

## Functional Analysis of Genes Implicated in Down Syndrome: 2. Laterality and Corpus Callosum Size in Mice Transpolygenic for Down Syndrome Chromosomal Region –1 (DCR-1)

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Received 1 Dec. 2004—Final 1 Feb. 2005

The association between atypical laterality and mental retardation has been reported several times, particularly in Down syndrome (DS). We investigated common genetic correlates of these components of the syndrome, examining direction (number of right paw entries in the Collins test) and degree (absolute difference between the number of right paw entries and the number of left paw entries) in mice that had incorporated extra-contiguous HSA21 fragments covering DCR-1 (Down Chromosomal Region-1). As corpus callosum size is substantially reduced in DS, and as the structure has been suspected of playing a role in atypical laterality, we also measured the corpus callosum in these mice. Extra copies of two regions (F7 and E6) have been associated with an atypical degree of laterality (strongly reduced degree). Extra copies of E8, G6 and E6 are also linked to the reduced size of the corpus callosum, indicating that the abnormal number of fibers linking the two hemispheres is not associated with atypical laterality in DS. Together, these results indicate that some of the genes involved in atypical laterality and in the reduced size of the corpus callosum in DS are present on DCR-1. An extra copy of F7 and, to a lesser extent, an extra copy of E6, are also associated with cognitive impairment. These results support the hypothesis of common genetic correlates in atypical laterality and mental retardation in DS.

**KEY WORDS:** Corpus callosum; Down syndrome; Down syndrome critical region-1; laterality; mouse model of Down syndrome.

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### INTRODUCTION

Typically developing persons (TDP) use the right hand for most manual tasks requiring strength, skill or accuracy, but in western populations about 9% prefer the left hand. The incidence of left-handedness varies according to the type of measurement (performance or preference—see, *inter alia*, Annett, 2003; Bishop, 1990; Calvert and Bishop, 1998; Doyen *et al.*, 2001). If we consider only the measurement of manual laterality as assessed by questionnaires, the incidence of left-handers differs according to the items used. Lee Ehrman's group conducted the most extensive epidemiological investigation on handedness. Perelle

and Ehrman (1994) and, most recently, Medland *et al.*, (2004) have illustrated one of three possible criteria for classifying the participants in the study: persons were classified as left-handed (1) if they stated that they wrote with their left hand; (2) if they used their left hand more frequently for the majority of the six primary handedness items in Annett's questionnaire; or (3) if they used their left hand more frequently in the majority of an extended 11-item handedness questionnaire. In 4.68% of cases, participants had conflicting classifications across the three criteria. The percentage would have been much higher if hand proficiency tasks had been included in the assessment of manual laterality.

To study not only the phenotype but also the physiological context in which it appears, a distinction must be made between non-syndromic and syndromic atypical laterality. Two arguments can be used to make the distinction.

First, the incidence of atypical laterality (left or inconsistent handedness) is higher in mentally retarded persons (MRP) compared to TDPs (Bishop, 1990). Studies published since Bishop's review have confirmed her conclusion (Cornish *et al.*, 1997; Grouios *et al.*, 1999; Devenny and Silverman, 1990; Lewin *et al.*, (1993; Morris and Romsky, 1993; di Nuovo and Buono, 2003; Vlacos and Karapetsas, 1999). Second, atypical laterality has recently been observed in a wide range of mental retardation disorders, confirming the older studies analyzed by Bishop (1990): Down (Carlier, personal communication; Vlachos and Karapetsas, 1999); Rett (Umansky *et al.*, 2003); Klinefelter (Itti *et al.*, 2003) or fragile-X syndromes (Cornish *et al.*, 1997); and in pervasive disorders associated with autism (Cornish and McManus, 1996). Dollfus *et al.*, (2002) reported a higher incidence of left-handedness in certain subtypes of schizophrenic patients. Given the different etiologies of these disorders, there could be different causes of such atypical laterality. This multiplicity of causes becomes apparent when comparing handedness in persons affected by disorders with different genetic origins such as Down, Rett, Klinefelter and fragile-X syndromes, leading to the conclusion that atypical laterality has different genetic correlates. *Post-mortem* brain investigations and, more recently, magnetic resonance imaging and spectrometry of patients affected by these disorders support this conclusion. The pattern of brain impairment and anatomical abnormality differ in these disorders, suggesting that different forms of brain disorganization may cause atypical laterality. Francks *et al.*,

(2002, 2003) reported, in TDPs, a replicated linkage with laterality in the 2q12-q11 regions. None of these regions corresponded to HSA21 or sexual chromosomes implicated in Down, Rett, Klinefelter and fragile-X syndromes. This difference of linkage supports the distinction between syndromic and non-syndromic atypical laterality.

