Neuroscience 154 (2008) 1255–1266

TRANSMEMBRANE PROTEIN 50B (*C21orf4*), A CANDIDATE FOR DOWN SYNDROME NEUROPHENOTYPES, ENCODES AN INTRACELLULAR MEMBRANE PROTEIN EXPRESSED IN THE RODENT BRAIN

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Abstract—*Transmembrane protein 50b*, *Tmem50b*, previously referred to as *C21orf4*, encodes a predicted transmembrane protein and is one of few genes significantly over-expressed during cerebellar development in a Down syndrome mouse model, Ts1Cje. In order to assess potential mechanisms by which *Tmem50b* could contribute to Down syndrome–related phenotypes, we determined the expression patterns of *Tmem50b* mRNA, as well as Tmem50b protein distribution, expression and subcellular localization. *In situ* hybridization in mice at embryonic day 14.5 showed cortical plate and spinal cord mRNA expression. By postnatal day 7, strong mRNA expression was seen in the cerebellum, hippocampus and olfactory bulb, with diffuse cortical expression. Quantitative PCR of adult mouse tissue showed *Tmem50b* mRNA expression in the brain, heart and testis. A rabbit polyclonal antibody was generated against Tmem50b and rat and mouse tissue screening by Western blot, and immunohistochemistry showed that protein expression concurred with mRNA expression. Double immunofluorescence revealed that Tmem50b is highly expressed in rat and mouse glial fibrillary acidic protein–positive cells *in vivo* and *in vitro*, but less so in neuronal MAP2- or β-tubulin II-positive cells *in vitro*. Tmem50b is invariably expressed in cultured mouse neural precursor cells. In adult mouse cerebellum sections, Tmem50b immunoreactivity was found in Purkinje and Golgi cell somata and in Bergmann glial processes. Electron microscopy confirmed that Tmem50b was present on endoplasmic reticulum (ER) and Golgi apparatus membranes. Results indicate that Tmem50b is a developmentally-regulated intracellular ER and Golgi apparatus membrane protein that may prove important for correct brain development through functions associated with precursor cells and glia.

Key words: Tmem50b, cerebellum, Bergmann glia, Down syndrome, endoplasmic reticulum.

Partial or complete trisomy of human chromosome (Hsa) 21 results in a plethora of pathological signs which are known as Down syndrome (DS; OMIM 190685). Approximately 345 genes have been identified from the sequence of Hsa21, but less than half (150) code for proteins with functional annotation (Gardiner and Costa, 2006). The detailed developmental and adult expression patterns, subcellular localization and functions of many Hsa21 genes are currently unknown, leaving their potential contribution to the DS phenotype unclear.

In an earlier study (Dauphinot et al., 2005; discussed also in Potier et al., 2006), we profiled the gene expression of the developing cerebellum in a mouse model of DS, T(12;16)1Cje (Ts1Cje; Sago et al., 1998). Unlike most genes present on the triplicated Hsa21 orthologue, Mmu16, only four were found to be over-expressed at each of the three time points of cerebellar development investigated: postnatal day (P) 0, P15 and P30. These genes included DS critical region 3 (*Dscr3*), downstream neighbor of SON (*Donson*), high mobility group nucleosomal binding domain 1 (*Hmgn1*) and the transmembrane protein 50b or chromosome 21 open reading frame 4 (*Tmem50b* or *C21orf4*). Of these four genes, little is known of the distribution, localization or expression of the mRNA or protein of *Tmem50b*. The selective over-expression of *Tmem50b* in the developing cerebellum of Ts1Cje mice suggests this gene as a potential key contributor to the hypocellular phenotype of the DS cerebellum (Moldrich et al., 2007).

The *Tmem50b* gene is one of two genes in the *transmembrane 50* group, with *Tmem50a* being the other, located on Hsa1. *Tmem50b* was first described as belonging to an evolutionary conserved cluster present on Hsa21 (Reboul et al., 1999; Lutfalla et al., 2003). This gene cluster was named the glycinamide ribonucleotide formyltrans-

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Abbreviations: a.a., amino acid; div, days *in vitro*; DS, Down syndrome; EEA1, early endosome antigen 1; ER, endoplasmic reticulum; E14.5, embryonic day 14.5; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GART-CRII, glycinamide ribonucleotide formyltransferase-cytokine receptor class II; GFAP, glial fibrillary acidic protein; IGL, internal granule cell layer; MAP2, microtubule-associated protein 2; NPC, neural precursor cell; NPCM, neural precursor cell medium; P, postnatal day; PBS-Triton, 0.1% Triton-X 100 in PBS; PBS-Tween-Milk, PBS with 0.1% Tween-20 and 5% skimmed milk; PC, Purkinje cell; SERT, 5-HT transporter; Ts1Cje, T(12;16)1Cje.

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0306-4522/08$32.00+0.00 © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2008.01.089

ferase-cytokine receptor class II (GART-CRII) gene cluster because in addition to *GART* it also included interferon α/β-receptor (*IFNAR1* and *IFNAR2*) and the second component of the interferon γ-receptor (*IFNGR2*) and of the interleukin-10 receptor (*IL10R2*). Among this cluster Reboul et al. (1999) identified *Tmem50b* (*C21orf4*), which was described as a seven-exon and five-intron gene with expressed sequences tag (EST) matches in humans and mice. The protein, Tmem50b, was estimated to contain four transmembrane-spanning domains with both the carboxy- and amino-terminals being cytoplasmic.

To date, the only other data that exist on Tmem50b have been presented by Rosen et al. (2005) who found *Tmem50b* to be a member of a group of six candidate genes, which based on their level of expression could differentiate benign from malignant thyroid nodules with high specificity and sensitivity. However, due to the unknown tissue specificity and function of Tmem50b, possible influences on thyroid tumor growth could not be ascertained. *Tmem50a* has not been studied apart from its original sequencing.

