generated by summing the time or count of the relevant behaviors in each bin. The sum of each category in each 12-h period was reported for each mouse (i.e., each mouse had two measures in light and three measures in dark for each of the eight categories). For hypothesis testing, each behavioral category was transformed (Dataset S3) to present an approximately normal distribution. Three-way ANOVA with light state, genotype, and sex as main factors was performed. The \( P_{\text{genotype}} \) value was reported.

**Multiple hypothesis correction.** Eight behavioral categories were compared between the cohorts. Each endpoint was measured twice: during the first 2 h and during the 60 h after the first onset of dark, making 16 comparisons. The 16 \( P_{\text{genotype}} \) values derived from these comparisons were subjected to the FDR linear step-up procedure (1), with an \( \alpha \) value of 0.05. Eleven of 16 null hypotheses were rejected, and therefore are significantly different between genotypes. To determine which groups differ from each other, we calculated Tukey confidence intervals for these 11 significant differences with an \( \alpha \) value of 0.05*(11/16) (3).

**Sociability test.** A cohort of 23 male mice, including +/- (+ = 6), df/dp diploid controls (\( n = 5 \)), df/+ (\( n = 6 \)), and dp/+ (\( n = 6 \)), was analyzed by using the three-channel sociability test as previously described (4). The time spent in each chamber was recorded. The difference between the time spent near a novel mouse vs. the time spent near a novel object (i.e., sociability) and between the time spent near a novel mouse vs. the time spent near a familiar mouse (i.e., novelty) was calculated for each mouse. The probability that a certain category does not distinguish between genotype groups (i.e., null hypothesis) was calculated using nonparametric ANOVA (Kruskall–Wallis; Matlab, statistics toolbox).

**Grip test.** A cohort of 28 male mice, including +/- (+ = 9), df/dp diploid controls (\( n = 6 \)), df/+ (\( n = 5 \)), and dp/+ (\( n = 8 \)), was analyzed. A grip-strength meter (Columbus Instruments) was used for the gripping test. Mice were allowed to grasp a triangular bar with their forelimbs, and gentle pressure was applied so that the mouse was pulled back horizontally. The test was repeated five times for each mouse, and the average value was recorded. The probability that a certain category does not distinguish between genotype groups (i.e., null hypothesis) was calculated using nonparametric ANOVA (Kruskal–Wallis; Matlab, statistics toolbox).

**MRI. Cohort.** Thirty-nine fixed mouse brains were examined, including male and female +/- (\( n = 9 \)); four male and five female), df/dp diploid controls (\( n = 8 \); six male and two female), df/+ (\( n = 11 \); five male and six female), and dp/+ (\( n = 11 \); six male and five female). This cohort was made up from progeny from df/+ × dp/+ crosses.

**Specimen preparation.** Mice were anesthetized with ketamine/xylazine and intracardially perfused with 30 mL of 0.1 M PBS solution containing 10 U/mL heparin and 2 mM ProHance, a gadolinium contrast agent (Bracco), followed by 30 mL of 4% paraformaldehyde containing 2 mM ProHance. After perfusion, mice were euthanized and the skin, lower jaw, ears, and the cartilaginous nose tip were removed. The brain and remaining skull structures were incubated in 4% paraformaldehyde plus 2 mM ProHance overnight at 4 °C, then transferred to 0.1 M PBS solution containing 2 mM ProHance and 0.02% sodium azide before MRI scanning (5).

**MRI acquisition.** A multichannel 7.0-T MRI scanner (Varian) with a 6-cm inner bore diameter insert gradient set (maximum gradient strength of 100 G/cm) was used to acquire anatomical images of brains within skulls. Three custom-built solenoid coils were used to image three brains in parallel (6). A 3D fast spin-echo sequence was used, optimized for the enhancement of gray/white matter contrast, with a repetition time of 325 ms, echo times of 10 ms per echo for six echoes, two averages, field of view of 14 × 14 × 25 mm\(^3\), and matrix size of 216 × 216 × 390, giving an image with 0.064 mm isotropic voxels. Total imaging time was approximately 1.5 h (5).

**Data analysis.** To visualize and compare brains with different dosages of 16p11.2, the brains were linearly (six-parameter followed by 12-parameter) and nonlinearly registered together, and a transform was created for each individual mouse. All scans were then resampled with the appropriate transform and averaged to create a population atlas representing the average anatomy of the sample. All registrations were performed by using the mni autoreg tools (7). The result of the registration is to have all scans deformed into exact alignment with each other in an unbiased fashion. This allows for the analysis of the deformations needed to take each individual mouse’s anatomy into the final atlas space, the goal being to model how the deformation fields relate to genotype (8). The determinants of the deformation fields were then calculated as measures of volume at each voxel. Significant volume changes are calculated by warping a preexisting classified MRI atlas onto the population atlas (9), which allows the volume of 62 segmented structures encompassing cortical lobes, large white-matter structures (i.e., corpus callosum), ventricles, cerebellum, brainstem, and olfactory bulbs to be assessed in all 39 brains. The 62 volumes were calculated as relative volumes (percentage of total brain volume). Family-wise error rate was controlled by using Holm correction for each genotype pair.