The present study investigated the function of HSA21 genes that may be linked to the atypical laterality described in Down syndrome (DS). DS occurs once in every 700 births and remains the main genetic cause of mental deficiency (Lejeune, 1990). Persons with DS have particular facial features (oblique eye fissures, epicanthic eye-folds, a flat nasal bridge, the mouth permanently open and tongue protruding), and morphology, being short and stocky. The degree of mental deficiency varies but remains the most prominent feature of the syndrome. Other traits occur with variable frequency and severity (Antonarakis *et al.*, 2004): cardiac and immune disorders, including abnormal levels of T-lymphocytes and leukemia. On the criterion of manual laterality, the higher percentage of left-handers, compared to TDPs has often been observed, reaching 18.8%, 28.9%, 13%, 20%, and 30%, respectively in Pickersgill and Panck (1970), Batheja and McManus (1985), Devenny and Silverman (1990), Lewin *et al.*, (1993) and Carlier (personal communication). Several authors have reported a higher incidence of mixed handedness in persons with DS, mixed handedness being defined as a lower degree of laterality when a number of items and tasks are used to assess handedness (Batheja and McManus, 1985; Grouios *et al.*, 1999; Vlacos and Karapetsas, 1999). Another difference in the development of manual laterality has also been described, with DS persons being slower to achieve stable laterality than TDPs (Vlacos and Karapetsas, 1999). The atypical laterality of DS persons may be considered in the light of the dramatic disorganization of brain structure in DS. Raz *et al.*, (1995) compared the volume measurements of twenty brain structures in DS and TDP groups. The volumes were substantially smaller in the DS group, reaching approximately 70% and sometimes less of TDP measurements for most structures (e.g., cerebellum, frontal cortex, hippocampus and amygdalum).

HSA21 has been sequenced (Hattori *et al.*, 2000) but most of the functions of its genes and their role in DS are still unknown. The aim of the present paper is to elucidate the function of genes

carried by HSA21 affecting laterality. Lejeune *et al.*, (1959) were the first to prove that DS was caused by an extra copy of HSA21 and since that discovery, two studies using partial 21 trisomy (Delabar *et al.*, 1993; Korenberg *et al.*, 1994) have suggested that two regions of HSA21 are linked to most of the Jackson signs (Jackson *et al.*, 1976), including mental deficiency. These regions were located around the D21S55 Site Targeted Sequence (STS), between D21S55 and the *MX1* gene and encom-

passing the 21q22.2 band. Korenberg *et al.*, (1994) studied a different population and observed that the proximal and distal regions of the 21q arm were also associated with the full DS phenotype. The region around 21q22.2 has been identified as being involved in most of the phenotypic traits used to define DS, with D21S17 and ETS2 as boundaries. The region was then labeled "Down syndrome Chromosomal Region-1" (DCR-1). As partial trisomies are too rare, mouse models were chosen here to investigate a possible link between HSA21 genes, in particularly DCR-1 genes, and laterality. Several reasons justify the choice of the mouse for this purpose. It has been established that brain and motor asymmetries are highly conserved across species (LaMendola and Bever, 1997; Zilles *et al.*, 1996), including the mouse (Collins, 1985). Measurements of paw preference in the mouse are available and provide reliable results (Collins, 1985; Roubertoux *et al.*, 2003). Comparative chromosomal mapping shows extensive synteny between HSA21 and MMU16. The present study reports on the screening of the DCR-1 genes for laterality, as measured by paw preference in the mouse. We also investigated the size of the corpus callosum. As the use of the right or left limb is controlled by the contra lateral left or right hemisphere (Hécaen, 1984), we hypothesized that with the corpus callosum providing the connection between the two hemispheres, there may be modifications to the corpus callosum in cases of syndromic atypical laterality.

