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TAG-1-Deficient Mice Have Marked Elevation of Adenosine A1 Receptors in the Hippocampus

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TAG-1 is a neural recognition molecule in the immunoglobulin superfamily that is predominantly expressed in the developing brain. Several lines of evidence suggest that TAG-1 is involved in the outgrowth, guidance, and fasciculation of neurites. To directly assess the function of TAG-1 *in vivo,* we have generated mice with a deletion in the gene encoding TAG-1 using homologous recombination in embryonic stem cells. Gross morphological analysis of the cerebellum, the spinal cord, and the hippocampus appeared normal in TAG-1-deficient mice. However, TAG-1 (−/−) mice showed the upregulation of the adenosine A1 receptors determined by [^{3}H]cyclopentyl-1,3-dipropylxanthine in the hippocampus, and their greater sensitivity to convulsant stimuli than that in TAG-1 (+/+) mice. We suspect that the subtle changes in neural plasticity induced by TAG-1 deficiency during development cause the selective vulnerability of specific brain regions and the epileptogenicity in TAG-1 (−/−) mice.

Key Words: **TAG-1; immunoglobulin superfamily; gene targeting; adenosine receptor; hippocampus, convulsive behavior.**

TAG-1 is a neural recognition molecule that belongs to the immunoglobulin (Ig) gene superfamily. TAG-1 structure consists of six Ig-like domains of the C2 class and four fibronectin type III-like repeats in its ectodomain, and is attached to the plasma membrane by a glycosylphosphatidylinositol lipid anchor (1). Axonin-1 glycoprotein is the chicken homologue of TAG-1 (2). TAG-1 has 95% homology between human and rat homologues at the amino acid sequence level. In addition, a 75% homology exists between rat TAG-1 and chick axonin-1 (3, 4). TAG-1 is a member of the contactin subgroup that includes contactin (also termed F3 or F11), BIG-1, BIG-2, NB-2, and NB-3 (5). Each of these molecules displays a distinct spatiotemporal expression pattern (6).

In addition to homophilic interactions, TAG-1 interacts with a number of different heterophilic binding partners, including two other members of the Ig superfamily, Ng-CAM/L1 and Nr-CAM, nervous tissue-specific chondroitin sulfate proteoglycans and several extracellular matrix molecules such as tenascin-C and -R. Through these interactions, TAG-1 is believed to play functionally important roles in the initial stages of axon outgrowth and in interactions between developing neurons. Some of these heterophilic interactions enable TAG-1 to induce or influence intracellular signaling processes (7–9).

Several reports suggest that TAG-1 plays a role in the establishment of specific axonal connections in the developing central nervous system (CNS). In particular, TAG-1 immunoreactivity is detected at high levels in the cerebellar molecular layer, the corpus callosum, hippocampal commissures, and crossed projections of the spinal cord (10, 11). To directly assess the function of TAG-1 *in vivo,* we introduced a null mutation into the TAG-1 gene by targeted mutagenesis and subsequently analyzed the TAG-1 knockout mice for developmental defects.

MATERIALS AND METHODS

Gene targeting by homologous recombination. A genomic DNA clone, containing exon II-VI of TAG-1 gene, was isolated from a genomic DNA library of 129/SvJ mouse (Stratagene, La Jolla, CA) by

