Abnormal social behaviors and altered gene expression rates in a mouse model for Potocki-Lupski syndrome

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The Potocki-Lupski syndrome (PTLS) is associated with a microduplication of 17p11.2. Clinical features include multiple congenital and neurobehavioral abnormalities and autistic features. We have generated a PTLS mouse model, *Dp(11)17/+*, that recapitulates some of the physical and neurobehavioral phenotypes present in patients. Here, we investigated the social behavior and gene expression pattern of this mouse model in a pure C57BL/6-*Tyr^{c-Brd}* genetic background. *Dp(11)17/+* male mice displayed normal home-cage behavior but increased anxiety and increased dominant behavior in specific tests. A subtle impairment in the preference for a social target versus an inanimate target and abnormal preference for social novelty (the preference to explore an unfamiliar mouse versus a familiar one) was also observed. Our results indicate that these animals could provide a valuable model to identify the specific gene(s) that confer abnormal social behaviors and that map within this delimited genomic deletion interval. In a first attempt to identify candidate genes and for elucidating the mechanisms of regulation of these important phenotypes, we directly assessed the relative transcription of genes within and around this genomic interval. In this mouse model, we found that candidates genes include not only most of the duplicated genes, but also normal-copy genes that flank the engineered interval; both categories of genes showed altered expression levels in the hippocampus of *Dp(11)17/+* mice.

INTRODUCTION

Autistic spectrum disorder (ASD) represents a group of neurodevelopmental disorders defined by three fundamental features: aberrant reciprocal social interaction, deficits in social communication and stereotypic and ritualistic behaviors. Although autism is recognized as an entity by itself, it is also present in several neurodevelopmental syndromes such as Rett, Fragile-X, Angelman, Prader-Willi and Joubert syndromes. Thus, the study of these syndromes holds promise for understanding the pathogenesis of autism. Another such syndrome that includes autistic features is the Potocki-Lupski Syndrome (PTLS; MIM 610883).

PTLS is associated with microduplication in chromosome 17 (p11.2p11.2) (1). The reciprocal 17p11.2 microdeletion is associated with Smith-Magenis Syndrome (SMS) (2), a well-characterized syndrome that comprises several congenital and neurobehavioral anomalies (3). Point mutations in the *Retinoic Acid Inducible* 1 gene (*RAI1*), a gene within the SMS and PTLS critical genomic interval, were identified in patients with clinical presentation of SMS but no molecular deletion found by fluorescent *in situ* hybridization (4–6), suggesting that *RAI1* is the dosage-sensitive gene causative for SMS. No single gene was yet associated with PTLS, but molecular data (7) and studies of a PTLS mouse model (8) suggest that *RAI1* could also be the dosage-sensitive gene responsible for most of the phenotypes observed in PTLS patients.

Autism is an important component of the clinical presentation of PTLS, observed in ~80% of evaluated patients

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(7,9). Autistic features seen in PTLS patients included decreased eye contact, motor mannerisms or posturing, sensory hypersensitivities, repetitive behaviors, lack of appropriate functional or symbolic play and joint attention. Moreover, qualitative impairment of reciprocal social interaction, poor communication, repetitive behaviors and stereotyped patterns and abnormal development were objectively observed before 36 months. Autism Diagnostic Interview–Revised (ADI-R), Autism Diagnostic Observation Schedule–Generic (ADOS-G) and ADOS-module 2 tests reached the criteria for autism in each case examined. Apart from autism, other clinical findings in PTLS include multiple congenital and neurobehavioral abnormalities, such as CNS abnormalities by magnetic resonance imaging, microcephaly, cognitive impairment, low adaptive function and language impairment (1,7,9).

Human chromosome 17p11.2 is syntenic to the 32–34 cM region of murine chromosome 11 (10). We have generated a mouse model for PTLS syndrome, $Dp(11)17/+$, that carries a duplication of a region of ~3 Mb, syntenic to the PTLS region. $Dp(11)17/+$ mice proved to be a valuable model in which to identify *Rai1* as the dosage sensitive gene whose copy number variation (CNV) is responsible for different phenotypes observed in PTLS (8,11,12).

The objective of this study was to assess the social behavior in a PTLS mouse model and to investigate the level of expression of genes within the rearranged genomic interval to identify candidate genes potentially related to PTLS social phenotypes as a first attempt to relate gene CNV and expression levels of the involved genes.

RESULTS

General health, reflexes, home-cage behavior and olfactory capabilities of *Dp(11)17/+* mice

General health and reflexes were previously tested for $Dp(11)17/+$ mice in a mixed C57BL/6-Tyr^{c-Brd} × 129S5/SvEvBrd genetic background, and no significant differences were found between them and wild-type littermates. We re-tested these parameters in a pure genetic background (C57BL/6-Tyr^{c-Brd}) to assess the potential impact of genetic background on phenotype. No differences were observed between $Dp(11)17/+$ and wild-type littermates in coat condition, in the presence of whiskers and piloerection. Reactions to a gentle touch from a cotton swab to the whiskers and the visual placing reflex were within the normal range (Table 1). While performing these experiments, we noticed that 100% of wild-type mice vocalized during handling, compared with only 55% of $Dp(11)17/+$ mice ($P = 0.023$). As vocalization is part of a normal response to stress, we tested a new batch of wild-type mice ($n = 10$) with a different researcher handling them to investigate if the stress factor was introduced by the particular experimenter. Nevertheless, we again observed a high percentage of vocalization (70%) [no significant difference between the two wild-type groups ($P > 0.05$)].