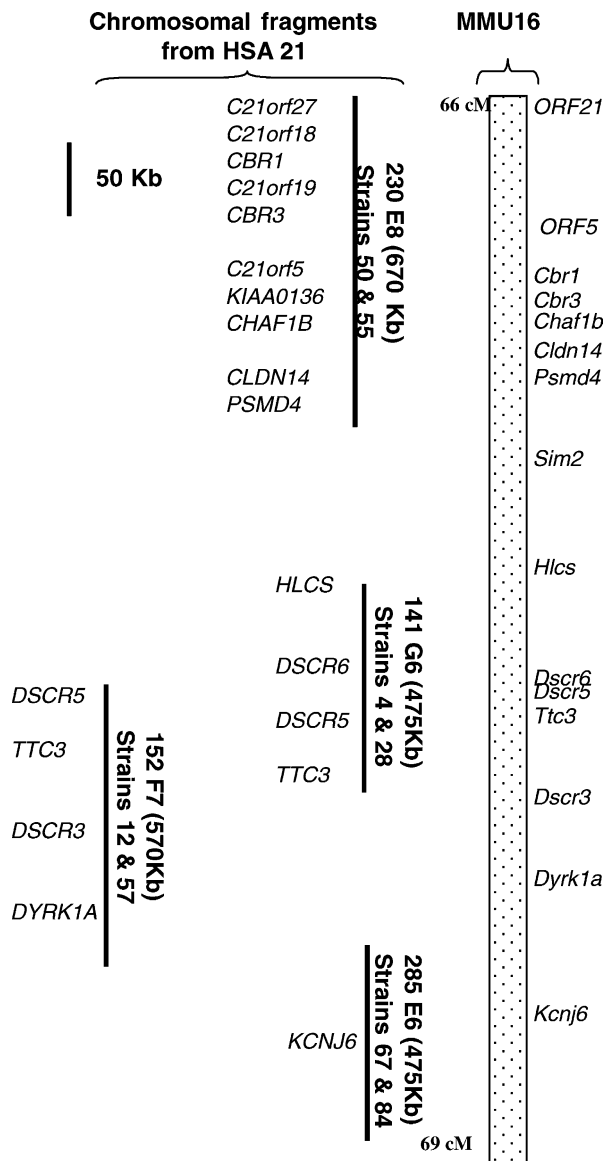


Fig. 1. Genes carried by the four YACs incorporating HSA21 fragments and corresponding regions on MMU16. Most of these genes reaches orthology criteria (amino acid sequence and nucleotide sequence comparisons).

## MATERIALS AND METHODS

### Mouse Model for DCR-1

Smith *et al.*, (1995, 1997) selected short chromosomal fragments of HSA 21. These chromosomal fragments were contiguous and covered DCR-1. Smith *et al.*, (1995) incorporated these short fragments of DCR-1 into Yeast Artificial Chromosomes (YACs); each fragment contained between 1 and 10 genes (Fig. 1). Each YAC was then incorporated into a mouse genome. Most of the genes carried by the four YACs are found on the syntenic region of MMU16 beginning with *Orf 21* (homologue of human *C21ORF18*). The murin homologues of the human *C21ORF27* and *C21ORF5* are more centromeric on MMU16, whereas *2600005C20Rik*, the murin homologue of *KIAA0179*, is on MMU17. Incorporation of each YAC induced partial trisomy for the genes that it carried.

So as to detect a possible insertion effect, Smith *et al.* (1995) developed several strains for each YAC. We used two strains for each of the four YACs: strains 50 and 55 for YAC 230E8 (abbreviated here to E8), 4 and 28 for YAC 141G6 (G6), 12 and 57 for YAC 152 F7(F7) and 67 and 84 for YAC 285E6 (E6). The integrity of the transgene, the number of copies and the expression of the genes have previously been reported (Smith *et al.*, 1995 and 1997). All strains carried one copy of the corresponding HSA 21 chromosomal fragment, except strains 28 and 57 with 2 and 3 copies, respectively; the number of copies carried by strain 84 was unknown. The FVB strain of mice used to micro-inject the transgenic fragments carries a recessive mutation (*rd*) inducing retinal degeneration. To avoid any impact of *rd* affecting visual cues, we used F<sub>1</sub> male offspring born from C57BL/6J females and transgenic FVB males (Smith *et al.*, 1997). This strategy permitted us to minimize differences due to genetic and environmental maternal effects. We individually screened the F<sub>1</sub> offspring from each litter using PCR. The primers and conditions of amplification were similar to those indicated by Chabert *et al.*, (2004).

