Tbx1: identification of a 22q11.2 gene as a risk factor for autism spectrum disorder in a mouse model

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Although twin studies indicate clear genetic bases of autism spectrum disorder (ASD), the precise mechanisms through which genetic variations causally result in ASD are poorly understood. Individuals with 3 Mb and nested 1.5 Mb hemizygosity of the chromosome 22q11.2 represent genetically identifiable cases of ASD. However, because more than 30 genes are deleted even in the minimal deletion cases of 22q11.2 deficiency, the individual 22q11.2 gene(s) responsible for ASD remain elusive. Here, we examined the impact of constitutive heterozygosity of Tbx1, a 22q11.2 gene, on the behavioral phenotypes of ASD and characterized the regional and cellular expression of its mRNA and protein in mice. Congenic Tbx1 heterozygous (HT) mice were impaired in social interaction, ultrasonic vocalization, memory-based behavioral alternation, working memory and thigmotaxis, compared with wild-type (WT) mice. These phenotypes were not due to non-specific alterations in olfactory function, exploratory behavior, motor movement or anxiety-related behavior. Tbx1 mRNA and protein were ubiquitously expressed throughout the brains of C57BL/6J mice, but protein expression was enriched in regions that postnatally retain the capacity of neurogenesis, and in fact, postnatally proliferating cells expressed Tbx1. In postnatally derived hippocampal culture cells of C57BL/6J mice, Tbx1 levels were higher during proliferation than during differentiation, and expressed in neural progenitor cells, immature and matured neurons and glial cells. Taken together, our data suggest that Tbx1 is a gene responsible for the phenotypes of 22q11.2 hemizygosity-associated ASD possibly through its role in diverse cell types, including postnatally and prenatally generated neurons.

INTRODUCTION

Autism spectrum disorder (ASD) is behaviorally defined by a constellation of deficits in social interaction, language and communication and a range of behavior, interests and activities, and is comorbid with variable degrees of cognitive development. Owing to its early onset in childhood, it has a major negative impact on the development of children, but symptoms affect the lives of adolescents and adults as well. As the precise mechanisms underlying ASD remain poorly understood, mechanism-based therapies are not available.

Twin studies have amply implicated gene variations as the predominant causative factor (1,2), and there are genetically identifiable cases of ASD (3). Individuals with 22q11.2 hemizygosity exhibit social interaction deficits (4–9), delayed development of vocal volume, vocalization and language (10,11), repetitive behaviors (12) and impairments in cognition, including working memory (9,13–16). These deficits are symptomatic elements and comorbid traits of ASD, and in fact 14–50% of individuals with 22q11.2 hemizygosity tested for ASD have met diagnostic criteria (6,12,17–19), collectively totaling in 29% of 22q11.2 hemizygous cases in
these studies. When screened for copy number variation (CNV) in individuals with ASD, 22q11.2 is one of many CNV sites (20–23).

A major obstacle in furthering our understanding of the genetic mechanisms of 22q11.2-associated ASD is that it is difficult to ascertain the precise manner by which individual genes cause ASD in humans. As hemizygosity of 22q11.2 minimally includes more than 30 genes (24), the impact of each of these genes on ASD cannot be isolated. Association of alleles of individual genes on the remaining copy of 22q11.2 with ASD cannot be isolated. Association of alleles of 22q11.2 hemizygosity, but does not identify individual genes whose hemizygosity ‘causes’ phenotypes in humans.

We have focused on Tbx1, one of the 22q11.2 genes, for two reasons. First, we have previously identified a ~200 kb 22q11.2 region, including Tbx1, whose gene dose alteration induces antipsychotic-responsive stereotyped behavior (25). Second, a rare case of Tbx1 mutation—not 22q11.2 hemizygosity—was associated with ASD in one family (26). Tbx1 belongs to a phylogenetically conserved family of genes that share a common DNA-binding domain, the T-box. The human TBX1 protein and its mouse ortholog Tbx1 share a highly conserved amino acid sequence, and thus it is amenable to studies in the mouse.

In this study, we first evaluated the impact of constitutive Tbx1 heterozygosity on ASD-related behavioral phenotypes. Although any attempt to model ASD in animals is at best a proxy for the real events, a number of behavioral paradigms to model symptomatic elements have validity (27). Second, we identified the localization of Tbx1 protein in the mouse brain at the regional and cellular levels. Although Tbx1 mRNA steadily increases in the whole mouse brain sample during the postnatal period toward adulthood (26), the regional and cellular distributions of its protein product in the mouse brain are not known. Our data suggest that constitutive Tbx1 heterozygosity contributes to 22q11.2-associated ASD presumably through its expression in diverse brain regions, some of which include postnatally generated neurons.

