Mild impairment of learning and memory in mice overexpressing the mSim2 gene located on chromosome 16: an animal model of Down’s syndrome

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Human Sim2 is a product of one of the genes located on human chromosome 21q22 and is a homolog of Drosophila single-minded (sim) which is a critical player in midline development of the central nervous system of the fly. Since Sim2 mRNA is expressed in facial, skull, palate and vertebra primordia in human and rodent embryos, features that are associated with phenotypes of Down’s syndrome (DS), its trisomic state is suspected to contribute to the symptoms of DS. Here we describe that mSim2 mRNA is expressed in hippocampus and amygdala of adult mice, and that while mice overexpressing mSim2 under the control of the β-actin promoter are viable and fertile and have superficially normal skeletal, brain and heart structures, they exhibit a moderate defect in context-dependent fear conditioning and a mild defect in the Morris water maze test. Taken together, our data show that overdosage of Sim2 may be important for the pathogenesis of Down’s syndrome, especially mental retardation.

INTRODUCTION

Down’s syndrome (DS) is the congenital disease caused by the trisomy of human chromosome 21. Patients with DS show a variety of symptoms such as characteristic craniofacial anomalies, skeletal defects, short stature, mental retardation, endocardial cushion defect and a high incidence of leukemia (1,2). Although the genetic basis of DS has not been fully investigated, many genes have been found in a region associated with DS on human chromosome 21 and several genes have been proposed for linkage with pathogenesis of DS by studies of transgenic and knockout mice (1,2). Thus far, it has been reported that overexpression of amyloid precursor protein (APP) causes progressive formation of Alzheimer-like plaque in the brain of transgenic mice. Ubiquitous overexpression of Ets2 under the control of the metallothionein promoter results in craniofacial defects and skeletal anomalies in transgenic mice (3). Overexpression of Sod1 also causes anomalies of the tongue, neuromuscular junction, neurotransmitter uptake and induction of apoptosis in the thymus (4–6). Recently, a novel ‘in vivo’ library approach has shown that trisomy of minibrain causes a mild learning and memory defect (7). Taken together, all the results suggest that specific symptoms of DS may result from the trisomy of one or a few gene(s) located on chromosome 21.

mSim2 is a murine homolog of Drosophila single-minded, whose product plays a pivotal role in the central nervous system (CNS) midline development (8–11), and is located on the C3.3–C4 band of mouse chromosome 16 which is syntenic with human chromosome 21q22 (12–16). In addition, human Sim2 maps to a position between Tiam and CBR of chromosome 21 (17,18). Interestingly, Sim2 mRNA is expressed in ventral diencephalon, branchial arches, ribs and limbs in developing rodent and human embryos, which are apparently associated with the sites of symptoms of DS (12–16). mSim2 protein is also known to act as a transcriptional repressor and has been suggested to work antagonistically to an unidentified murine ortholog of Drosophila Sim which is a positive regulator of CNS development (19,20). Here, we report overexpression of mSim2 mRNA in adult brain regions including hippocampus and amygdala, and initial characterization of two lines of transgenic mice overexpressing the mSim2 gene under the control of the chicken β-actin promoter. Two lines of the transgenic mice were fertile and revealed superficially normal phenotypes. Analysis of these mice, however, has demonstrated mild impairment in learning tasks such as fear conditioning and the spatial water maze test, which are considered to depend on the function of the hippocampus and amygdala, and suggests that mSim2 plays a causative role in mental retardation in DS, similar to the previously reported product of the gene minibrain.

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RESULTS

Creation of transgenic mice overexpressing mSim2

In order to investigate whether overdosage of mSim2 influences the skeletal structure and learning performance of transgenic mice, we tried to create mice carrying the pCAGGS-mSim2 transgene and established two independent transgenic lines (Fig. 1). Quantitative RT–PCR analysis showed that in wild-type adult mice, mSim2 mRNA is expressed abundantly in skeletal muscle and kidney and weakly in lung, stomach, thalamus, hippocampus and amygdala (Fig. 2). On the other hand, potentiated expression of mSim2 mRNA was observed in two independent transgenic lines, because the time of exposure in autoradiography was 2.5-fold longer with wild-type (30 h) than with the transgenic mice (13 h) in Figure 2A. The patterns of the expressed mSim2 mRNA were not the same in the two lines of transgenic mice, nor did they show a proportional increase in intensity over that of the normal mice. This is probably because the chromosomes sites for insertion of the mSim2 gene may be different in the two lines, and because an illegitimate promoter of the chicken β-actin gene was used for construction of the expression plasmid. Since mSim2 mRNA was expressed in the facial, skull, palate and vertebra primordia in developing embryos (12–14,16), alteration of the F
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mRNA was expressed abundantly in skeletal muscle

and kidney and weakly in lung, stomach, thalamus, hippocampus and amygdala, were also conducted on hematoxylin–

eosin-stained specimens, and no abnormalities were found in these tissues (Fig. 2C).