The olfactory capacity of these mice was tested by assessing their ability to find buried food. Fasted mice from both genotypes were successful in locating and retrieving hidden food with no significant differences ($P > 0.5$), indicating that both groups are capable of smelling and finding food. Both

Table 1. General health, reflexes, home-cage and nesting behavior and olfactory capabilities observed in $Dp(11)17/+$ mice

Physical characteristics	Wild type	$Dp(11)17/+$
Poor coat condition (%)	22	0
Whiskers (% individuals with full whiskers)	100	100
Piloerection (%)	0	0
Neurological reflexes		
Visual placing (%)	100	100
Vibrissae orienting (%)	100	89
Vocalization during handling (%)	100	55*
Olfaction		
Uncover buried food (% individuals)	100	100
Latency to uncover buried food (sec)	32.7 ± 7.6	26.8 ± 5.6
Home-cage behaviors		
Sleeping (no. of episodes)	33	24
Together (no. of observations)	9	6
Tight (no. of observations)	23	24
Huddled (no. of observations)	6	0
Feeding (no. of episodes)	10	8
Activity (no. of episodes)	12	18
Self-grooming (no. of episodes)	9	12
Grooming others (no. of episodes)	3	5
Nest building		
1 h later (%)	29	0
6 h later (%)	100	50*

Physical characteristics, neurological reflexes and olfaction capability were measured for wild type ($n = 9$) and $Dp(11)17/+$ ($n = 9$) mice. The number of episodes of each action is shown in the table. For sleeping patterns, the following parameters were followed: 'together', mice in the same spot sleeping but no physical contact between them; 'tight', mice sleeping side by side with physical contact; 'huddled', mice sleeping one on top of the others. *$P < 0.05$.

groups of mice were equally motivated to eat, since every mouse continued eating when the test finished.

We systematically evaluated home-cage behavior as described in the Materials and Methods section. Nineteen wild-type mice housed in seven cages, and 16 $Dp(11)17/+$ mice housed in six cages were observed. Overall $Dp(11)17/+$ mice seemed more active than their wild-type littermates, displaying over the observation period 42 episodes different from sleeping (feeding, walking, self grooming or grooming other cage mates) compared with only 34 episodes displayed by their wild-type littermates. The 'episodes' do not pertain to a specific cage or animal but are a summary of the activities observed in all the mice of a specific genotype. No overt aggressive behaviors were ever observed. When sleeping, mice from both genotypes tend to group together in the same place in the cage and stay tightly 'huddled'. As a measure of home-cage activity related to social behavior, we evaluate nesting behavior by adding a paper towel to each cage. After the first hour, 29% of the cages housing wild-type mice showed clear evidence of nest building, while none of the cages housing $Dp(11)17/+$ mice did ($P > 0.05$). After six and a half hours, a nest was observed in 100% of the cages housing wild-type mice but only in 50% of the cages with $Dp(11)17/+$ ($P = 0.026$). Despite these differences in nest building velocity, nesting behavior was normal for both genotypes as every mouse was inside the nest during resting periods.
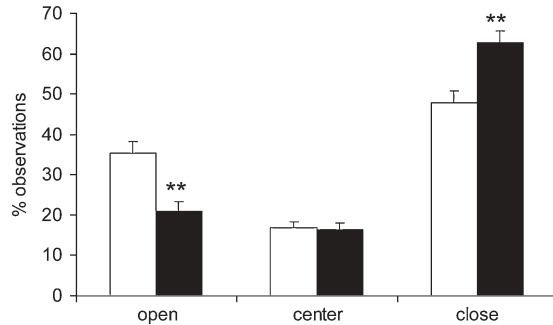
Figure 1. Plus maze test. The percentage of observations in each arm or the center of the plus maze is represented. White column: wild type (*n* = 19), black column: *Dp(11)17/+* animals (*n* = 16). Values represent mean ± SEM. The asterisk denotes significant differences from their wild-type littermate (**$P = 0.001$).

Figure 2. Sociability and social novelty preference in *Dp(11)17/+* mice. (**A**) Percentages of observations in the chamber side with stranger 1 (black columns) or with the empty container (white columns) during the sociability test are shown. (**B**) Percentages of observations in the chamber side with stranger 1 (black columns) or with stranger 2 (grey columns) during the preference for social novelty test are depicted. The mean ± SEM values are presented. Asterisk denotes significantly different from their respective wild type (*$P < 0.05$, **$P < 0.01$) (*n* = 14 for each genotype).

Increased anxiety-like behavior in the plus maze test

Anxiety is a feature commonly presented by individuals with autistic spectrum disorders (13) and is a prominent symptom in PTLS patients. We subjected *Dp(11)17/+* mice to the elevated plus maze test, a well-characterized test to measure anxiety-related behaviors in mice, that takes advantage of the conflict faced by the mice between their tendencies to actively explore a new environment versus the aversive properties of an elevated open runway. Analysis of the data indicate that *Dp(11)17/+* mice spend more time in the closed arms (62 ± 3%) than their wild-type littermates (47.8 ± 2.8%) [$t(33) = 3.58$, $P = 0.001$]. In addition, the percentage of observations in the open arms was significantly diminished for *Dp(11)17/+* mice (21 ± 2.3%), compared with wild types (35.3 ± 3) [$t(33) = 3.6$, $P = 0.001$] (Fig. 1), indicative of increase anxiety in *Dp(11)17/+* mice and fortifying previous findings (Supplementary Material, Table S1).

Sociability and social novelty

Mice are highly social individuals. Sociability in mice, as well as their preference for social novelty, could be assessed by the use of the three-chamber test (14), based on the tendency of a subject mouse to approach and engage in social interaction with an unfamiliar mouse. We tested *Dp(11)17/+* and wild-type littermates in the three-chamber test. To exclude any environmental interference within the social test chamber, we evaluated the percentage of observations for each genotype in each compartment during the habituation period, and no chamber preference was evident ($P > 0.05$).