**RESULTS**

**Congenic Tbx1 heterozygous mice exhibit ASD-related behavioral phenotypes**

Congenic Tbx1 heterozygous (HT) mice exhibited lower levels of active and passive affiliative social interaction; aggressive behavior was rarely seen in our experimental setup (Fig. 1A). This phenotype was not due to a specific reduction in approach behaviors. HT and wild-type (WT) mice were indistinguishable in olfactory investigation of the non-mouse odor (i.e. peanut butter, Fig. 1B); HT mice initially exhibited higher levels of contact with a novel, non-mouse object (Fig. 1C), compared with WT mice. Social interaction includes an element of anxiety. HT and WT mice were indistinguishable in anxiety-related behaviors in the elevated plus maze (Fig. 1D), although HT mice exhibited a higher degree of thigmotaxis than did WT mice (Fig. 1E). The social interaction requires motor movement, but HT and WT mice were indistinguishable in motor activity in an open field (Fig. 1F).

Compared with WT mice, HT mice exhibited vocalization less frequently in complex, two-syllable, composite, frequency steps and flat (Fig. 2A) and for shorter duration in harmonics, two-syllable, composite and frequency steps (Fig. 2B).

Compared with WT mice, congenic HT mice exhibited lower levels of spontaneous alternations at 0 and 30 s delays; both WT and HT mice reached a chance level at a 60 s delay (Fig. 3A). HT mice visited the same arms across trials more often than WT mice (Fig. 3B). Interestingly, this tendency was significantly different from WT mice when HT mice showed spontaneous alternation (e.g. 0 s delay) but not when they do not at 60 s delay (Fig. 3A and B).

**Tbx1 is expressed throughout the mouse brain but is enriched in postnatally generated cells**

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 4A) showed that Tbx1 mRNA was expressed in all regions examined in C57BL/6J mice at 2 months of age.

Although low levels of Tbx1 protein were ubiquitously seen in the whole brain of C57BL/6J mice, higher levels were present in the olfactory bulb (Fig. 4B), rostral migratory stream at the levels of olfactory bulb and nucleus accumbens (Fig. 4B and C), the subventricular zone (Fig. 4C) and the granular layer of the dentate gyrus (Fig. 4D).

Because these structures exhibit postnatal/adult neurogenesis (28), we next determined the localization of Tbx1 in postnatally proliferating cells. Almost all bromodeoxyuridine (BrdU)-positive cells (green) were colocalized (yellowish green) with Tbx1-positive cells (red) in the rostral migratory stream (Fig. 4E), the subventricular zone (Fig. 4F) and the subgranular zone of the dentate gyrus (Fig. 4G).

Because BrdU was given within a 7-day window and is expected to label a fraction of proliferating cells, there were BrdU-negative, Tbx1-positive cells within the regions known to exhibit postnatal neurogenesis (Fig. 4F and G). Tbx1 is also probably expressed in prenatally generated neurons, as Tbx1 was additionally seen in regions not known to exhibit postnatal/adult neurogenesis (Fig. 4B–D). On the other hand, there were a few BrdU-positive cells without detectable levels of Tbx1 staining in the rostral migratory stream (see arrows pointing at green cells in Fig. 4E), suggesting the possibility that Tbx1 expression might have declined after proliferation in these cells.

To confirm this possibility, we examined Tbx1 levels in proliferating and differentiated cells in hippocampal cell culture derived from the postnatal (P) day 0 hippocampus of C57BL/6J mice. Tbx1 levels were high during proliferation in the presence of epidermal growth factor (EGF), and declined as differentiation progressed following EGF withdrawal (Fig. 5).