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screening with a rat 5′ cDNA probe and was partially sequenced before constructing targeting vectors. Targeting vectors were designed to replace a 5 kb DNA fragment of the TAG-1 gene between the *Hinc*II site in exon II and the *Eco*RI site in exon V with the neomycin resistance gene (Neo) cassette. A diphtheria toxin A fragment gene (DT) cassette, under the control of the MC1 promoter, was ligated at the 3′ end of the vector for negative selection (12) (Fig. 1A). The *Sac*II-linealized targeting vector DNA (50 μg) was transfected by electroporation (500 μF, 250 V; Gene Pulsar; Bio-Rad Laboratories, Hercules, CA) into 1×10^7 R1 embryonic stem (ES) cells (13). Cells were selected by survival in the presence of G418 (250 μg/ml; GIBCO BRL, Gaithersburg, MD), and screening of the resistant colonies was conducted by using nested PCR. Outer primers (P1: 5′-ATGGTGCTCTAAGACGGACAGACT-3′; antisense, P2: 5′-CGTTCCACAGGCAGGAGCTGTGGA-3′) were used for the first round PCR and inner primers (P3: 5′-TTGGGAAGACAATAGCAGGCATGC-3′; antisense, P4: 5′-CTCTATGGCTTCTGAGGCGGAAAG-3′) were used for the second round PCR (Fig. 1A). Chimeric mice were produced from the targeted ES clones using an aggregation method originally described by Nagy *et al.* (13). The contribution of ES cells to the germline of chimeric mice was identified by breeding with C57BL/6 mice and screening for agouti offspring. Germline transmission was confirmed by Southern blotting of tail DNA, and mice heterozygous for the mutation were interbred to homozygousity. The mice were maintained under specific pathogen-free conditions at the Laboratory Animal Research Center, Institute of Medical Sciences, University of Tokyo. All experiments were performed in accordance with the institutional ethical guidelines for animal experiments and the safety guidelines for gene manipulation experiments in Japan.

Southern and Western blot analysis. For Southern blot analysis, genomic DNA was extracted from mouse tail and digested with *Sca*I. After agarose gel electrophoresis, the separated DNA was transferred to nylon membranes and hybridized with probes corresponding to intron I (0.5 kb of *Sca*I-*Eco*RI fragment) (Fig. 1A). Southern blot hybridization analysis was performed according to standard methods. In the case of Western blot analysis, the SDS polyacrylamide gel electrophoresis was performed by the method of Laemmli (14), and detection was performed by ECL, as described in the manufacturer's manual (Amersham Pharmacia Biotech, Japan).

Preparation of rabbit anti-TAG-1 antibodies. cDNA fragments encoding Ig domains I–II (amino acids: 31–143) of rat TAG-1 were amplified by PCR using the following primers: 5′-TCATATGCAGGGAACCCCAGCTACCTTTGG-3′ and 5′-TGAGGAGCCAGCGGTAGGACAAACC-3′. Details were described by Kasahara *et al.* (15). A rabbit antiserum against mouse TAG-1 peptides corresponding to the carboxyl terminus of the amino acid positions 723–933 of rat TAG-1 was also prepared in the same manner using PCR primers (5′-TTCATATGCAGACTGCCCGGGTGCCTGGCGCT-3′ and 5′-ATGGATCCCTTAAGGCTGAGACTGGAGCT-3′) and the pET-15b expression vector (Novagen, Madison, WI).

Immunohistochemical analysis. The cerebrum and the spinal cord of TAG-1 knockout mice at postnatal day 2 (P2) and embryonic day 15 (E15) were examined histologically. Some sections were stained with 0.5% cresyl violet for Nissl staining, while others were processed for the immunohistochemistry. For immunohistochemical staining, the following antibodies were used: [1] a rabbit antiserum against neurofilament (1:5000) (16) and [2] a rabbit antiserum against calbindin D-28k (1:5000; Swant antibodies, Bellinzona, Switzerland). Bound antibody was visualized using appropriate peroxidase conjugated secondary antibodies, followed by incubation in 50 mM Tris buffer (pH 7.4) containing 0.01% diaminobenzidinetetrahydrochloride and 0.01% hydrogen peroxide at 37°C for 5–15 min.

Autoradiographic studies of adenosine A1 receptors. The mice (P34–38) were killed by decapitation, and the brain was rapidly removed and frozen in isopentane chilled to −45°C. Coronal cryostat sections (10 μm in thickness) were cut at −20°C, and thaw-mounted onto chrome-gelatin coated glass slides. Adenosine A1 receptors were visualized using [^{3}H]cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective high affinity antagonist for A1 adenosine receptors, as a radioactive ligand (17).