The analysis of the sociability data showed a significant main effect of chamber side ($F_{(1,26)} = 9.08$, $P = 0.005$). *Post hoc* analysis demonstrated that wild-type mice spend more time in the chamber side that contains the stranger 1 versus the side with the empty container ($P = 0.018$) (Fig. 2A). *Dp(11)17/+* mice showed no significant difference in side preference, suggestive of a subtle impairment in the preference for a social target versus an inanimate target for *Dp(11)17/+* mice ($P = 0.07$). To exclude any environmental interference during the sociability part of the test, the stranger 1 and the empty cage were placed alternatively in the left or right side of the test chamber. There was no significant
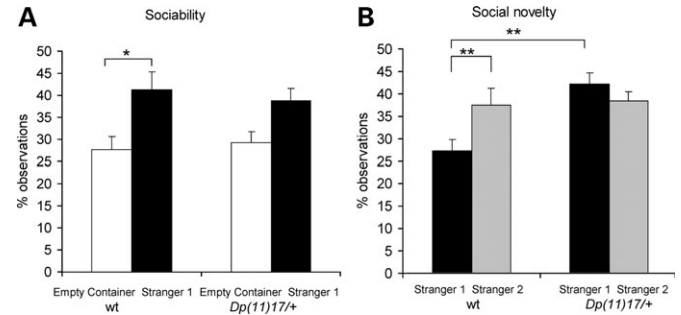
difference in the number of observations with the stranger 1 for both genotypes if it was placed in the compartment 1 or 3 of the test chamber ($P > 0.05$).

We then analyzed the preference for social novelty data and observed a significant main effect for genotype ($F_{(1, 26)} = 7.121$, $P < 0.021$). *Post hoc* analysis revealed that wild-type mice spent significantly less time in the side of the stranger 1 than the *Dp(11)17/+* mice ($P = 0.00003$). Wild-type mice tend to spend significantly more time with stranger 2 than with stranger 1 ($P = 0.03$), but *Dp(11)17/+* mice spent the same amount of time with the stranger 1 as with the stranger 2 ($P > 0.05$), showing an impairment in response to social novelty (Fig. 2B).

When the number of sniffing episodes were analyzed, significant differences were found with a main effect of chamber side ($F_{(3,78)} = 13.5$, $P < 0.0000001$). *Post hoc* analysis showed that wild-type and *Dp(11)17/+* mice sniff significantly more times the stranger 1 than the empty container in the sociability test ($P = 0.0025$ and 0.012, respectively) (Fig. 3A). In the preference for social novelty test, both genotypes showed more sniffing episodes towards the stranger 2 than stranger 1 ($P = 0.0066$ for wild type and $P = 0.0025$ for *Dp(11)17/+* mice) (Fig. 3B).

Increased dominance in *Dp(11)17/+* mice

To further evaluate social interactions, we used the tube test, a paradigm previously found to be useful in predicting impairments in social interaction (15,16). Two mice of different genotypes are positioned at the opposite end of an acrylic tube and released to meet inside. After a period of time during which the animals explore each other, one mouse backed out of the tube, ending the test. This test is repeated with the same pair of mice for a second round, switching the side into which each mouse starts in order to avoid bias. As can be seen in Figure 4, amazingly *Dp(11)17/+* mice backed out 10% of the times (1/10) in the first round, and 0% of the times (0/10) in the second round when confronted with wild-type mice (Supplementary Material, video), demonstrating a dominant behavior for *Dp(11)17/+* mice. No aggressive behaviors
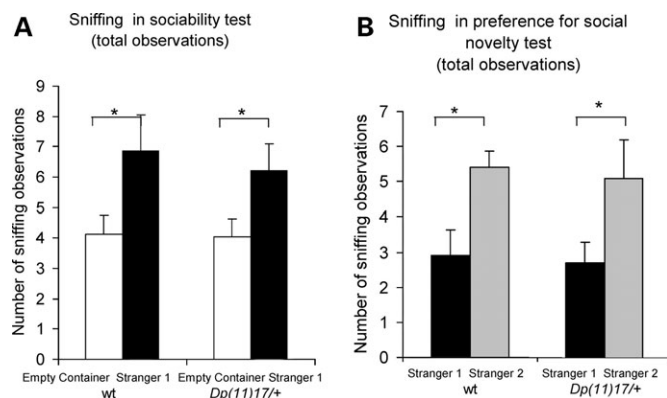
Figure 3. Sniffing episodes during the sociability and social novelty preference test. Representations of the total number of sniffing observations (**A**) in the chamber side with stranger 1 (black column) or with the empty cage (white column) during the sociability test and (**B**) in the chamber side with stranger 1 (black column) or with stranger 2 (grey column) during the preference for social novelty test. The mean ± SEM values are presented. Asterisk denotes significantly different from their respective wild type. (*$P \le 0.01$) ($n = 14$ for each genotype).
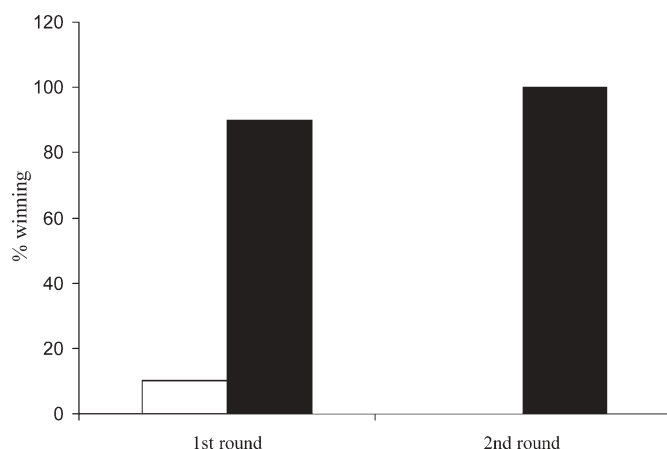

Figure 4. Tube test for social dominance. The results for the first and second round are depicted as the percentage of winning for each genotype. Wild type mice (white columns, $n = 10$) and $Dp(11)17/+$ mice (black columns, $n = 10$) are represented.

during the encounters was observed, and we confirmed that $Dp(11)17/+$ were capable of backing-up. We also noted that, during the training part of the test, wild-type mice tend to walk out of the tube on their own more often than the $Dp(11)17/+$ mice, but this was not systematically evaluated.