Although Tbx1 levels were very low during differentiation (see Fig. 5, bar graph, day 7), western blotting uses samples that include many cell types that may or may not express Tbx1, potentially underestimating the level of Tbx1. Thus, we histochemically examined Tbx1 in postnatally generated neurons during proliferation and differentiation. Tbx1 was expressed in neuronal progenitor cells (labeled by nestin) (Fig. 6A) during proliferation (labeled by Ki67) (Fig. 6B).
Tbx1 was expressed in cells positive for neuron-specific class III β-tubulin (Tuj1), an early neuronal marker (Fig. 6C) 7 days after EGF withdrawal. As Tuj1 is expressed in both immature and mature neurons, we also examined the colocalization of Tbx1 with microtubule-associated protein 2 (MAP2), a marker of mature neurons (29), at the time point when cells differentiate into mature neurons (i.e. 14 days after EGF withdrawal). Tbx1 was expressed in differentiated, mature neurons (Fig. 6D). Tbx1 was also expressed in glial fibrillary acidic protein (GFAP)-positive astroglia 7 days after EGF withdrawal (Fig. 6E). Tbx1 signals without MAP2 (Fig. 6D) or GFAP (Fig. 6E) are likely to represent glial cells and neurons, respectively. These data demonstrate that Tbx1 is present in proliferating cells, immature and mature neurons and glial cells of the postnatal origin.

DISCUSSION

Constitutive Tbx1 heterozygosity in mice genetically recapitulated constitutive human 22q11.2 hemizygosity at a single gene and isolated the impact of this 22q11.2 gene on behavior. Our analysis demonstrates that constitutive heterozygosity of Tbx1 results in ASD-related behavioral phenotypes, and that Tbx1 protein is ubiquitously expressed throughout the mouse brain, but is enriched in postnatally proliferating cells.
Defective social interaction, the core symptom of ASD, appears in childhood but persists into adolescence and adulthood (30). Mice begin to show signs of sexual maturation around 1 month of age (31), but many biological processes in the brain continue to change afterwards (32–36) until they reach mature adulthood at 3 months of age (37). Congenic HT mice were impaired in affiliative social interaction when they reach mature adulthood at 3 months of age (37). Congenic HT mice were impaired in affiliative social interaction at 2 months of age without confounding alterations in aggression, olfactory investigation or motor behavior. This behavioral phenotype in social interaction is not due to a non-specific reduction in exploratory behavior, as HT mice showed elevated levels of interaction with a non-mouse object. Interestingly, this phenotype in interaction with an object is consistent with the fact that children with ASD often prefer to interact with non-human objects.

HT mice were not impaired in the elevated plus maze, but exhibited higher levels of thigmotaxis in the inescapable open field. This apparent discrepancy might be due to a floor effect. Because WT and HT mice exhibited robust anxiety-related behaviors in our elevated plus maze, as shown by low levels (~4%) of time spent in the open arms of the elevated plus maze, it might have been difficult to detect a further reduction in time spent in the open arms. However, much lower levels of time in the open arms have been demonstrated in various mouse strains (38). Alternatively, although these two tasks reflect the level of anxiety, stress levels are much lower when mice have a choice to escape from an open arm to a closed arm in the elevated plus maze than when they do not in the inescapable open field (39,40). Although anxiety is not the symptomatic element of ASD in itself, it is highly prevalent and exacerbated by stress in children, adolescents and adults with 22q11.2 deletion (5,16,41–43). Tbx1 deficiency might additionally increase the vulnerability to stress-related anxiety in 22q11.2 deletion cases.
When mouse pups are separated from mothers, they emit ultrasonic vocalization, which elicits their retrieval by mothers. This behavior is regarded as a form of social communication in rodents (44). Compared with WT pups, HT pups were impaired in complex patterns of vocalization (e.g. complex, harmonics, two-syllable, composite and frequency steps); WT and HT were indistinguishable in simple patterns (e.g. upward, downward, hump and short). This deficit pattern in vocalization might signify an autism-related phenotype in pups. One caution is that, like 22q11.2-deletion patients (45,46), constitutive Tbx1-deficient mice show hearing impairments (47) and velopharyngeal dysfunction (47–50). It could be that Tbx1 HT mice emit less vocal calls partly due to these physical abnormalities. However, HT mice selectively showed lower levels of vocal sounds in complex vocal patterns only, and were indistinguishable from WT mice in simple vocal patterns; HT mice emitted even more frequently—although statistically non-significantly—upward and downward vocal sounds. These considerations notwithstanding, this is not an issue of whether this mouse faithfully recapitulates the ASD-related phenotypes—it does—but what processes underlie defective vocal sounds in both 22q11.2-deletion patients and Tbx1 HT mice.