RESULTS

Production of TAG-1 Mutant Mice

The targeting vector DNA was transfected into ES cells, and transfectants were subjected to positive (Neo) and negative (DT) selection. DNA, isolated from surviving colonies, was screened for the desired homologous recombinants. A total of 624 double-resistant colonies were selected and screened by PCR (see Materials and Methods). The targeting event was evaluated by Southern blot analysis. The predicted sizes for the *Sca*I-digested fragments associated with the wild-type allele and mutant alleles are 5.2 and 8 kb, respectively (Fig. 1A). Southern blot analysis confirmed that four clones had a correctly targeted TAG-1 allele. Mice obtained from the recombinant ES cells were tested for the TAG-1 mutation by Southern blot analysis (Fig. 1B). Western blot analysis of brain tissue confirmed a complete absence of the 130 Kd TAG-1 band in TAG-1 (−/−) mice (Fig. 1C). This observation was verified by two antisera raised to both N- and C-terminal peptides, as described under Materials and Methods. The TAG-1 gene was deleted in the mutant mice between exon II and V, which encoded the amino acid sequence from the translation initiation site to the first Ig-like domain. However, the antiserum against the second and third fibronectin type III-like regions did not show any band on the Western blot analysis. This indicates that TAG-1 protein is not produced in TAG-1 (−/−) mice. Two lines of germline-transmitting mice, produced from two independently derived mutants from the R1 ES cell clones, were obtained and used for further experiments.

General Appearance of Mutant Mice

The genotype of the offspring produced from TAG-1 (+/−) mating was approximately 25% (−/−) mutant, 25% (+/+) and 50% (+/−) mutant. These results are compatible with Mendelian ratios and indicated that homozygosity did not result in lethality either in utero or shortly after birth. Grossly, the mutant mice were indistinguishable from the wild-type mice with respect to the food and water intake, body weight, and posture, and did not display any signs of ataxia.

Histological Analysis of the CNS

The development of the brain and spinal cord in TAG-1 (+/+) (Figs. 2 and 3, left panels) and TAG-1 (−/−) (Figs. 2 and 3, right panels) mice was histologically and immunohistochemically examined at P2 and E15. In Nissl-stained sections, both the size and struc-