Contextual fear conditioning and general activity

Fear conditioning. On the training day, mice were placed in a conditioning chamber and subsequently received three foot shocks. Transgenic and wild-type mice displayed a gradual and comparable increase in freezing during the training period, demonstrating that the transgenic mice did not have a performance deficit such as an inability to freeze. On the next day, the transgenic and wild-type mice showed the conditioned freezing response to the chamber where foot shocks had been given (Fig. 3A).

Two-way ANOVA on the mean percentage freezing per min showed that there were significant differences among groups [F(2,150) = 18.81, P < 0.001] and no significant differences in the time process [F(4,150) = 1.10, P = 0.35]. The least significant difference (LSD) test for groups indicated that T1-4 mice exhibited significantly fewer freezing responses than did wild-type mice (P < 0.001) and T3-7 mice (P < 0.01), and also that T3-7 mice showed significantly less freezing than wild-type mice (P < 0.002). These results revealed that T1-4 and T3-7 mice have a moderate deficit in contextual fear conditioning.

Vocalization and jumping. For the foot shock sensitivity test (Fig. 3B), we measured the minimal level of current required to elicit vocalization and jumping after both general activity and light–dark choice tests. One-way ANOVA showed that there was no difference in the mean threshold for the vocalization [F(2,15) = 0.11, P = 0.89] or jumping [F(2,15) = 0.35, P = 0.71] among the wild-type, T3-7 and T1-4 mice.
Hidden platform test. Both transgenic and wild-type mice showed significant improvement in mean escape latency during trials (Fig. 4A) \( F(7,240) = 151.40, P < 0.001, \text{two-way ANOVA} \), but there was a significant difference among groups \( F(2,240) = 29.61, P < 0.001, \text{two-way ANOVA} \). T1-4 mice initially tended to perform less well on the first two training days than wild-type mice \( P < 0.001, \text{LSD test} \), while T3-7 mice were not significantly different from wild-type mice.

Probe test. Both transgenic and wild-type mice showed a highly spatial bias for the quadrant where the platform had been fixed (training quadrant) in the pool (Fig. 4B), 30 min after the completion of the hidden platform test. There was no significant difference in percentage time spent in the training quadrant among groups \( F(2,30) = 0.67, P = 0.52, \text{one-way ANOVA} \).

Reversal learning. All the groups of mice showed significant learning over trials (Fig. 4A) \( F(3,120) = 13.05, P < 0.001, \text{two-way ANOVA} \). There was a significant difference among groups \( F(2,120) = 86.34, P < 0.001, \text{two-way ANOVA} \). T3-7 and T1-4 mice took a significantly longer time to locate the hidden platform during training than wild-type mice \( P < 0.001, \text{LSD test} \).

Visible platform test. Each group easily acquired the habit of finding the visible platform (Fig. 4A) \( F(2,90) = 49.71, P < 0.001, \text{two-way ANOVA} \), and there was no significant difference among groups \( F(2,90) = 1.73, P = 0.18 \).

Reversal probe test. In the reversal probe test (Fig. 4C), T1-4 mice failed to search selectively the quadrant where the reversed platform had been situated, while wild-type and T3-7 mice searched the training quadrant, spending significantly more time there than in the other three quadrants \( F(3,52) = 23.27, P < 0.001; F(3,32) = 10.29, P < 0.001 \), respectively, one-way ANOVA; \( P < 0.001 \) and \( P < 0.02 \), respectively, LSD test).

Speed of swimming. The longer time taken to locate the hidden platform during original and reversal training in the transgenic mice might reflect their swimming performance (Fig. 4D). Thus, we compared the swimming speed during training sessions for the groups of mice. On blocks 1–4, the mean speeds of the wild-type, T3-7 and T1-4 mice were \( 20.60 \pm 0.39 \), \( 21.05 \pm 0.49 \), and \( 20.10 \pm 0.40 \) cm/s, respectively. The speeds of the wild-type, T3-7 and T1-4 mice were slightly faster in the later blocks \( F(1,282) = 30.26, P < 0.001 \); and \( F(3,32) = 10.29, P < 0.001 \), respectively, one-way ANOVA; \( P < 0.001 \) and \( P < 0.02 \), respectively, LSD test).