Children and adolescents with 22q11.2 hemizygosity—like idiopathic autism—are weak in working memory (9,13–16). Tbx1 HT mice showed lower levels of spontaneous alternation in the T-maze. Given the lack of anxiety-related phenotypes in a similar choice paradigm (i.e. elevated plus maze), the deficit in spontaneous alternation is unlikely to be a result of altered anxiety levels. Although spontaneous alternation requires working memory to recall an arm previously visited (51), performance could also be negatively impacted by a repetitive behavioral tendency. Interestingly, when HT mice showed a sign of memory at 0 s delay, they had a higher degree of...
repetitive choices than WT mice; at a 60 s delay, HT and WT mice were indistinguishable in repetitiveness and HT mice did not increase the degree of repetitiveness beyond 50% (Fig. 3B). These data suggest that the repetitive behavioral tendency manifests itself in the form of memory-guided behavior, but not as a simple motor repetitiveness, in HT mice. Interestingly, individuals with idiopathic ASD have difficulty in inhibiting context-inappropriate behavior based on working memory, which is thought to underlie actions and verbalizations that are inappropriate in timing or to the circumstances; they are not impaired in simple response inhibition that are not dependent on memory (52,53).

A gene dose alteration of a ∼200 kb 22q11.2 region, including Tbx1, induces antipsychotic-responsive repetitive motor behavior (25), and 22q11.2-associated ASD includes motor stereotypy (12). We did not find motor stereotypy in HT mice. As deficiency in Sept5, another gene in the 200 kb region, impairs social interaction but does not cause motor stereotypy either (54), Gnb1 and Gp1bβ, the remaining genes encoded in the segment, might contribute to motor stereotypy. A corollary of the existing data so far (25,54,55) is that some but not all 22q11.2 genes contribute to behavioral phenotypes and each of these genes affects its own set of behaviors, which could partially overlap or be totally distinct in their phenotypic targets.

Tbx1 protein was present in regions not known to have postnatal/adult neurogenesis (e.g. prefrontal cortex and amygdala) as well as in postnatally proliferating cells in the rostral migratory stream, subventricular zone and hippocampus. Tbx1 deficiency could cause signaling alterations in prenatally or postnatally generated mature neurons in the mouse brain. Alternatively or additionally, Tbx1 deficiency might alter prenatally/postnatal/adult neurogenesis. However, not all of the behavioral phenotypes of congenic Tbx1 HT mice are likely to occur through low levels of Tbx1 in postnatally generated neurons or alterations in postnatal/adult neurogenesis. First, our data show that Tbx1 could additionally be expressed in cells that differentiate into glial cells. As various environmental stimuli (e.g. enriched environment, physical activity and electroconvulsive seizures) induce reactive glial proliferation in the prefrontal cortex and amygdala of adult rats and mice (56–60), Tbx1 might be additionally involved in reactive glial proliferation in areas with no known postnatal/adult neurogenesis. Second, the phenotype in ultrasonic vocalization was detected at P7–8, a time point too early for postnatally generated neurons to mature and function. Third, spontaneous alternation in the T- or Y-maze is not affected by inhibition of adult neurogenesis (61,62) (but see 63). Fourth, inter-gender interaction (but not intra-gender affiliative social interaction) in female mice is ‘increased’ (not decreased) by inhibition of adult neurogenesis in the subventricular zone (64). However, as inhibition of adult neurogenesis was targeted at 2 months of age or older in these studies, the role of earlier, postnatal neurogenesis in these ASD-related behaviors remains unexplored. A future challenge is to selectively target Tbx1 deletion in prenatally or postnatally generated neurons and glial cells, and determine the precise role this transcription factor plays in ASD-related behavioral phenotypes and postnatal/adult neurogenesis.

Individuals with 22q11.2 hemizygosity are also diagnosed—at elevated rates compared with the general population—with schizophrenia (41,65–70). A recent estimate indicates that one-fourth of individuals with 22q11.2 deletion are diagnosed with schizophrenia (71). Defective social cognition, poverty of speech and working memory deficits are prodromal symptoms and symptomatic elements of schizophrenia (72,73). When individuals with 22q11.2 hemizygosity reach adulthood, defective working memory is worse in those with schizophrenia than in those without it (15). Higher and increasing levels of anxiety are a risk factor for later developing schizophrenia in 22q11.2 cases (74). Our data suggest a tantalizing hypothesis that Tbx1 deficiency underlies specific prodromal and symptomatic elements of schizophrenia, as well as ASD. Such an action of a gene is consistent with the suggestion that the way genes influence neuropsychiatric disorders does not necessarily follow the DSM-based diagnostic boundary (55,75,76).