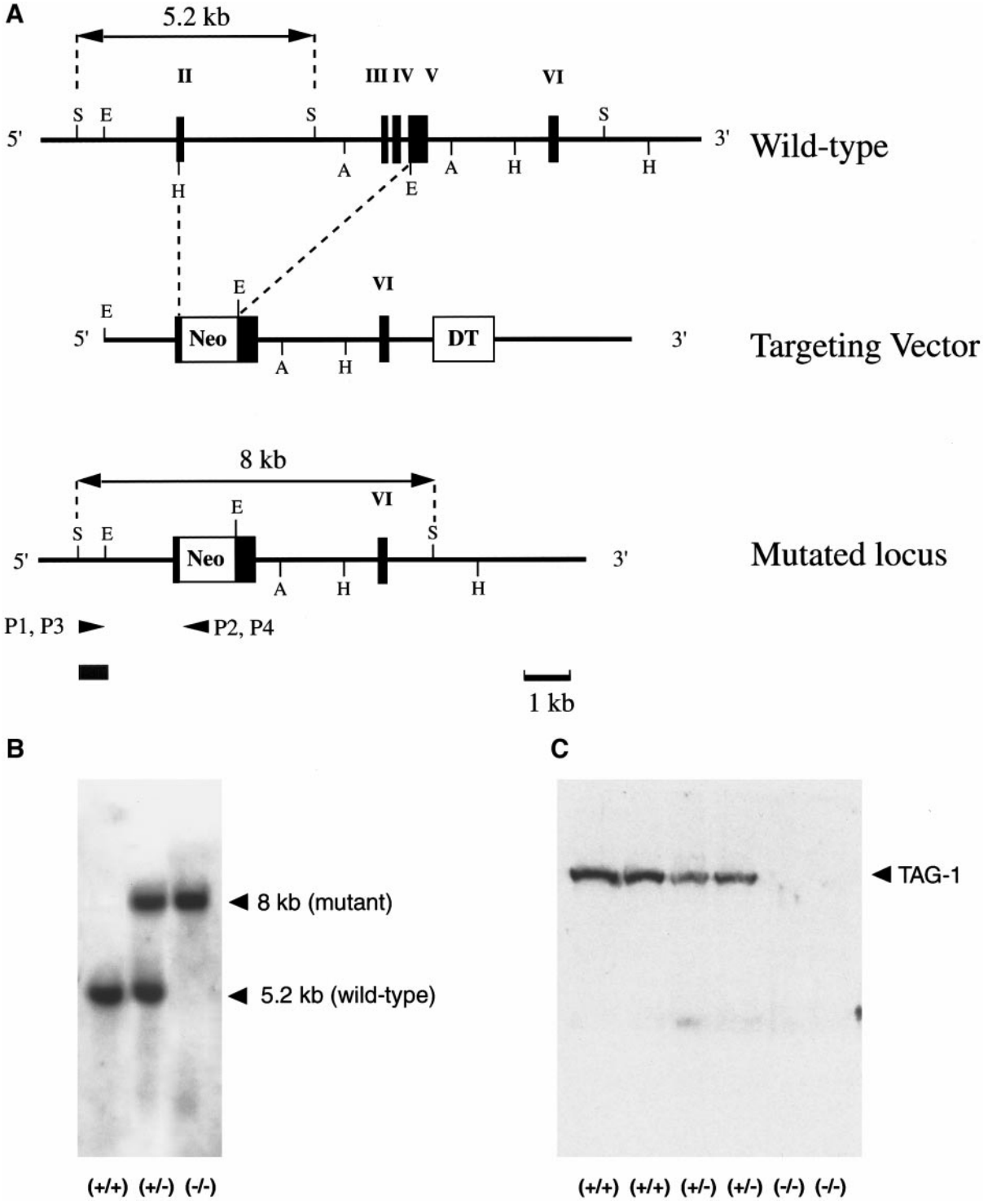

FIG. 1. Inactivation of TAG-1 gene by homologous recombination. (A) Schematic diagram of the strategy used to target TAG-1 gene. Black boxes represent exons for the TAG-1 gene. Translation initiation codon ATG was encoded in exon II. A part of the TAG-1 gene (exons II–V) was replaced with the neomycin resistance gene (Neo). The diphtheria toxin A fragment gene (DT) fragment was ligated at the 3′ end of the vector for negative selection. The Neo and DT are inserted in the reverse orientation to the TAG-1 gene. In the targeting vector, pBluescript KS(+) was shown by a hatched bar. 5.2 and 8 kb DNA fragments obtained by *Sca*I digestion of the wild-type and mutant allele DNAs are indicated by arrows, respectively. Arrowheads indicate the positions for nested PCR primers (P1, P2, P3, and P4). Shaded box: the probe for Southern blot analysis. (A, *Apa*I; E, *Eco*RI; H, *Hin*cII; S, *Sca*I). (B) Genomic Southern blot analysis of tail DNAs of a littermate from heterozygous intercrosses. The probe used for hybridization was a 0.6 kb *Sca*I-*Eco*RI fragment shown in Fig. 1A (bottom). This probe hybridized to a 5.2 and a 8 kb fragment from wild-type and mutant alleles, respectively. Wild-type (+/+), heterozygous (+/−), and homozygous (−/−) alleles are indicated. (C) Western blot analysis of the TAG-1 protein. Brain extracts were prepared from TAG-1 (+/+), (+/−) and (−/−) mice. Two extracts from each genotypes were applied to the SDS–PAGE. TAG-1 protein was detected at 130 Kd in (+/+) and (+/−), using a rabbit antiserum as described in the text. Band intensities from TAG-1 (+/−) mice were approximately half of those from the wild-type mice. In contrast, no band detected at 130 Kd in the TAG-1 (−/−) mice.