### Mice

Male mice were tested between 85 and 107 days of age. Sample sizes for laterality measurements were as follows: 27 mice for YAC E8 (12 for strain 50 and 15 for strain 55), 31 for YAC G6 (13 for strain 28 and 18 for strain 4), 29 for YAC F7 (15 for strain 12 and 14 for strain 57), 32 for YAC E6 (15 for strain 67 and 17 for strain 84). We obtained neither transgenic females nor non-transgenic males in the 4 and 57 strains, suggesting that the YACs were carried by the Y-chromosome. We observed no difference between non-transgenic littermate males from 50, 55, 28, 12, 67 and 84 strains and F<sub>1</sub> offspring males from FVB non-carrier males and C57BL/6J females. We decided to use the same controls for all the strains, and we derived 30 F<sub>1</sub> offspring males as controls. We maintained the mice with food and water *ad libitum* and a 12/12 light cycle with lights on at 8 a.m.

### Paw Preference Measurement

Laterality was assessed using the protocol developed by Collins (1968) which, is the best situation for assessing paw laterality in mice (see Maarouf *et al.*, 1999). The mice were deprived of food at

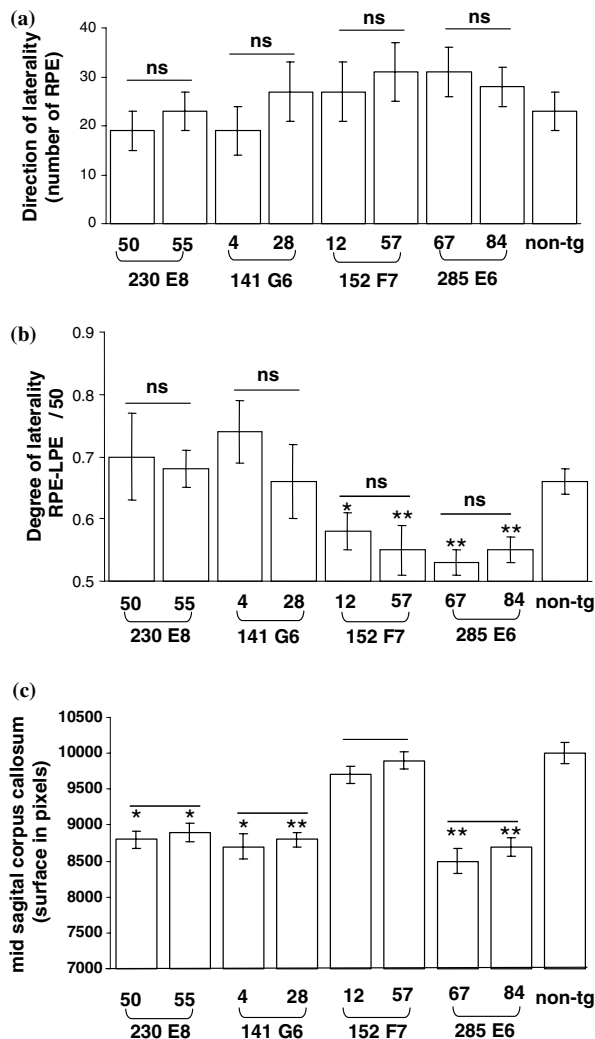
approximately 5.30 p.m. and tested  $17 \pm 2$  h later. Each mouse was placed in a chamber (10.5 × 6 × 6 cm) where the usual food was available in a tube half-way up the front wall and equally accessible from both right and left. The mouse could obtain the food by placing just one of its forepaws inside the tube. Each testing session observed fifty reaches and recorded the sequence of paws used. Two values were calculated: “direction” and “degree”. The number of right paw entries (RPE) in the course of a session gave the direction of laterality: the higher the score, the more right-pawed the mouse. The degree of laterality was the absolute difference between the number of right paw entries and the number of left paw entries (LPE). The mice with the highest |RPE-LPE| were the most lateralized, either right or left. Individual scores were transformed into logit values ( $\ln |RPE-LPE|$ ) (Collins, 1985) for homoscedasticity.