These results obtained from the water maze learning test demonstrated that T1-4 mice had a mild deficit in acquisition on the hidden platform test and reversal test, and that T3-7 mice had a similar deficit only on the reversal test.

**DISCUSSION**

The gene encoding mSim2 is localized on the C3.3–C4 band chromosome 16 which is syntenic with human chromosome 21q22, and its mRNA is expressed in the hypothalamus, ventral thalamus, branchial arches, ribs and limbs in developing embryos (12–16). These regions are apparently related to those where the major...
symptoms of DS, namely mental retardation, dysmorphic face, ribs and limbs, are observed. It is interesting to clarify whether and how overdose of the mSim2 transcriptional factor, with a repressive nature, plays a causative role in DS. Here, we have demonstrated that mSim2 mRNA is not overexpressed in all the tissues examined, but rather in specific tissues of the two lines of mSim2 transgenic mice. When the exposure time of RT-PCR analysis is taken into account as described in Figure 2, mSim2 mRNA was overexpressed in heart, lung and brain of the transgenic mice. In particular the hippocampus and amygdala in the two lines of transgenic mice showed overexpression of mSim2 mRNA. Microscopic observations of stained specimens, however, revealed that there was no apparent anomaly in skeletal, brain and heart structures (Fig. 2C).

Interestingly, both lines of the transgenic mice showed mild deficits in two learning tasks, the contextual fear conditioning task, which requires the integrated neural circuit of the hippocampus and amygdala (22,23), and the water maze spatial learning task, which is dependent on hippocampal function (24), while these mice showed a performance comparable with the wild-type mice in terms of general activity and light–dark preference tests. Apparently a more severe learning deficit was observed in the context-dependent fear conditioning than in the water maze test. This may be due to the fear conditioning task requiring the function of the two brain regions. In the water maze, T1-4 mice showed impairment in both acquisition and reversal learning, while T3-7 mice showed impairment only in reversal learning. This difference may be dependent on the sensitivity of acquisition and reversal learning to the dysfunction of the hippocampal formation, although the level of mSim2 overexpression in this tissue does not appear to be related directly to the severity of the impairment. As it is known that the effects of a hippocampal lesion are greater in reversal learning than in initial acquisition on spatial tasks (25), T3-7 mice may have a less disrupted function of the hippocampal system than T1-4 mice.

So far, several studies using Ts65Dn mice, which have partial trisomy of mouse chromosome 16 from app to the telomere, have found severe spatial learning deficits and skeletal anomalies in these mice (26,27). Very recently, it has been reported that new trisomic mice (Ts1Cje), with partial overdosage from sod1 to mx1, have learning deficits (28). Transgenic mice carrying an extra copy of minibrain seem to exhibit a milder defect than the above two partial trisomic mice, suggesting that some other genes may contribute to the spatial learning defects in DS. It is interesting to note that mSim2 and hSim2 are located on the genomic loci between sod1 and mx1 and between Tiam and Chr, respectively. Although Smith et al.
CMV immediate early enhancer. pCAGGS plasmid was digested with HindIII and ligated with a ClaI linker. Full-length mSim2 cDNA was subcloned into the XhoI site of pCAGGS. The resultant pCAGGS-mSim2 plasmid DNA was prepared in large quantities and purified by CsCl centrifugation. After digestion with ClaI and SalI, the DNA was electrophoresed on an agarose gel and the transgene was separated from the vector backbone DNA. After purification by the glass Max DNA isolation spin cartridge system (Gibco BRL, Rockville, MD), the transgene DNA was injected into eggs prepared from female DBA/2J mice mated with male C57BL/6J mice. Tail DNA from F1 founder mice was extracted, digested with EcoRI and electrophoresed on an agarose gel. The separated DNA was transferred onto a nylon membrane, (Hybond-N; Amersham, Little Chalfont, UK) and the blot was hybridized with mSim2 cDNA according to standard protocols (30). Genotyping was performed routinely by following PCR protocols using the primer pair: CMV1 primer 5'GGGTACCATGTTTGATAGC-3' and CMV2 primer 5'GGATCATGATACTTGATG-3'. Each cycle (30 cycles) consisted of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. Two independent transgenic lines were created and maintained by crossing with BDF1 mice.