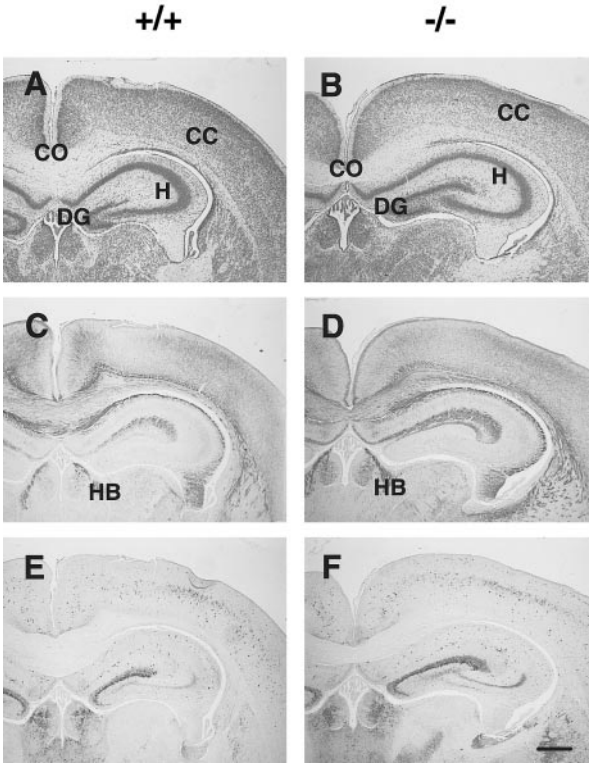

FIG. 2. Histological analysis of TAG-1 (+/+) and TAG-1 (−/−) mice. Coronal sections of the hippocampus from P2 (+/+) (left panels) and P2 (−/−) (right panels) mice. Stained for Nissl (A and B), and immunostained for neurofilament (C and D), and calbindin (E and F). The hippocampus of TAG-1 (−/−) mice seems to develop normally. Anatomical regions are abbreviated: CC, cerebral cortex; CO, corpus callosum; H, hippocampus; DG, dentate gyrus; HB, habenula. Scale bar = 200 μm.

ture of the CNS were similar between TAG-1 (+/+) and TAG-1 (−/−) mice at P2 in the hippocampus (Figs. 2A–2F), cerebellum (Figs. 3A–3D) and spinal cord (Figs. 3E and 3F). Neuronal cells, such as cortical plate neurons (18) and cerebellar granule cells (10), which intensely express TAG-1 during development, were present in the brain of TAG-1 (−/−) mice. Some of the neurons of the hippocampus (Figs. 2E and 2F) and cerebellum (Figs. 3C and 3D) were immunoreactive for calbindin.

Immunohistochemical analysis using the anti-neurofilament antibody revealed no remarkable changes in the appearance of axonal pathways in the brain, including TAG-1 positive axonal tracts, such as the optic chiasm, lateral olfactory tract, corpus callosum, and hippocampal commissure (Figs. 2C and 2D). In the ventral midline of the spinal cord, the appearance of neurofilament-positive crossing fibers was similar between wild-type and TAG-1-deficient mice (Figs. 3E and 3F), although the perturbation of axonin-1, avian TAG-1 homolog, has been reported to result in abnormal trajectory of commissural axons in chick embryos (19). When immunostained with anti-TAG-1 antibodies, no positive neurons or axon tracts were detected in TAG-1 (−/−) mouse brains at both P2 and E15 (data not shown), although TAG-1 immunoreactivity decreases at this age in wild-type mice compared to that in fetal stages (10).