### Corpus Callosum Anatomy

The midsagittal surface area of the corpus callosum was measured at 3 months of age. The mice were killed by cervical dislocation; the brain was removed and weighed, post-fixed for at least 1 week in 6% paraformaldehyde, cut along the midsagittal plane using a razor blade and stained with 0.2% gold chloride solution (Wahlsten *et al.*, 2003), then fixed in 2.5% sodium thiosulfate solution and placed again in 6% paraformaldehyde fixative until measured. This procedure was done blind with respect to strain. The left and right hemispheres of the brains were taken at random. The midsagittal surface area provides a good estimates of the corpus callosum volume (Wahlsten, 1982). This surface was measured using an image analyzing system (Scion image, developed by NIH) and the measurement was expressed in pixels. Five mice per each group were used.

### Statistical Analysis

We transformed raw data to fulfill normal distribution assumptions and homoscedasticity using Bartlett's test. We performed inter-strain comparisons within YAC comparisons using Student's *t*-test to ensure there was no insertion effect. Then each strain was compared to the non-transgenic group for the indices of laterality and corpus callosum measurements. The effect sizes of the differences were calculated using Cohen's *d* statistics (1977).



**Fig. 2.** Estimation of laterality in mice with incorporated HSA21 fragments covering DCR-1. 50 and 55 duplicated strains for YAC 230E8; 4 and 28 for YAC 141G6; 12 and 57 for YAC 152 F7 and 67 and 84 for YAC 285E6, non-tg (non-transgenic controls). Mean  $\pm$  SEM. ns indicates no difference between strains within YACs (duplicated strains); \* $p$  < 0.05 to  $p$  < 0.01; \*\* $p$  < 0.005 difference between transgenic and non-tg. Numbers under X-axis indicate corresponding strains and YACs. (a) Direction of laterality in the 8 strains plus non-tg; (b) Degree of laterality in the 8 strains plus non-tg; (c) Midsagittal surface of corpus callosum in the 8 strains plus non-tg.

**RESULTS**

The direction of laterality, expressed as the number of right-paw entries, is shown in Figure 2a. No inter-strain difference appeared within each YAC and none of the eight strains differed from the non-transgenic group for these measurements ( $p$  > 0.10).

No difference in the degree of laterality was detected between strains within each YAC ( $p$  > 0.10), but strains 12 and 57 from the F7 YAC, on the one hand, and strains 67 and 84 from the E6 YAC, on the other hand, differed from the non-transgenic mice ( $F$  = 5.67,  $p$  < 0.03, for strain 12;  $F$  = 8.23,  $p$  < 0.01, for strain 57;  $F$  = 9.55,  $p$  < 0.005, for strain 67 and  $F$  = 11.65,  $p$  < 0.005, for strain 84) (see Fig. 2b). The effect sizes were large (between 0.76 and 1.06). The scores obtained for the mice carrying the same HAS 21 fragment were then pooled for degree and compared to the non-transgenic controls. F7 and E8 mice differed from controls ( $p$  < 0.001 and 0.0005, respectively), but no deviation from the control value was observed for either E8 or G6 mice.

For the midsagittal corpus callosum surface area no difference between the strains was shown within each YAC ( $p$  > 0.10). A link was observed between the E8, G6 and E6 regions and a reduction in the surface area of the corpus callosum mice ( $F$  = 7.5,  $p$  < 0.025 for E8 (strain 50);  $F$  = 8.23,  $p$  < 0.025 for E8 (strain 55);  $F$  = 9.4,  $p$  < 0.025 for G6 (strain 4);  $F$  = 7.96,  $p$  < 0.025 for G6 (strain 28);  $F$  = 11.45,  $p$  < 0.01 for E6 (strain 67);  $F$  = 10.82,  $p$  < 0.01 for E6 (strain 84) (Fig. 2c). The effect sizes were large (between 1.95 and 2.18). The E8, G6 and E6 YACs differed from controls ( $p$  < 0.001;  $p$  < 0.001 and  $p$  < 0.007, respectively), after pooling the strains within each YAC.

**DISCUSSION**

We performed a genotype–phenotype correlation between four chromosomal regions overlapping human DCR-1 and measurements of laterality and midsagittal corpus callosum surface area in transgenic mice. First, we did not detect any insertion effect (differences between strains within YACs) for these measurements. In a previous study using the same strains and cognitive assessments, only one insertion effect appeared in 28 comparisons (Chabert *et al.*, 2004). Second, the insertion of an extra fragment of DCR-1 did not modify the direction of laterality, although the insertion of the F7 and E6 fragments strongly reduced the degree of laterality, as shown by effect sizes. It should be noted that F7 and, to a lesser extent, E6 display cognitive impairments. Third, the insertion of extra chromosomal regions from DCR-1 E8, G6 and E6 strongly modified the midsagittal corpus callosum surface area, while an extra copy of F7 region did not contribute to this phenotype.