Decreased brain weight in adult $Dp(11)17/+$ mice

Increased brain weight in childhood of some autistic patients has been reported. Also, brain size in adulthood has been reported as normal, thus implying an abnormal growth rate of the brain for some autistic patients (17). We evaluated brain weight in our mouse model. At 6 weeks of age, significant difference was found in total body weight between $Dp(11)17/+$ (17.8 ± 1.2 g) and wild-type mice (20.7 ± 0.5 g) ($t(13) = 7.8$, $P = 0.04$), but no significant differences
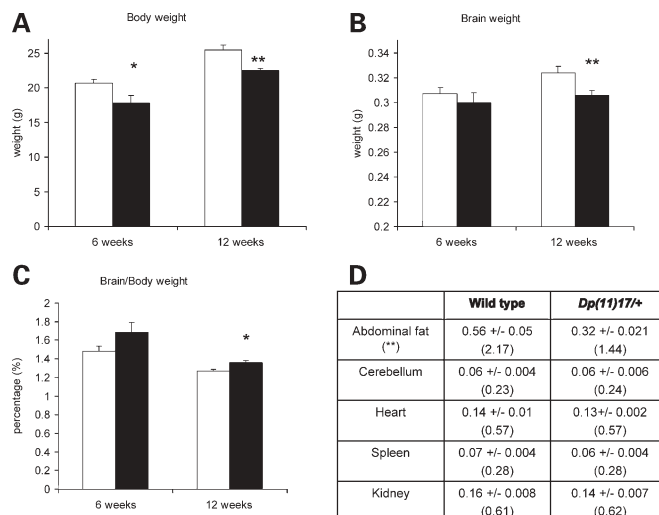

Figure 5. Weight differences in $Dp(11)17/+$ mice. (**A**) The total body weight and (**B**) brain weight were obtained when the animals were 6 weeks old, wild type ($n = 7$) and $Dp(11)17/+$ ($n = 8$) or 12 weeks old, wild type ($n = 7$) and $Dp(11)17/+$ ($n = 7$). (**C**) Representation of brain weight as a percentage of body weight. For all the figures, white columns represent the wild type values while $Dp(11)17/+$ values are represented by black columns. (**D**) A table showing the average weight (in g) found for each genotype at 12 weeks [wild type ($n = 7$) and $Dp(11)17/+$ ($n = 7$)] for every weighed organ. The values in brackets indicate the percentage of the total body weight for each organ. The values represent mean ± SEM, *$P < 0.05$, **$P < 0.01$.

were found in any of the organs weighed ($P > 0.05$). At 12 weeks, there is a significant difference in body weight between $Dp(11)17/+$ (22.5 ± 0.34 g) and wild-type mice (25.5 ± 0.72 g) ($t(12) = 3.7$, $P = 0.05$) (Fig. 5A). The brain was the only organ showing a significant difference between $Dp(11)17/+$ (0.3 ± 0.04 g) and wild-type mice (0.32 ± 0.05 g) ($t(12)=3.06$, $P = 0.01$) (Fig. 5B). Total abdominal fat weight was significantly decreased in $Dp(11)17/+$ mice. The percentage of total body weight that corresponds to brain weight was significantly different ($P = 0.017$) at 12 weeks of age between $Dp(11)17/+$ mice and wild-type mice (1.36 versus 1.27%, respectively) (Fig. 5C and D).

PTLS mice gene profiling

To identify gene(s) that are modified in their relative expression levels in the PTLS mouse and map to the rearranged region, i.e. possible candidate dosage-sensitive genes potentially responsible for the PTLS-like phenotypes, we compared the transcriptome of these mice with that of normal littermates. The medial temporal lobes of the brain perform primary roles in the formation and storage of emotional memories. Because some of the structures that comprise the amygdalae and hippocampi were shown to be altered in autistic patients (18), we profiled the hippocampi of three $Dp(11)17/+$ and three wild-type males by hybridize them to GeneChip mouse genome 430 2.0 Affymetrix arrays. These arrays assess the relative expression levels of 45 101 probe sets. The data discussed in this paper have been deposited in NCBIs Gene Expression Omnibus (GEO,

http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE11013.