RT–PCR and tissue analysis
Quantitative RT–PCR was performed as follows. Total RNAs were prepared by the CsCl–guanidium centrifugation method (31) from various tissues of wild-type and transgenic mice. The total RNAs (1 µg) were reverse-transcribed by Superscript reverse transcriptase (Gibco BRL) and finally adjusted to 100 µl. A portion (2 µl each) of cDNA solution was used for quantitation of mRNAs for mSim2 and β-actin. β-Actin mRNA content was determined as a standard. The cDNA solution (2 µl) was subjected to 30 cycles of PCR in a reaction mixture (15 µl) with GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT); each cycle consisted of 94°C for 30 s, 60°C for 30 s and 72°C for 2 min with a pair of mSim2 primers: 5'-CTTGGGTCTGCTGAAGACTT-3' and 5'-GTCTGAGAACCACCTTGT-3', in the presence of [α-32P]dCTP (0.2 µl, 3000 Ci/mmol, 10 mCi/ml). For determination of β-actin mRNA content, the RT–PCR method consisted of 22 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, using the primers 5'-CCCGAGACACAGCTTTT-3' and 5'-ATGAGGTAGTGCTGACGGG-3'. The PCR cycles for quantitation of these mRNAs were proven to ensure a linear relationship between the product and the mRNA content.

Staining of embryos for cartilage and bone was performed essentially as described (32). Hematoxylin–eosin staining was performed according to the standard procedure (33). Isolated tissues such as brain and heart fixed in formalin (15%) were embedded in paraffin. Coronal sections of brain and heart were cut with a microtome and subjected to hematoxylin–eosin staining. The stained specimens were observed under the microscope.

Behavioral tests
All experiments were done on two independent lines of transgenic mice (T3-7 and T1-4) and on wild-type mice. Littersmates of the transgenic mice were used, and their genotypes were determined by Southern blot analysis of tail DNA samples. All the mice were male and 3–5 months old. The mice were kept

MATERIALS AND METHODS

Creation of transgenic animals and genotyping
pCAGGS vector (29) (a kind gift from Dr J. Miyazaki, Osaka University, Japan) was used for the regulated expression of mSim2 under the control of the chicken β-actin promoter and

Figure 4. Performance of wild-type and transgenic mice in the Morris water maze. (A) Hidden platform, reversal and visible platform tests. (a) Hidden platform test. The tests are described in detail in Materials and Methods. Wild-type (n = 14), T3-7 (n = 9) and T1-4 mice (n = 10) were used. During the training trial days 1–4, the system automatically measured the time and swim path until the mouse mounted the platform. These data were transferred to a computer. The times presented are average values of the mice used for each group. (b) Reversal platform test. On day 5, the hidden platform was switched to the opposite quadrant for reversal training. The mice received six trials on day 5 and two trials on day 6. The times presented are average values of the mice used for each group. (c) Visible platform test. The position of the platform was signaled by the presence of a white flag (10 × 10 cm) above the platform. The platform position was changed among four possible positions and the mice were tested on a total of six trials at an inter-trial interval of 30 min with a different starting point. The times are presented as in (b). (B) Speed of swimming. To compare the swimming performance of the mice, we calculated the speed of swimming (swimming distance to platform/swimming time) during the trial in all training sessions.

(7) tried to examine the effects of overdosage of the genes on part of chromosome 16 on the neuropathology of transgenic mice by an ‘in vivo’ library approach, the yeast artificial chromosome (YAC) transgenes used were unfortunately considered to lack the region containing mSim2. Our data show that mSim2 is a new candidate for the causative gene for mental retardation. The Sim2-overexpressing mice will provide a new model for studying the pathogenesis of DS, especially the mental retardation.
on a 12 h light–dark cycle under a constant temperature (23 ± 1°C). The tests were always conducted between 13:00 and 18:00 h. One week before the beginning of behavioral tests, the mice were housed one per cage and were handled once a day for 5 days.

**Contextual fear conditioning**

The conditioning chamber was an observation box (20 × 20 × 20 cm) made of clear and gray vinylchloride plates. The floor of the chamber consisted of 26 stainless steel rods through which foot shocks were delivered by a shock scrambler (SGS 002; Muromachi, Tokyo, Japan). The chamber was placed in a lighted and sound-attenuating room. A video camera placed in front of the chamber allowed the behavior of each mouse to be observed and recorded by two experimenters in an adjacent room. The shock scrambler and controller for conditioning were operated by a remote switch in the same room.