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Fig. S1. Molecular details of chromosome engineered df/+ and dp/+ mouse models of human 16p11.2. (A) In step 1, gene targeting at the 135k15 locus generates ES cells that are singly targeted. DNA fragments used for detecting homologous recombinants via Southern blot (blue) and PCR (green) are shown. (B) In step 2, gene targeting at the 216k15 locus in the singly targeted ES cells shown in A generates doubly targeted ES cells. The trans event, in which each targeting event had occurred on different chromosome 7 homologues, is depicted. DNA fragments used for detecting homologous recombinants via Southern blot (purple) and PCR (gold) are shown. (C) In step 3, Cre-mediated recombination in doubly targeted trans ES cells generates df and dp alleles in the same cell. df/dp ES cells were used to generate chimeric mice via blastocyst injection. Size of vector and targeted loci are shown in kilobases, and are drawn approximately to scale. Drug resistance at each step is depicted (HAT, puromycin-, and G418 resistance are shown as H⁺, P⁺, G⁺, respectively). XhoI (X) and EcoRI (E) restriction enzyme sites are indicated. LoxP sites (yellow triangles), puromycin (P) and neomycin (N) cassettes, 5’ and 3’ portions of the Hprt gene, as well as Agouti (Ag) and tyrosinase (Ty) coat color markers.
Fig. S2. Molecular details of Cre-mediated recombination in cis-targeted clones. Cre-mediated recombination in doubly targeted cells in the cis orientation, in which each targeting event had occurred on the same chromosome 7 homologue, generates the df allele. LoxP sites (yellow triangles), puromycin (P) and neomycin (N) cassettes, 5′ and 3′ portions of the Hprt gene, as well as Agouti (Ag) and tyrosinase (Ty) coat color markers. Size of vector and targeted loci are shown in kilobases, and are drawn approximately to scale. Drug resistance at each step is depicted (HAT-, puromycin-, and G418 resistance are shown as HR, PR, G R, respectively). Xhol (X) and EcoRI (E) restriction enzyme sites are indicated.

Fig. S3. Gene expression in the brain of 16p11.2 models depends on dosage. Brains from 16p11.2 mice and sibling controls were dissected into four regions: olfactory bulbs, cerebellum, brainstem, and cortex. RNA extracted from each brain region was subjected to genome-wide expression profiling using microarray. (A) Expression of genes mapping within the engineered region depends on copy number. The y axis shows the difference between dp/+ and +/+ (plotted as log₂ (intensity in mice with three copies / intensity in mice with two copies) of the region corresponding to 16p11.2), whereas the x axis shows the difference between df/+ and +/+ (plotted as log₂ (intensity in mice with one copy / intensity in mice with two copies) of the region corresponding to 16p11.2). Cyan dots represent each individual gene on the microarray; black circles represent mouse genes within the manipulated region. (B) Principal component analysis of genes within 16p11.2 indicates that dosage has the largest effect on expression. The first component (x axis) captures dosage effect, and the second component (y axis) captures brain region effect. Colors represent the genotype (red, df/++; green, +/+; blue, dp/+), and shape represents the different brain regions analyzed (asterisk indicates the cerebellum; plus sign the cortex; circle the olfactory bulb; star the brainstem). Note that the effect of 16p11.2 dosage on gene expression is much greater than the effect of brain region. In addition, gene expression is in opposite polarities in df/+ and dp/+, with deletion having the most pronounced effect. Dataset S2 and SI Experimental Procedures include the genes affected, more detail on the microarray procedure, and statistical analyses.
Fig. S4. Comparison of social behavior and grip strength in 16p11.2 CNV models. Comparisons of three-chambered social approach and grip strength in df/+, +/+, df/dp, and dp/+ cohorts of male mice are shown. (A) Box plots show the difference in time spent near novel mouse vs. time spent near novel object for each cohort. The genotype effect was not significantly different between the cohorts ($P = 0.3725$, Kruskal–Wallis test). (B) Box plots show the difference in time spent near familiar mouse vs. time spent near novel mouse for each cohort. The genotype effect was not significantly different between the cohorts ($P = 0.1229$, Kruskal–Wallis test). (C) Box plots show grip strength for each cohort. The genotype effect was not significantly different between the cohorts ($P = 0.7821$, Kruskal–Wallis test).