We ranked the most differentially expressed probe sets between *Dp(11)17/+* and wild-type genotypes (Supplementary Material, Table S2). Fourteen of the top 50-, 17 of the top 100- and 25 of the top 500-ranked differentially expressed probe sets are mapping to the engineered MMU11B2 interval. A highly significant propensity ($P < 1.8 \times 10^{-15}$) in all three cases was found. These probe sets correspond to the following genes from centromere to telomere: *Nt5m, Med9, Rai1, Srebf1, Tom1l2, Atpaf2, 4933439F18Rik, Drg2, Alkbh5, AW215868, Llgl1, Flii, Smcr7, Smcr8, Dhrs7b, Tmem11, Gtlf3b, Map2k3, Usp22* and *Slc47a1*. An excellent correlation was found in the five cases where multiple probe sets targeted the same gene. All but one (RIKEN cDNA 1300013J15/*Slc47a1*) of these genes are over expressed in the hippocampal structures of *Dp(11)17/+* mice (Fig. 6). They are expressed an average of 1.42 ± 0.13-fold more in the *Dp(11)17/+* animals (range, $1.25-1.66$-fold; *Slc47a1* excluded), a value close to the theoretically 'expected' 1.5-fold value. Other genes mapping to the PTLS region are either below the detection range of the array (*Rasd1, Pemt, Top3a, Myo15, Gtlf3a, Tnfrsf13b* and *Aldh3a1*), over expressed in the PTLS model animals albeit not in a statistically significant way (*Lrrc48, Shmt1, Kcnj12* and *Zfp179*; average, 1.38 ± 0.05-fold; range, $1.34-1.45$-fold) or expressed at the same level in both genotypes (*Aldh3a2*) (Fig. 6, Supplementary Material, Table S2). The *loxP* site inclusion necessary for the mouse engineering induced a loss-of-function of one *Cops3* copy (11), thus *Dp(11)17/+* animals have only two active copies of this gene. Consistently, we found no differences in *Cops3*-relative expression level between the PTLS model and control littermates.

To confirm the expression array results, the relative expression levels of seven genes mapping to the rearranged region were measured by Taqman QPCR. Four were significantly overexpressed, two were overexpressed but not significantly and one showed no differences in the array readout (Fig. 7). We found an excellent reproducibility of the data for the three genes that were quantified with two different Taqman assays. The real-time amplification results confirm the overexpression of *Rai1, Srebf1, Drg2, Llgl1, Shmt1* and *Zfp179* in *Dp(11)17/+* males, although the differences between the studied genotypes sometimes are magnified with this technology. On the contrary, *Aldh3a2* that appeared unchanged in the arrays, by QPCR, has a significantly increased relative expression level in animals with the duplication. Furthermore, we confirmed the lack of change in *Cops3* expression. We were able to replicate these experiments in a second population: hippocampi from *Dp(11)17/+* and wild-type females (data not shown). Thus, all but one of the genes duplicated and expressed in the hippocampus (RIKEN cDNA 1300013J15/*Slc47a1*) show ~50% increase in relative expression in the *Dp(11)17/+* mice.

Interestingly, 6, 12 and 37 genes mapping to the flanks of the engineered interval (up to 20 Mb from the breakpoints) were also part of the top 50-, 100- and 500-ranked genes in the hippocampus microarrays (e.g. *Zfp39, Mrpl55, Fbxo39, Cyb5d2, Gosr1, Gemin4, Alox8, Zfp207, Ccnjl, Dlgh4* and *Vtn*) (Supplementary Material, Table S2). A proportion
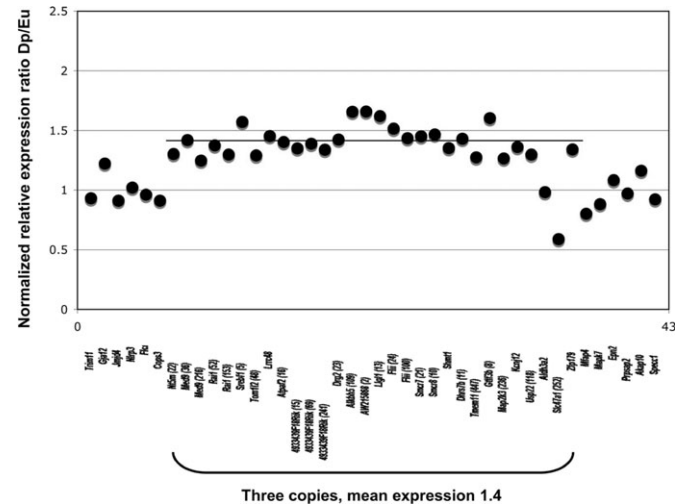

Figure 6. Gene expression levels of genes duplicated in the PTLS mouse model. The ratio of average relative expression levels measured in the hippocampus from *Dp(11)17/+* and wild-type male mice using Affymetrix Gene-Chip arrays. All the genes mapping to the engineered interval and detected are shown. The horizontal line denotes their 1.4-fold mean expression. Numbers in brackets specify the ranking of significantly differently expressed genes. Note that some genes were surveyed by multiple probe sets. Dp, *Dp(11)17/+*; Eu, euploid.
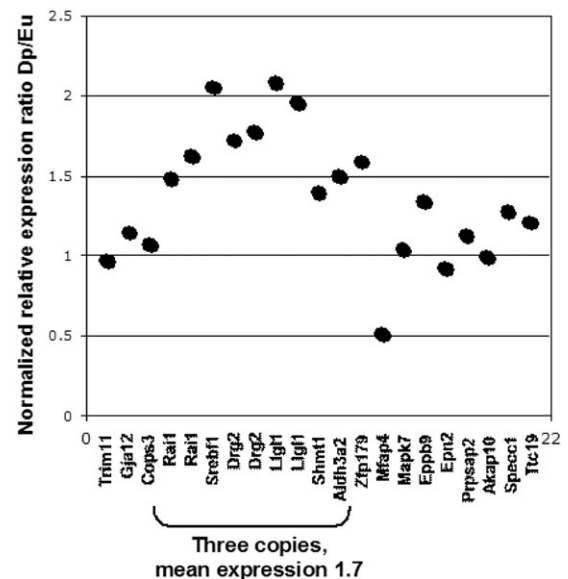

Figure 7. Gene expression levels of genes duplicated in the PTLS mouse model. The ratio of average relative expression levels measured in *Dp(11)17/+* and wild-type male mice hippocampus with Taqman real-time PCR. All the assessed duplicated genes are overexpressed by 50% in the PTLS mice. Note that some genes were surveyed by multiple probe sets. Dp, *Dp(11)17/+*; Eu, euploid.

significantly higher than expected by chance ($P < 2 \times 10^{-5}$) in all three cases was found. To confirm these results, 10 genes mapping close to the breakpoint, but not altered in copy number [two positioned centromerically, *Trim11* and *Gja12*, and eight positioned telomerically, *Mfap4, Mapk7, Eppb9, Epn2, Prpsap2, Akap10, Specc1* and *Ttc19*, were assessed by Taqman real-time (Fig. 7)]. Three of these

neighboring genes, *Mfap4, Ttc19* and *Gja12*, showed altered expression in PTLS mice hippocampi, consistent with the data obtained by microarray expression profiling. Thus, some of the normal-copy genes likely in *cis* with the duplication are affected in their expression.