On the training day, each mouse was placed in the chamber for 2.5 min, and subsequently received three foot shocks (0.5 mA intensity, 1 s duration, 1 min interval). The mice were removed from the chamber 1 min after the last foot shock and returned to their home cages. Twenty-four hours after the training, the mice were placed back into the chamber and tested for 5 min without foot shocks. The amount of fear conditioned to the chamber was assessed by scoring freezing behavior. Freezing was defined as the absence of visible movement, except for respiration. During the test period, two observers who were blind to the experimental conditions scored the tendency of the mice to freeze by watching the TV monitor. Observations were carried out using a time sampling procedure. Every 5 s, each mouse was judged as either freezing or being active during the test. An unbiased estimate of the actual time spent in freezing (the percentage of the observations) was calculated per minute.

**General activity**

Locomotion and rearing behavior were measured by the method described previously (32) in an open-field box (32 × 32 × 20 cm) placed in another sound-attenuating room which was different from the room used for fear conditioning. Two pairs of 7 × 7 array infrared photosensors were set against the outer wall and equally spaced in the lower and upper rows at intervals of 2 and 4.5 cm above the floor. The frequency of photobeam interruption caused by animal movement was recorded by the computer. Each mouse was kept in the box for 30 min.

**Light–dark choice test**

The apparatus comprised two compartments and was placed in a darkened and sound-attenuating room. One was a bright (250 lux) chamber (16 × 32 × 20 cm) illuminated by a white bulb (60 W) and the other was a dark (1 lux) chamber of the same dimensions. The two compartments were separated by a wall and connected by seven small openings (3 × 9 cm) through which the photobeam of sensors passed. Each mouse was placed in the center of the light chamber and its behavior was recorded for 15 min by a video camera. The mouse was considered to have entered the new area when all four feet were in this area. The following behavioral measures were scored: the time spent in the light and dark compartments, the number of transitions between the two compartments and the latency of the initial movement from the light to the dark room.

**Electric shock sensitivity test**

Because foot shock sensitivity may affect freezing responses, we measured the minimal level of current required to elicit vocalization and jumping by the method described previously (34) after both general activity and light–dark choice tests.

Each mouse was placed in the chamber used for contextual fear conditioning and then delivered 1 s shocks of increasing intensity. The interval between shocks was 10 s. The sequence of current used was as follows: 0.05, 0.08, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.8 mA. The minimal level of current required to elicit vocalization and jumping was determined. These experiments were performed blind.

**Water maze task**

About 2 months after the contextual fear conditioning, mice were trained in the water maze. The apparatus consisted of a circular tank (120 cm diameter) of water (depth, 25 cm; temperature, 26 ± 1°C) made opaque with the addition of skimmed milk powder. A transparent platform (10 cm diameter) for escape was submerged 0.6 cm below the water surface. Several salient cues were placed around the testing room to enable the animals to learn the platform location. A video camera was set above the center of the pool and connected to a color video tracking system (CAT10; Muromachi) and a video recorder that allowed on- and off-line automated tracking of the swim path of the mice in the pool. During the training trial, the system automatically measured the time, swim distance and swim paths until the mouse mounted the platform. These data were transferred to a computer and stored.

Mice were first trained to find the hidden platform and escape onto the platform fixed in the center of one of the four quadrants of the pool for six trials per day with an inter-trial interval of 30 min over 4 consecutive days. The start positions were selected semi-randomly from seven of eight equally spaced wall locations, excluding the point nearest the platform. The animals were allowed to swim until they mounted the platform and spent 30 s on it before being returned to their cages. If the mice failed to find to the platform within the 120 s limit, they were placed on to the platform for 30 s. A probe test was given 30 min after the last trial on day 4. For this test, the platform was removed from the pool, and the mouse was allowed to swim freely for 60 s. The time spent in each of the quadrants was measured by the automatic tracking system. On day 5, the hidden platform was switched to the opposite quadrant for reversal training. The mice received six trials on day 5 and two trials on day 6 followed by a reverse probe test 30 min later. On day 7, for a visible platform test, the position of the platform was signaled by the presence of a white flag (10 × 10 cm) above the platform. The platform position varied among four possible positions, and the mice were tested on a total of six trials at an inter-trial interval of 30 min with a different starting point.

**Data analysis**

Data were analyzed by one- or two-way ANOVA, and comparison of paired groups was carried out by Fisher’s LSD test. All values in the text and figure legends are expressed as means ± SEM, and n is the number of mice tested.
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