**MATERIALS AND METHODS**

**Mouse**

We generated congenic Tbx1 HT and WT mice by backcrossing non-congenic Tbx1 HT mouse (77) to C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) for 10 generations. Congenic, male Tbx1 WT and HT mice were behaviorally tested at 2 months of age. Because ultrasonic vocalization is detectable at P7–8 but is rare and difficult to detect before and after this time window (44), this behavior was recorded at P7–8 in congenic, female Tbx1 WT and HT mice. For anatomical analyses, male C57BL/6J mice were used at 5 weeks or 2 months of age. For cell culture, C57BL/6J mice were sacrificed at P0. Animal handling and use followed a protocol approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine, in accordance with NIH guidelines.
Behavioral analysis

Social interaction. Our experimental protocol (54,55) was designed to optimally elicit affiliative social interaction and minimize aggressive behaviors. Age-matched, male C57BL/6J inbred mice (Jackson Laboratory) were paired with either WT or HT mice.

Olfactory investigation. A plastic home cage (28.5 cm long × 18 cm wide and 13 cm high) was divided into two compartments (19.5 cm long and 9 cm long) with a partition wall. A 1.5 ml Eppendorf tube containing peanut butter (50% in water) was attached on the wall of the smaller compartment 2.5 cm above the floor. The lid of the tube had seven holes to release the odor. Two 3 min sessions were given with a 30 min intersession interval. All activity was recorded with a digital camera. An observer blinded to genotype and experimental groups scored the amount of time each mouse spent sniffing the odorant-containing tube.

Novel object contact. An individual mouse was placed in a new home cage for 30 min. The mouse was then moved into another home cage that contained a modified falcon tube (3 cm diameter × 8.5 cm length) during two 5 min sessions with a 30 min intersession interval. The amount of time each mouse spent contacting the novel object, in the forms of sniffing and exploratory investigation, was analyzed. Otherwise, the procedure was identical to that of olfactory investigation.

Figure 6. Tbx1 colocalization in postnatal hippocampal cell culture. (A and B) Tbx1 localization in postnatal progenitor cells of hippocampal cell culture during proliferation with EGF. The cell nucleus is visualized with the nuclear marker DAPI (blue). Tbx1 (red) is shown with the neural progenitor cell marker nestin (green) (A) or the cell proliferation marker Ki67 (green) (B). All Tbx1-positive cells are colocalized with nestin or Ki67, but the intensity of each color differed from cell to cell, thereby giving different tones of merged color. Tbx1 localization in postnatal progenitor cells of hippocampal cell culture during differentiation into neurons (C and D) and GFAP-positive glial cells (E) 7 (C and E) and 14 days (D) following EGF withdrawal. Tbx1 is shown with the neuronal markers TUJ1 and MAP2, the glial marker GFAP and the nucleus marker DAPI. The experiment was repeated two or three times. Scale bars, 20 μm.
Elevated plus maze. The apparatus and procedure for this anxiety-related behavior were identical to those described in our previous studies (54,55,78).

Motor activity. Motor activity was recorded for 30 min in an inescapable open field (Truscan, Coulbourn Instruments, Allentown, PA, USA) (54,55,78).

Ultrasonic vocalization. Each pup was separated from its mother and placed onto a plastic tray with cage bedding at P7–8. The tray was placed in a styrofoam box with the ultrasonic microphone (Avisoft UltraSoundGate condenser microphone capsule CM16, Avisoft Bioacoustics, Berlin, Germany) on the lid 12.5 cm above the tray. This microphone is sensitive to frequencies between 10 and 200 kHz. Immediately after each pup was placed onto the tray, the vocalization sound was recorded and analyzed for 5 min at 300 kHz sampling rate (format 16 bit) using AVISOFT SASLABPRO (version 5.1.0). For acoustical analysis, a fast Fourier transformation (FFT) was conducted. Spectrograms were generated with an FFT length of 256 points and a time window overlap of 50% (100% frame, flat-top window). The spectrogram was produced at a frequency resolution of 488 Hz and a time resolution of 1 ms. A lower cut-off frequency was set at 20 kHz. The frequency and average duration of 10 distinct types of calls (79) were analyzed.

Spontaneous alternation. A T-maze was used to evaluate spontaneous alternation, as described in our previous publications (54,55).