DISCUSSION

PTLS is associated in humans with duplication of chromosome 17(p11.2p11.2) [dup (17)(p11.2p11.2] and presents multiple clinical symptoms, including autistic features, several behavioral abnormalities, mild to borderline mental retardation, attention deficit disorder and hyperactivity, and reduced insight. We developed and described a mouse model for PTLS, *Dp(11)17/+* mice, carrying a genomic duplication of the mouse genomic region syntenic to the human duplicated region. This mouse model recapitulates several physical and behavioral aspects present in PTLS patients and has proven to be very useful to assess *Rai1* as the dosage-sensitive gene that confers most of the phenotypes, including low body weight, increased anxiety and learning and memory deficits (8). Most behavioral phenotypes previously described in *Dp(11)17/+* mice could be corrected when the gene dosage within the interval was normalized *(Dp(11)17/ Df(11)17)* or by the sole normalization of *Rai1* gene copy number within the interval *(Dp(11)17/Rai1⁻)* in a mix genetic background. In spite of this identification of *Rai1* as the dosage-sensitive gene, the actual expression levels of *Rai1* on the engineered mice were never determined. In this study, we report that *Rai1* shows increased expression in the hippocampus of *Dp(11)17/+* mice, suggesting that overexpression of *Rai1* might underlay the gene copy number effect involved in the major pathogenic features of PTLS (see below).

To study the validity of this mouse model in recapitulating the autistic component of PTLS, we challenged *Dp(11)17/+* animals in a variety of tests designed to assess social behaviors in mice, such as the elevated plus maze test, the sociability and preference for social novelty test and the tube test, in addition to observing home-cage and nesting behaviors.

The analysis of the home-cage behaviors exhibited by the mice revealed no differences between wild-type and *Dp(11)17/+*, except for the fact that *Dp(11)17/+* mice seemed more active. Anxiety is a feature commonly presented by individuals with ASD (13). We found that *Dp(11)17/+* mice presented increased anxiety-related behaviors in the elevated plus maze, compared with their wild-type littermates. This result reinforces the presence of elevated anxiety in *Dp(11)17/+* mice, prompting us to perform more specific measurements of social interactions.

We therefore subjected the mice to the sociability test, in which the *Dp(11)17/+* mice showed a subtle impairment in the preference of a social target versus an inanimate target. Further, *Dp(11)17/+* mice had a clearly abnormal response in a social novelty test. This lack of preference for social novelty demonstrated by *Dp(11)17/+* mice could be analogous to the reported aberrant reciprocal social interactions in some autistic individuals, including an indiscriminate approach to strangers and family or friends (19). Another

possible explanation is that these mice have a defect in short-term memory and thus are not able to remember that they had previously engaged in interactions with the stranger 1 mouse. Interestingly, a similar phenotype to the one observed by us was recently found in a complexin 1 (*Cplx1*) knockout mouse (20). Abnormal expression of complexin 1 (a presynaptic protein that modulates neurotransmitter release) is seen in several neurodegenerative and psychiatric disorders in which disturbed social behavior is often found, including schizophrenia. In this context, it is interesting to note that CAG repeat polymorphisms in *RAI1* were shown to be associated with both the severity of the phenotype and the response to medication in schizophrenic patients (21).

Despite the lack of preference for social novelty, *Dp(11)17/+* mice sniffed more frequently the stranger 1 than the empty cage, indicating their ability to discriminate another mouse versus an inanimate object. Furthermore, *Dp(11)17/+*, like wild-type mice, exhibited more sniffing episodes toward the unfamiliar mouse than toward the familiar one, demonstrating recognition of a previously encountered mouse. These data rule out a prominent short-term memory deficit and could be interpreted as if *Dp(11)17/+* mice engage in some form of social interaction. However, although sniffing is a common component of social interactions, it is also an important part of the investigatory strategy for mice, and thus the observed difference could originate more from the richness of the olfactory stimuli than from the social cue itself. In the report by Moy *et al.* (22), some strains of mice also presented this dissociation between the time spent in a certain chamber side and the sniffing episodes.

Another interesting social phenotype found in *Dp(11)17/+* mice was that in the tube test *Dp(11)17/+* mice always stayed inside the tube, forcing the wild-type mice to retreat. The tube test was originally developed to evaluate dominance hierarchies in mice (23). While mice typically use aggression to establish dominance, it is unclear whether the tube test performance is directly related to aggression. As the tube we used was of clear plastic, we could directly observe the interactions in our tests and did not see any aggressive behaviors. Only pushing, or just standing there without letting the wild-type pass, was what we observed. Also, we observed that *Dp(11)17/+* mice sometimes showed 'backing-up' behavior. Thus, we suggest that the tendency of *Dp(11)17/+* mice to stay inside the tube is not likely due to differences in aggressive behavior or to the impossibility of retreating, but more consistent with immobility related to a failure in recognizing the social cues that usually guide encounters between unfamiliar mice or increased anxiety.