The objective of the present study was to establish the tissue distribution and subcellular localization of *Tmem50b* mRNA and protein with a particular focus on the cerebellum. We expect these results to serve as a reference for future investigation into Tmem50b protein function under normal and disturbed physiological and developmental conditions such as DS.

EXPERIMENTAL PROCEDURES

Animals

Animals used in the present study were Wistar rats, NMRI or C57BL/6 mice. Pregnant Wistar rats or Wistar pups were used for *in vitro* studies while male Wistar rats were used for protein extraction for Western blot. All other experiments were performed with C57BL/6 mice with the exception of embryonic *in situ* hybridization where NMRI mice were used to ensure maximum comparability with large-scale ongoing studies. Experiments were carried out in accordance with institutional guidelines that aim at minimizing animal use and suffering, and according to the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Cultures

Neural precursor cells (NPCs) were derived from C57BL/6 mouse embryos at day 14 and grown as 'neurospheres' in neural precursor cell medium (NPCM; consisting of DMEM:F12 containing Glutamax, 1× B27 supplement, 10 ng/μl epidermal growth factor, 10 ng/μl basic fibroblast growth factor, 20 ng/μl leukemia inhibitory factor, and penicillin/streptomycin (Invitrogen, Cergy Pontoise, France)). Briefly, the neocortex was dissected and dissociated to a single cell suspension using a syringe and needle of 18 gauge. The cells were then placed in a flask of NPCM and incubated for up to 5 days *in vitro* (div) at 37 °C in the presence of 5% CO_2. After this period of time, neurospheres containing NPCs were split by trituration with the aid of warmed Accumax (Sigma) and a small pipette tip. Such passaged neurospheres were plated at half the density of their precursors. NPCs were cultured in this way for at least five passages (~25 div) before being employed for immunocytochemistry.

Mixed neuronal–glial cultures of the hippocampus were established following dissection and dissociation of embryos at 18 days for rat and at 16 days for the mouse with the aid of 0.25% trypsin. Cells were counted and plated at a density of 60,000–80,000 cells per 12 mm poly-D-lysine (50 μg/ml) -treated glass coverslip in Neurobasal medium (Invitrogen) with 1× B27 supplement (Invitrogen), penicillin/streptomycin and 10% fetal calf serum. Cultures were left to adhere to coverslips for 4 h at 37 °C. Afterward, the medium was replaced with Neurobasal medium with 1× B27 supplement and penicillin/streptomycin that had been previously conditioned in the presence of glial cultures for 2 days. Cultures were grown to 14 div without medium change.

Rat and mouse glial cultures were established from dissected cerebral cortex of between the ages of P3 to P5 for rats and at E16 for mice and grown in DMEM with 10% fetal calf serum (Invitrogen) in flasks at 37 °C with 5% CO_2. After reaching confluence, cells were passaged with the aid of 1× Trypsin-EDTA (Invitrogen). Glial cultures had been passaged no more than five times before being used for immunocytochemistry or for conditioning neuron culture medium.

RNA *in situ* hybridization on tissue sections

Collection, preparation, and cryosectioning of mouse tissue was performed as previously described (Visel et al., 2006). Brains were dissected from P7 C57BL/6 strain mice and embryos were dissected from NMRI strain mice at embryonic day 14.5 (E14.5). Animal breeding and tissue collection were performed according to institutional guidelines. Non-radioactive *in situ* hybridization with digoxigenin-labeled probes was carried out on a GenePaint robotic *in situ* hybridization platform (Visel et al., 2004; Yaylaoglu et al., 2005). Templates for riboprobe synthesis were generated by reverse transcription and PCR of a fragment that corresponded to positions 20–2120 of GenBank entry NM_030018 for *Tmem50b* and NM_027935 for *Tmem50a*, and finally, NM_007430 for nuclear receptor subfamily 0, group B, member 1 (*Nr0b1*) (also known as *Dax1*) used as an antisense negative control which is expected to show no expression in brain. Digoxigenin-labeled antisense riboprobe was generated by standard methods (Visel et al., 2006). Image acquisition of tissue sections was carried out with a customized Leica DM RXA2 robotic microscope connected to a Hitachi HV-C20A digital camera (Herzig et al., 2001).

Tmem50b gene expression analysis by quantitative PCR

A multiple tissue screen of *Tmem50b* gene expression was performed on mouse cDNA using the Multiple Tissue kit (Clontech). The manufacturer's protocol was adapted for SYBR® Green qPCR in a Roche Lightcycler by diluting (1:5) the initial cDNA stocks. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control: the forward and reverse primers used for GAPDH were TGTTAGTGGGGTCTCGCTCCT and GGTCGGTGTGAACGGATTTGG, respectively. Forward and reverse primers for *Tmem50b* were GCACTCTGATCTACAAATT and AATTTGTAGATCAGAGTGC, respectively.

Tmem50b antibody

An antibody for Tmem50b was created with the assistance of Eurogentec (Seraing, Belgium). First, a 14 amino acid (a.a.) peptide was constructed against the N-terminal portion of Tmem50b (a.a. 8–21), which possesses a highly unique sequence that shares little similarity with the protein, Tmem50a, the gene of which is found on Hsa1. The peptide sequence used was as follows: FRWPECECIDWSER. The peptide was purified and conjugated to KLH. Immunization was performed in two rabbits over the course of 3 months. ELISA titers and affinity purification were performed. Aliquots of the purified antibody (hereafter anti-Tmem50b) used in this study were stored at −20 °C in the presence of glycerol. To verify the specificity of anti-Tmem50b, a