Anatomical Analysis

Semiquantitative RT-PCR. Total RNA was extracted from the prefrontal cortex, nucleus accumbens, caudate-putamen, hippocampus, amygdala, ventral tegmental area and substantia nigra of 2-month-old C57BL/6J mice using RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). DNA was eliminated by on-column treatment with RNase-Free DNase I (QIAGEN). A total of 250 ng RNA was used for cDNA synthesis by SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Primers used were: Tbx1, forward, ATGATCTCCCGCTGTCTAG, reverse, CGTGGGGACA TTCGTCTGCCTG, band size, 420 bp; Gapdh, forward, AC CACCATGGAGAAGGC, reverse, GGCATGGACTGTGGT CATGA, band size, 234 bp. RT-PCR was run for 35 cycles.

Immunofluorescence. C57BL/6J mice were sacrificed at 4 months of age and their brains were coronally or sagittally sectioned at 10 μm using a cryostat and placed onto silane glass slides. Sections were fixed for 5 min with 4% paraformaldehyde in phosphate-buffered saline (PBS) with Tween 20 (0.2%), washed in PBS three times, treated with 0.1% Triton X in PBS for 15 min, washed with PBS three times, blocked for 1 h with 10% fetal bovine serum in PBS and incubated at 4°C overnight with a polyclonal rabbit anti-Tbx1 antibody (1:50 or 1:100, Abcam, see Supplementary Material, Figure S1, for the selectivity of the antibody). Sections were then incubated for 90 min with donkey anti-rabbit IgG conjugated with Texas red (1:200, Jackson ImmunoResearch, West Grove, PA, USA) and cover-slipped.

BrdU labeling. C57BL/6J mice were given the thymidine analog BrdU (50 mg/kg/day, intraperitoneal, 10 mg/ml) for 7 days starting at the age of 5 weeks, and were killed 24 h after the last injection. Following the basic procedure (see Immunofluorescence), sections were incubated for 48 h at 4°C with a cocktail of rabbit anti-Tbx1 antibody (1:100, Abcam) and a mouse anti-BrdU (1:12, Roche, Indianapolis, IN, USA). The sections were incubated with a cocktail of goat anti-rabbit IgG conjugated with Alexa Fluor-594 (red, 1:200, Invitrogen) for Tbx1, and goat anti-mouse IgG conjugated with Alexa Fluor-488 (green, 1:200, Invitrogen) for BrdU in PBS for 90 min at room temperature. Fluorescent signals were detected using confocal microscopy (Nikon, Melville, NY, USA).

Cell culture preparation. We followed our standard procedure with modifications (80). Briefly, hippocampal tissues were dissected out from the brains of C57BL/6J mice at P0. Cells were maintained in a culture medium containing EGF; cell differentiation was initiated by removing EGF from the culture. Cells were culled and fixed for western blotting and immunofluorescent staining, respectively, while they were maintained in an EGF-containing medium or 1, 2, 7 or 14 days after the removal of EGF from the culture. We used our routine western blotting protocol (39) to quantitatively evaluate Tbx1 expression in postnatally derived and cultured hippocampal cells. We used a rabbit polyclonal antibody to Tbx1 (1:500, Abcam, Cambridge, MA, USA) and goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (1:5000, Pierce, Rockford, IL, USA). Additionally, immunofluorescent staining was used to evaluate Tbx1 expression in various cell types, according to our routine procedure (80). Briefly, we used rabbit anti-Tbx1 antibody (1:100, Abcam), mouse anti-nestin antibody (1:50, BD Pharmingen, Franklin Lakes, NJ, USA), mouse anti-Ki67 antibody (1:100, Abcam), mouse anti-Tuj1 antibody (1:100, Sigma), mouse anti-MAP2 antibody (1:100, Thermo Scientific) and mouse anti-GFAP antibody (1:200, Sigma). These markers were then labeled with goat anti-rabbit IgG conjugated with Alexa Fluor-594 (1:200, Invitrogen) for Tbx1, and goat anti-mouse IgG conjugated with Alexa Fluor-488 (1:200, Invitrogen) for nestin, Ki67, Tuj1 MAP2, and GFAP.

Statistical analysis

Group means were compared using analysis of variance, followed by Newman–Keuls post hoc comparisons. When only two groups were compared, we used the t-test. A probability of 0.05 or less was considered to be significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.
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