There are reports relating brain size and autistics behaviors. We explored this parameter in our mouse model and found no difference in brain weight for *Dp(11)17/+* mice at 6 weeks of age, but at 3 months the absolute brain weight in *Dp(11)17/+* mice was significantly lower than their wild-type littermates. Interestingly, this was the only organ that showed weight reduction. In spite of the *Dp(11)17/+* mice brains weighting less than wild-type brains in absolute values, when the brain weight/total weight ratio was analyzed, the percentage of weight corresponding to the brain in *Dp(11)17/+* mice was significantly higher than the wild-type mice. Since, at both ages studied,

Dp(11)17/+ mice presented a lower body weight than their wild-type littermates, these results suggest an overgrowth of the brain in this mouse model.

There are numerous examples of animal models for autism that give insights into the complexity related to this disorder reviewed by Moy and Nadler (24). Classical syndromes associated with autistic features such as Rett, Fragile-X (FXS) and Angelman syndromes have their counterparts in mouse models that in most cases presented social impairments. In the case of the FXS mouse model, the loss of *Fmr1* gene function resulted in altered anxiety and social behavior in mice (16,25). In the case of Rett syndrome, several mouse models have been produced, each presented abnormalities of social interactions and home-cage behavior (26,27). For both mouse models, the performance in the test tube for dominance was abnormal (15,16). These mice represent a powerful tool for further defining the pathogenesis of the disease and the molecular basis of the social abnormalities observed both in mice and humans.

An enormous amount of effort has been placed on discovering associations between genetic variants, both SNP and gene CNV, and ASD by several groups around the world (28–30). Nevertheless, none of the specific genes undergoing CNV have been identified. Interestingly, in these screenings, the association between CNV at 17p12 and autism has been reported. Currently, it is not known whether the autistic phenotype observed in the PTLS [17(11.2p11.2p)] patients is due to one or several dosage-sensitive genes within this delimited genomic region. There are ~23 genes within the critical genomic interval. Compound heterozygous mice carrying a duplication *Dp(11)17* along with a null allele of *Rai1* (*Rai1⁻*) were used to study the relationship between *Rai1* gene copy number and the *Dp(11)17/+* phenotypes. Normal disomic *Rai1* gene dosage is sufficient to rescue the complex physical and behavioral phenotypes observed in *Dp(11)17/+* mice, despite altered trisomic copy number of the other ~20 genes present in the rearranged genomic interval (8), indicating that *Rai1* gene CNV is involved in complex traits such as obesity and behavior. However, despite *RAI1* seemingly being the major contributor to the phenotypes observed in SMS and PTLS, several lines of evidence suggest that other genes or regulatory elements in the region serve as modifiers of the phenotypes observed both in human and mice (3,8,31,32). Consistently, we observe that all but one of the genes duplicated in the PTLS models present significantly elevated relative expression levels in the hippocampus. Only the RIKEN cDNA 1300013J15/Slc47a1 gene deviates from this general pattern, demonstrating that tissue-specific changes are not always directly correlated to copy number, suggesting an underlying complexity that might involve the size of the duplication, altered structure of chromatin, a dosage compensation mechanism or a combination of these factors (33).

Furthermore, we found that not only the aneuploid genes but also flanking genes that map up to several megabases away from the engineered interval are affected in their relative expression level. We reported similar results for the human chromosome 7 DNA deletion that causes Williams–Beuren syndrome (33). Hence, normal-copy genes that map either to the flank of a microdeletion or a microduplication should

also be considered as possible contributors to the phenotypic variation in genomic disorders (34).

We have presented here an association between gene copy number of a specific genomic interval, alterations in the expression of genes mapping both within and flanking the rearranged genomic interval and social behavior abnormalities in mice. Our findings represent a first step toward recognizing dosage-sensitive gene(s) and pathways regulating social behavior.

MATERIALS AND METHODS

Animals

Heterozygous mice carrying a duplication, *Dp(11)17/+*, were analyzed in a pure genetic background C57BL/6-*Tyr^c-Brd* (more than 14 backcrosses to wild-type C57BL/6-*Tyr^c-Brd*). Mice were genotyped visually by the presence of Agouti coloration in the coat color or by PCR in selected mice, with a concordance of 100% (11).

At weaning age *Dp(11)17/+* and wild-type littermate control male mice were grouped by genotypes and housed two to four per cage in a room with a 12 h light:dark cycle (lights on at 7 AM, off at 7 PM) with access to food (Teklad Global 19% Protein Extruded Rodent Diet from Harlan) and water *ad lib*. Behavioral testing was performed between 9 AM and 1 PM. All behavioral testing procedures were approved by the CECS Institutional Animal Care and followed the NIH Guidelines, 'Using Animals in Intramural Research'.

Order of tests

At 10 weeks of age, mice were tested with a battery of test with 0–2 days between each test in the following order: (i) home-cage and nesting behaviors, (ii) general health and neurological reflexes, (iii) elevated plus maze, (iv) sociability and preference for social novelty, (v) olfactory test and (vi) dominance test tube. A batch of six animals (four wild type and two *Dp(11)17/+*) were excluded from the social test because of noise outside the testing environment. The dominance test was added latter at the end of the battery and performed in only 10 mice of each genotype. For each test, the number of mice (*n*) tested is indicated in the respective figure caption.

Home-cage and nesting behaviors

During the 10th week of life, observations such as those regarding activity, fighting, sleeping and any other behavior were recorded at 8:30 AM, 10:00 AM, and 6:30 PM for 20 min each time for a total of 60 min. To assess nest-building behavior at noon, a paper towel was added to each cage and observations (presence of nest and sleeping behavior) were performed 1 and $6\frac{1}{2}$ h later.