Fig. S5. Comparison of voxel-wise changes among three genotype cohorts. Voxel-wise differences (contraction, red; expansion, blue) are shown between df/+ and +/+ cohorts (Left) and between df/+ and dp/+ cohorts (Right). Brighter colors depict a greater difference, as indicated by the FDR bar in the middle. Note that the same regions are affected in both comparisons, but that the differences are much greater between df/+ and dp/+ than between df/+ and +/+. 
Fig. S6. Expression of MapK3 in the brain of +/- mice. Parasagittal sections were prepared from brains of +/- mice and analyzed for MapK3 expression by immunohistochemistry by using a monoclonal antibody against the C-terminal region of MapK3 (EM2331; ECM Biosciences). Shown is a representative section of MapK3 expression (brown). Subcortical gray matter structures and tracts that show the strongest expression at this plane of section and that are relevant to the volumetric changes noted by MRI and behavioral abnormalities described in the text include the caudate-putamen (c-p) or striatum, a combined structure that receives inputs from the substantia nigra (sn); the nucleus accumbens (na), a structure associated with reward, pleasure, addiction, aggression, and fear via connections with the basal ganglia, amygdala, and substantia nigra; the stria terminalis (st), a major output pathway of the amygdala that runs to the ventral medial nucleus of the hypothalamus; the nigrostriatal bundle (ns) that connects the substantia nigra with the striatum; and the lateral hypothalamus (lh), predominantly associated with hunger and a region showing significant enlargement in the df/+ mice in this study. This pattern of expression in structures and their subcortical projections suggest a possible role in the movement disorders seen in the df/+ mice. Other regions of strong MapK3 expression include the molecular layer of cortex, primarily motor (c); molecular layer of the hippocampal dentate gyrus (dg) and mossy fiber projections (mf); and the external plexiform layer of the olfactory bulb (ep) and olfactory tubercle (ot).

Table S1. Transmission of 16p11.2 deficiency and duplication alleles

<table>
<thead>
<tr>
<th>Mating Stage</th>
<th>Expected (%)</th>
<th>Observed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/-</td>
<td>df/+ or dp/+</td>
</tr>
<tr>
<td>df/+×C57BL/6N</td>
<td>Weaning</td>
<td>33</td>
</tr>
<tr>
<td>df/+×129Sv</td>
<td>Weaning</td>
<td>65</td>
</tr>
<tr>
<td>df/+×C57BL/6N</td>
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</tr>
<tr>
<td>dp/+×C57BL/6N</td>
<td>Weaning</td>
<td>51</td>
</tr>
</tbody>
</table>

NS, not significant.

*M=Studs were F1 C57BL/6N×129Sv hybrids (50% C57BL/6N, 50% 129Sv).
†=Studs were F1 129Sv (100% 129Sv).
‡=Studs were N2 (F1 C57BL/6N×129Sv hybrid)×C57BL/6N (75% C57BL/6N, 25% 129Sv).
§=Includes embryos at E13.5 (two litters) and E17–E18.5 (three litters).

Movie S1. Nonprogressive behavior of 16p11.2 deletion mice. Climbing episodes of +/- (Right) and df/+ (Left) mice during the first 10 h of the experiment. At the beginning of the trial, both +/- and df/+ mice climb to the lower part of the cage, returning to the floor with their hindlimbs leading. Later on in the trial, +/- mice progress until they reach the highest point of the ceiling of the cage, returning to the floor from different spots with their forelimbs leading. In contrast, df/+ mice lack this progressive behavior, exhibiting the same behavior as earlier in the trial. Quantification of behavior in individual mice is provided in Table 1.

Movie S1
Movie S2. Behavioral trap on the ceiling of 16p11.2 deletion mice. Four female sibling mice (+/+, Upper Left; df/+, Upper Right; dp/+, Lower Left; df/dp, Lower Right) housed in individual cages were recorded simultaneously. Shown is a 21-min episode (with speed increased by a factor of four). Controls (+/+ and df/dp) readily adapt their climbing abilities and move freely around the larger cages, and readily go up to and down from the ceiling. In contrast, df/+ mice frequently become trapped on the ceiling, and are unable to climb down. Eventually, the df/+ mouse comes down from the lower part of the ceiling.

Movie S3. Repetitive behavior of 16p11.2 deletion mice. Shown are 15 sequential climbing episodes in which the df/+ mouse uses a repetitive, stereotypical behavior to climb down from the ceiling; this behavior is not observed in control mice.

Dataset S1. Genes mapping to the engineered region of mouse chromosome 7 and their human homologues

Dataset S2. Genes differentially expressed in brains of 16p11.2 mice

Dataset S3. Behaviors affected in 16p11.2 mice

Dataset S4. Volumetric changes in the brain detected by MRI