Increase in A1 Adenosine Receptors and Neuroexcitability

Although TAG-1 deficient mice were observed to be normal in their behavior, a few mutant mice displayed spontaneous epileptic seizures. This observation prompted us to examine the levels of adenosine receptors in the brain.

The autoradiographic distribution of [^{3}H]DPCPX binding sites in TAG-1 (+/+) mouse brain is shown in Fig. 4A. The highest density of binding sites in TAG-1 (+/+) mice was found in the dentate gyrus molecular layer, hilar region, polymorphic, and molecular layers of Ammon's horn and the subiculum. In the brain of TAG-1 (−/−) mice, the density of [^{3}H]DPCPX binding sites was increased by 40% in the entire hippocampus area, compared to that of TAG-1 (+/+) mice (Figs. 4B

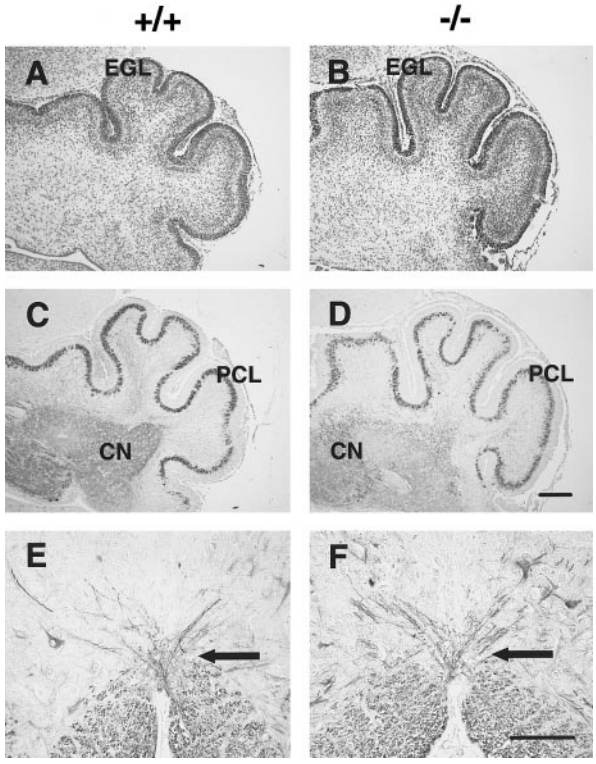

FIG. 3. Coronal sections of the cerebellum (A–D) and the lumbar spinal cord (E and F) from P2 (+/+) (left panels) and P2 (−/−) (right panels) mice. Stained for Nissl (A and B), and immunostained for calbindin (C and D) and neurofilament (E and F). Arrows indicate crossing fibers of the spinal cord at the higher magnification. Anatomical areas are abbreviated: EGL, external granular layer; PCL, Purkinje cell layer; CN, cerebellar nucleus. Scale bars = 100 μm in A–D; 50 μm in E and F.