To the best of our knowledge, there are no reports of either observed or experimentally induced genetic events in mice modifying the direction of laterality in mice. Collins (1985, 1991) showed that no intra-strain difference in direction can be attributed to residual genetic variation, as he failed to select left or right-handers from a population of C57BL/6J well known for its U-shaped distribution of right-paw entries (direction) in the food reaching test used in the present study; he also showed that direction did not respond to selection in a segregating population (Collins, 1991). In previous studies no quantitative trait loci (QTL) was detected for the same measurement in an F<sub>2</sub> intercross generation derived from C57BL/6J and NZB/BINJ mice, but the degree of laterality responded to selective breeding (Collins, 1985, 1991), indicating that degree and not direction was inherited. Performing a wide genome scan in the population where no QTL for direction was detected, a QTL was identified for the degree of laterality as defined and measured in the present study (Roubertoux *et al.*, 2003). The possibility for detecting genetic correlates for degree but not for direction may result from a specific brain control of laterality found in mice but not in humans. In the human species, the left hemisphere is usually over-developed, compared to the right one, leading to the preferential use of the right contra lateral limb. In the mouse, a mild degree of laterality, i.e. an almost equal preference for the left or right paws, is associated with reduced asymmetry of the brain hemispheres (Cassells *et al.*, 1990; Collins, 1985; Lipp *et al.*, 1984; Ward and Collins, 1985). The direction of laterality in the mouse may depend on randomly distributed environmental factors, whereas degree would be modulated by the genes. The findings of the present study provide further experimental evidence for this hypothesis. This view would mean that a low degree of laterality (or increased ambidexterity) in mice may be a model of a higher incidence of left-handedness in humans. The extra copies of F7 and E6 regions of HSA21 modulate degree but not direction. Both regions reduce degree, therefore increasing ambidexterity, in mice. Could this finding reveal an association between extra copies of genes encompassed by these chromosomal segments and a reduced degree of laterality, and pave the way for identifying genes involved in the atypical laterality of DS patients?

The G6 and F7 fragments overlap. Because F7 is associated with a reduced degree of laterality, the genes on the F7 fragment, but not on the G6 fragment, are therefore linked to the degree of laterality.

Two genes, *DSCR5* (Down syndrome chromosomal region protein 5) and *TTC3* (tetratricopeptide repeat domain 3) that are carried by G6 and F7 can therefore be eliminated. Two others, *DSCR3* (Down syndrome critical region gene 3, the function of which remains unknown) and *DYRK1A* (dual-specificity tyrosine-regulated kinase) are carried by F7 but not by G6. Both *DSCR3* and *DYRK1A* are expressed in the embryonic territories corresponding to the regions of the adult cerebrum (Ngimbous, personal communication). *DSCR3* and *DYRK1A* can therefore be considered as candidates for the degree of laterality. The study of gene expression can help in selecting from amongst the candidates. Although HSA21 genes are present in three copies, not all these genes are over-expressed. Differences in gene dosage alteration have been reported (Lyle *et al.*, 2004). Some of the HSA21 genes with three copies do not show more expression than would be expected with two copies; some genes are unexpressed (for a review, see Antonarakis *et al.*, 2004). This expression is tissue-dependent (Gitton *et al.*, 2002; Kahlem *et al.*, 2004). The difference in expression in embryos and after birth at different ages, indicates that the level of expression is also age-dependent (Reymond *et al.*, 2002; Lyle *et al.*, 2004). To identify genes modulating laterality in our model of transgenic mice, we selected over-expressed genes carried by the F7 and E6 region and referred to Lyle *et al.* (2004) for the expression of genes carried by the chromosomal segment of MMU16 that is in three copies in Ts65Dn mice. We selected the expression values in the brain. Since the normalized expression value for a gene with two copies is 1.00, the expected normalized value for a gene with three copies is 1.50. The normalized expression values for *Dscr3* range from 1.20 to 1.11 between 1 and 11 months of age respectively, and from 1.42 to 1.31 for *Dyrk1a*, under the same conditions. Although the function of *Dscr3* is still unknown, *Dyrk1a*, (Altafaj *et al.*, 2001) is linked to motor disorders and abnormal neuronal morphology (Fotaki *et al.*, 2004). Studies with punctual transgenics should indicate whether or not *Dyrk1a* reduces the degree of laterality. *Kcnj6* (potassium inwardly rectifying channel, subfamily J, member 6) is the sole gene known on the E6 fragment. It can be therefore be hypothesized that it contributes to a reduction in the degree of laterality. The normalized expression value of *Kcnj6* is slightly higher than the expected expression of two copies at 1 month of age (1.21) and nil at 11 months (adult), suggesting a linear age-dependent reduction in the expression of *Kcnj6*. As