General health and neurological behavior

Mice were evaluated for general health, including appearance of fur and whiskers, reflexive reactions to a gentle touch from a cotton swab to the whiskers and the visual placing reflex.

Elevated plus maze test for anxiety-like behaviors

The elevated plus maze (50 cm above the floor) consists of two closed arms (with 20 cm height walls) and two open arms (without walls). Each arm is 33 cm long. The light level at the center of the maze was ∼300 lux. Animals were placed in the center of the maze and allowed to freely explore in a 5 min trial. The position of the animal was recorded every 10 s having a total of 30 observations/mouse. Percentage of time for the mice in the open (or close) arm was estimated as: number of observations in the open (or close) arm × 100/total observations.

Social interaction and social recognition

The social behavior apparatus consisted of a rectangular three-chambered cage made of clear carbonate (14). Dividing walls have retractable doors allowing access to each section of the cage. The test consisted of three intervals of 10 min each. In the first 10 min (habituation period), the mouse was placed in the center chamber and allowed to explore the entire cage (doors open) and its' position was recorded by an observer every 10 s. After the habituation period was finished, the test mouse was enclosed in the center compartment, and an unfamiliar mouse (stranger 1) was placed into a plastic container with openings that allow for visual and olfactory recognition, but prevent direct contact, in one side of the chambers, and an empty container in the other chamber. The doors were open and the position of the test mouse was recorded for another 10 min. Notes were taken when sniffing to either the stranger 1 or the empty container took place as one sniffing episode (only one sniffing episode can be recorded per observation). To measure the preference for social novelty at the end of the 10 min interval, the mouse was enclosed again in the center chamber and a second unfamiliar mouse (stranger 2) was placed in the empty container. The doors were open again and the position and sniffing or not of the tester mouse was recorded for an extra 10 min. Data were analyzed as a percentage of total time spent in each of the chamber sections in each of the 10 min intervals.

The stranger mice were adult C57BL/6-Tyr^{c-Brd} housed far away from the tester mice and habituated to the container for period of 10 min during 5 days before the test.

Olfactory test

The mouse was placed in a $28 \times 28 \times 11$ cm^3 cage that contained 3 cm of bedding material and allowed to explore for 5 min. Then, the animal was removed and a food pellet (∼2 cm long) was buried at the bottom of the bedding material. The subject was placed again in the cage, and the time latency to find the food was recorded. Animals were fasted for 16–20 h prior to the test, to shorten the latency period (22).

Social dominance tube test

In a 30 cm long × 3.5 cm diameter tube, two age-matched males of different genotype were released toward each other from the opposite ends of the tube (35). A subject was declared a 'winner' when its opponent backed out of the tube. All matches resolved within the first 5 min. Each pairing ($n = 10$ wild types and $n = 10$ $Dp(11)17/+$) was performed twice (20 trials in total), each of the mice entering the tube using alternative ends and one trial followed by the other with no intertrial interval. Mice were trained to enter the tube alternatively at both ends prior to the test.

Statistical analysis

The plus maze, olfactory and weight data were analyzed using the independent samples t-test. Vocalization during handling and nest building data were analyzed by utilizing the χ^2 statistical test. Sociability and social novelty preference data were analyzed using two-way (genotype X side) ANOVA with repeated measure (side) followed by a Fisher–LSD analysis when a significant F-value was determined.

Gene expression profiling

Whole hippocampus of three male and three female $Dp(11)17/+$ and wild-type mice were dissected and immediately frozen in TRIzol reagent (Invitrogen, Carlsbad, NM, USA). Total RNA was extracted in the presence of TRIzol and purified on RNeasy columns (Qiagen), according to the manufacturers' protocols. cDNA and cRNA synthesis, labeling, hybridization and samples scanning were performed as described by Affymetrix (Santa Clara, CA, USA). GeneChip mouse genome 430 2.0 arrays, each containing 45 101 probe sets (Affymetrix), were used to hybridize the labeled cRNA. Each sample was processed individually. Expression data were normalized using RMA and MAS 5.0 methods implemented in the Bioconductor 'Affy' package (36).

Probe set detections were calculated using Bioconductor implementation of Affymetrix presence/absence detection algorithm (function 'mas5calls'). This implementation has been validated against the original MAS 5.0 and 1% of the calls differ from the original implementation of MAS 5.0.

Taqman real-time quantitative PCR

Total RNA was converted to cDNA using Superscript III (Invitrogen) primed with a mix of oligo(dT) and random hexamers. Oligos and probes were designed using the Primer Express program (Applied Biosystem) with default parameters (Supplementary Material, Table S3). Nonintron-spanning assays were tested in standard $+/-$RT reactions of RNA samples for genomic contamination. HPLC-purified Yakima-yellow dark-quencher-labeled double-dye Taqman probes and qPCR mastermix (RT-QP2X-03) were obtained from Eurogentec (Seraing). The efficiency of each Taqman assay was tested in a cDNA dilution series as described previously (37). All RT–PCR reactions were performed in a 10 μl final volume and three replicates per sample and set up in a 384-well plate format using a Freedom EVO robot (TECAN) and run in an ABI 7900 Sequence Detection System (Applied Biosystems, Foster city, CA) with the following amplification conditions: 50°C for 2 min, 95°C for 10 min and 50 cycles of 95°C 15 s/60°C for 1 min. Each plate included the appropriate normalization genes to control

for any variability between the different plate runs. Raw threshold cycle (Ct) values were obtained using SDS2.2 (Applied Biosystems). To calculate the normalized relative expression ratio between *Dp(11)17/+* mice and wild-type littermates, we followed the method as described in (37), and exploited the geNorm method to select *Eef1a1, Gapdh, Rps9* and *Tbp* as the four normalization genes (38).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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