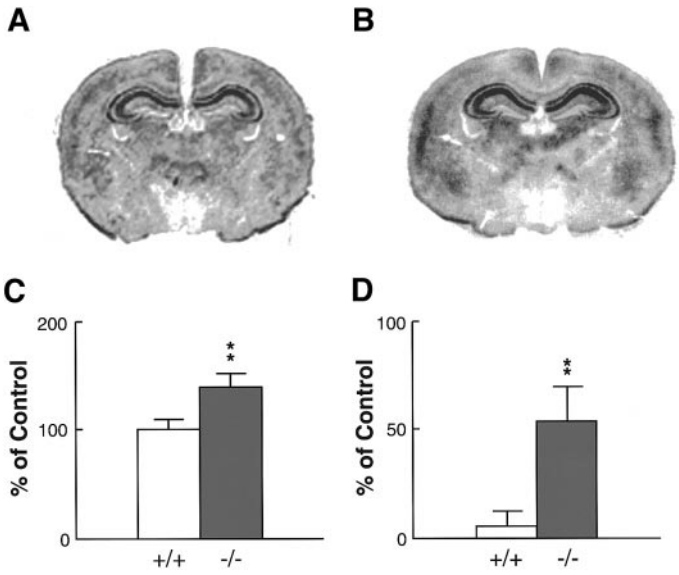

FIG. 4. Representative autoradiograms of [^{3}H]DPCPX binding at the hippocampal level of (A) TAG-1 (+/+) and (B) TAG-1 (−/−) mouse brain. (C) Densitometry of autoradiograms and (D) the percentage of seizure-onset mice by 30 mg/kg of PTZ. Data in (C) was calibrated by the internal microscale. Statistical difference between (+/+) (n = 25) and (−/−) (n = 20) were made using the Student's t-test and values are expressed as mean ± SEM (**$P < 0.01$).

and 4C). No statistically significant differences were observed in the other regions of the brain. In contrast, the levels of dopamine D1 and D2 receptors, evaluated by [^{3}H]SCH23390 (0.5 nM) and [^{3}H]spiperone (0.5 nM), respectively, were virtually identical between (−/−) and (+/+) (data not shown).

To evaluate the substantial neuroexcitability in the mutant mice, mice (P34-38) were treated once intraperitoneally with convulsant stimuli; pentylenetetrazole (PTZ), and kainic acid. A dose that failed to induce convulsive behavior in TAG-1 (+/+) mice (30 mg/kg of PTZ) induced clonic-tonic seizures, as evaluated by the Student's t-test in the 52% of TAG-1 (−/−) mice (Fig. 4D). On the other hand, the mice injected with kainic acid (20 mg/kg) initially exhibited characteristic changes in motor behaviour, including hunched posture with crossed fore-paws and hind-legs spread apart, body tremor, occational jerking of the extremities, and marked catatonic posture in most animals of both TAG-1 (+/+) and TAG-1 (−/−) groups. Myoclonic twitching, which was restricted to the head, face and one or both fore-limbs, often developed into generalized clonic-tonic seizures involving the whole body. In TAG-1 (+/+) mice, the convulsions subsided 2 h after injection and after 24 h the animals appeared physically recovered. However, TAG-1 (−/−) mice had significantly shorter latencies to generalized seizures compared with those in TAG-1 (+/+) mice (Table 1), and the convulsive phase in TAG-1 (−/−) mice often lasted for several hours with frequent rearing and falling over to one side, and the foam from the mouth. The mortality rate of the kainic acid-induced convulsive mice was more than 75% in TAG-1 (−/−) mice within 24 h of the administration, compared to less than 10% in TAG-1 (+/+) mice.

DISCUSSION

TAG-1 is abundantly expressed in the cerebellum, spinal cord, and hippocampus of newborn mice. In this study, we have explored the role of the TAG-1 gene in development by disrupting its expression. The following findings were obtained by the analyses of the TAG-1 deficient mice.

First, TAG-1 is generally expressed on granule cells in the deep external granule layer of the postnatal cerebellum. TAG-1 exhibits a complementary expression pattern in the developing cerebellar cortex to calbindin, which is a useful cell specific marker for Purkinje cells (10, 20). The dendrites of Purkinje cells make synaptic contact with the parallel fibers of granule cells. However, a histological analysis of the cerebellum revealed that the thickness and density of both the granule cell layer and the Purkinje cell layer were normal in TAG-1 deficient mice (P2 and E15). These findings are truly surprising because null-mutant mice that lack contactin, a member of the Ig superfamily subgroup, show drastic changes in both granule cell axons and Purkinje cells (21).