*Kcnj6* is expressed more during pre and post-natal life, we could assume that the over-expression of *Kcnj6* contributes to atypical early brain development with consequences on laterality.

Magnetic resonance imaging shows a reduced size of most brain structures in patients with DS (Kieslich *et al.*, 2002), including the corpus callosum (Teipel *et al.*, 2003). We have demonstrated here that three chromosomal fragments (E8, G6 and E6), within the boundaries of DCR-1, reduce the mid-sagittal surface area of the corpus callosum. Of the ten genes carried by E8, three could be candidates for the reduction of the corpus callosum volume in DS. *CLDN14* (Claudin tight junction protein) plays a role in myelin sheaths in the brain (Morita *et al.*, 1999) and may modulate the volume of the corpus callosum, as myelin is a major component in the fibers comprising the corpus callosum. The normalized expression value of *CLDN14* in the brain is high at both 1 and 11 months of age (2.09 and 2.03, respectively). *CBR1* and *CBR3* (carbonyl reductase 1 and 3) modulate NADPH activity, and NADH is involved in neuronal functioning. *CBR1* is over-expressed at both ages (1.43 and 1.84 at 1 and 11 months of age, respectively), whereas *CBR3* is over-expressed at 11 months only (1.47). Two genes, *HLC6* (holocarboxylase synthetase, modulating the frequency, rate and/or extent of the cell cycle), and *DSCR6* (DCSR protein 6) are on the part of G6 not common to F7. No information is available for *DSCR6*. *HLC6* is over-expressed at 1 and 11 months (1.57 and 1.84) and may be involved in neuronal development *via* its function in the cell cycle. The *KCNJ6* gene, associated with atypical laterality, is also associated with a reduced size of the corpus callosum. An over-expression of *Kcnj6* (which is higher at month 1 than month 11) might suggest that the *KCNJ6* gene is involved in the first stage of development of the corpus callosum.

The association of chromosomal regions of DCR-1 with atypical laterality plus the under-development of the corpus callosum indicate that several of the genes of HSA21 that are linked with these phenotypes may also be present on this region. The distinction between syndromic and non-syndromic phenotypes means they have different etiologies. But do these observations tally with the distinction between syndromic and non-syndromic laterality or syndromic and non-syndromic variation in the size of the corpus callosum? The present results are not sufficient to prove the distinction. A QTL for the degree of laterality measured on both forepaw and hindpaw (Roubertoux *et al.*, 2003) was found on

MMU4 in a F<sub>2</sub> population derived from C57BL/6By and NZB/BINJ. In the same population, we discovered two QTL for the midsagittal corpus callosum surface area (Le Roy *et al.*, 2001). One was on the MMU1, the other was on MMU4. None of these phenotypes had links with genes on MMU16. This difference can be seen as a confirmation of the difference of the genetic basis of the phenotype in a typically developing population of mice and in a population with a genetic disorder. It may be also seen as the result of differences in genetic background (FVB versus NZB/BINJ).

In a previous investigation, we studied mice from the same strains, using different learning measurements in a Morris water-maze and a fear-conditioning task (Chabert *et al.*, 2004). We observed that the F7 mice showed cognitive defects in most of the tasks. The E6 mice were slightly affected (long-term memory and discriminating learning). Here we have shown that F7 and E6 fragments are also implicated in laterality, an association supporting the link between atypical laterality and mental retardation.

## ACKNOWLEDGMENTS

ZS was granted a fellowship from the Fondation Jérôme Lejeune. PLR and MC also had grants from the Fondation Jérôme Lejeune. ZB received fellowships from Scientific Council of Région Centre and SESEP. The work was supported by CNRS and Université de la Méditerranée (UMR CNRS 6196 - P3M, Marseille), Université de Provence, Institut Universitaire de France and Université d'Orléans.

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Edited by Yong-Kyu Kim