TAG-1 and contactin are highly expressed on the axons of cerebellar granule cells during the first postnatal week. It has been suggested these molecules are important for neurite outgrowth and fasciculation and/or defasciculation events in the cerebellar development (22). In addition, Buttiglione *et al.* (23) reported that the inhibitory effect of contactin on the neurite outgrowth of cerebellar neurons was completely blocked by the presence of TAG-1 *in vitro.* Nevertheless, the occurrence of no manifest changes in the cerebellum of TAG-1 (−/−) mice in our study strongly suggests that some mechanisms compensate for the lack of the TAG-1 gene.

TABLE 1

Latencies of TAG-1 (+/+) and TAG-1 (−/−) Mice to Kainic Acid-Induced Generalized Convulsions

Mice	(n)	Latency (sec)
TAG-1 (+/+)	(12)	2684 ± 298
TAG-1 (−/−)	(12)	1702 ± 196*

Note. TAG-1 (−/−) mice had significantly shorter latencies to kainic-acid (20 mg/kg)-induced generalized seizures than did TAG-1 (+/+) mice. Values are mean ± SEM. Numbers in parentheses are numbers of experiments. Single asterisk denotes $P < 0.01$ (Student's t-test).

Second, TAG-1 stimulates neurite outgrowth of commissural neurons in spinal cord and dorsal root ganglia neurons (10). After commissural axons pass through the floor plate, they undergo an abrupt change in trajectory and begin to fasciculate and express L1. Floor plate cells secrete a diffusible factor that evokes the outgrowth of commissural axons from the dorsal horn towards the floor plate. Immunohistochemical analysis using an anti-neurofilament antibody revealed no remarkable changes in the pathways of the spinal commissural axons in mutant mice (Figs. 3E and 3F).

Third, no overt phenotype was observed in the hippocampus of the TAG-1 (−/−) mice. However, since a few TAG-1 (−/−) mice revealed spontaneous epileptic seizures, we performed adenosine A1 autoradiographic studies of adult mice (P34-38) using [^{3}H]DPCPX. Adenosine is a purine nucleoside that has many effects on the CNS through its action on adenosine receptors designated A1, A2, and A3 (24). Important roles of the A1 adenosine receptor have been assumed in the hippocampus, where this receptor is expressed at its highest levels. Radioligand autoradiographic studies have identified A1 adenosine receptors in the dentate gyrus located predominantly on axons. Adenosine analogs block experimentally induced seizures in animals, and adenosine may act as an endogenous anticonvulsant in humans (25). Here, we report a significant increase of the A1 adenosine receptors in the hippocampus, and the enhanced seizure susceptibility (i.e., seizure severity and higher mortality) of TAG-1 (−/−) mice to PTZ and kainic acid, despite the lack of histological abnormalities in the hippocampus of TAG-1 (−/−) mice (E15, P2, and P34). Taken together, these results suggest that the increase of adenosine A1 receptors can be attributed to a postsynaptic compensation mechanism for adenosine A1 hypofunction and the neuroexcitability (i.e., lower threshold to PTZ and kainic acid) is due to this adenosine hypofunction in neural plasticity induced by TAG-1 deficiency during brain development. In this context, TAG-1 (−/−) mice provide a valuable model to clarify a possible mechanism of the epileptic seizures.

A fundamental mechanism underlying the relationship between adenosine transmission and the function of TAG-1 remains to be identified. Interestingly, the inhibition of serotonin synthesis by 5,7-dihydroxytryptamine injections in the raphe nucleus has been reported to lead to a decrease in highly polysialylated neural cell adhesion molecules and, in contrast, to an increase in tenascin-C immunoreactivity in some brain regions (26). Moreover, tenascin-C mutant mice display an abnormal response to dopamine agonists, suggesting a role for tenascin signaling in dopamine transmission (27). These findings suggest that Ig superfamily adhesion molecules and the extracellular matrix in the CNS can modulate brain neurotransmission. In this respect, mice deficient for Ig superfamily molecules may prove to be powerful tools in elucidating mechanisms of neurotransmission.

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