Neurobiology of Disease

Impaired Feedforward Inhibition of the Thalamocortical Projection in Epileptic Ca\textsuperscript{2+} Channel Mutant Mice, \textit{tottering}

Sachie Sasaki,\textsuperscript{1,2} Kadrul Huda,\textsuperscript{1} Tsuyoshi Inoue,\textsuperscript{1,2} Mariko Miyata,\textsuperscript{1,2} and Keiji Imoto\textsuperscript{1,2}

\textsuperscript{1}Department of Information Physiology, National Institute for Physiological Sciences, and \textsuperscript{2}School of Life Sciences, The Graduate University for Advanced Studies, Okazaki 444-8787, Japan

The \textit{tottering} (tg) mice have a mutation in the Ca\textsubscript{v}2.1 (P/Q-type) voltage-dependent Ca\textsuperscript{2+} channel \alpha\textsubscript{2,1} subunit gene. tg mice show not only cerebellar ataxia but also absence epilepsy, which begins at ~3 weeks of age and persists throughout life. Similarities in EEG and sensitivity to antiepileptic drugs suggest that tg mice are a good model for human absence epilepsy. Although imbalance between excitatory and inhibitory activity in the thalamocortical network is thought to contribute to the pathogenesis of absence epilepsy, the effect of the mutation on thalamocortical synaptic responses remains unknown. Here we showed imbalanced impairment of inhibitory synaptic responses in tg mice using brain slice preparations. Somatosensory thalamocortical projection makes not only monosynaptic glutamatergic connections but also disynaptic GABAergic connections, which mediate feedforward inhibition, onto layer IV neurons. In tg mice, IPSC amplitudes recorded from layer IV pyramidal cells of the somatosensory cortex in response to thalamic stimulation became disproportionately reduced compared with EPSC amplitudes at later developmental stages (postnatal days 21–30). Similar results were obtained by local stimulation of layer IV pyramidal neurons. However, IPSC reduction was not seen in layer V pyramidal neurons of epileptic tg mice or in layer IV pyramidal neurons of younger tg mice before the onset of epilepsy (postnatal days 14–16). These results showed that the feedforward inhibition from the thalamus to layer IV neurons of the somatosensory cortex was severely impaired in tg mice and that the impairment of the inhibitory synaptic transmission was correlated to the onset of absence epilepsy.

Key words: absence seizure; thalamocortical projection; feedforward inhibition; synaptic transmission; Ca\textsuperscript{2+} channel; mutant mice

Introduction

Absence seizure is one type of generalized epilepsy. It is characterized by bilaterally synchronous spike-and-wave discharges (SWDs) in the electroencephalogram (EEG) over wide cortical areas. The generalized nature of the SWDs led to the hypothesis of a common central pacemaker and implied the thalamic contribution to the absence seizure (Jasper and Kershman, 1941). Conversely, analyses of the penicillin-induced generalized epilepsy suggested the importance of intracortical processes in the SWDs (Avoli and Gloor, 1982). Because the thalamus can generate strong synchronized oscillatory activities through interaction between thalamocortical relay cells and cells of the reticular thalamic nucleus (Steriade et al., 1993), these results, combined together, led to the hypothesis that an aberration of the interplay between the cerebral cortex and the thalamus causes the SWDs. However, the relative contribution of the thalamus and the cerebral cortex to the pathophysiological condition has been a matter of severe debate for decades.

The homozygous \textit{tottering} (tg) mice are an established genetic model of absence seizure (Noebels and Sidman, 1979). The tg mice have a mutation in the gene encoding the \alpha\textsubscript{2,1} subunit of the voltage-gated Ca\textsuperscript{2+} channel (Fletcher et al., 1996). The \alpha\textsubscript{2,1} subunit forms the channel pore of the Ca\textsubscript{v}2.1 (P/Q-type) channel (Mori et al., 1991), which plays essential roles in a variety of functions, including neurotransmitter release at presynaptic sites (Wheeler et al., 1994). Extensive studies on mutated Ca\textsubscript{v}2.1 channels in native neurons as well as in recombinant expression systems revealed that the tg mutation reduced the Ca\textsuperscript{2+} influx through the Ca\textsubscript{v}2.1 channel (Wakamori et al., 1998; Qian and Noebels, 2000). In the thalamocortical loop, excitatory but not inhibitory synaptic transmission was reduced in the somatosensory thalamus of tg mice (Caddick et al., 1999). However, synaptic transmission from the thalamus to the cortex was not examined in tg mice or other epileptic model animals.

In this study, we examined excitatory and inhibitory synaptic responses in layer IV pyramidal neurons in response to thalamic and cortical stimulation using somatosensory thalamocortical brain slice preparations. The results indicated that the mutation of Ca\textsubscript{v}2.1 calcium channel in tg mice results in a significant impairment of inhibitory, but not excitatory, synaptic transmission to layer IV pyramidal neurons in tg mice over 3 weeks of age. The impairment resulted in dysfunction of feedforward inhibition, which, in wild-type (wt) mice, suppressed excitability of layer IV neurons in response to thalamocortical inputs. The defective feedforward inhibition may play a role in the pathogenesis of absence epilepsy.
Parts of this work have been published previously in abstract form (Huda and Imoto, 2001).

Materials and Methods

Animals. The C57BL/6-tg strain of tg mice was introduced from The Jackson Laboratory (Bar Harbor, ME). Mice at postnatal day 14 (P14) to P30 were used for the experiments. Mice were provided with a commercial diet (CE-2; Nihon Clea, Tokyo, Japan) and water ad libitum under conventional conditions with controlled temperature, humidity, and lighting (22 ± 2°C, 55 ± 5%, and 12 h light/dark cycle with lights on at 6:00 A.M.). The air was maintained and purified by mixing between heterozygous mice. All animal studies described herein were reviewed and approved by the ethical committee in our institute and were performed according to the institutional guidelines concerning the care and handling of experimental animals.

Genotyping of tg mice. Genomic DNA was extracted from the tail. The genomic region encoding a part of the Ca2+ channel α2,1 subunit gene was amplified using the cDNA Polymerase Mix (Clontech, Palo Alto, CA) and two sets of PCR primers, which were 5'-TATAATTGTG-ATGAGGGGTC-3' (sense for wt), 5'-TATATTGTGATGAGGGAGCCTC-3' (sense for tg), and 5'-CCACCGCAATGAAAGAAACG-ATTCCCAAACG-3' (antisense for both). The DNA-extract solution (2 μl) was used as a template for PCR (28 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and final extension at 72°C for 7 min). The resulting PCR products were subjected to electrophoresis on a 2.0% agarose gel.

Slice preparation. Somatosensory thalamocortical slices were prepared as described by Agmon and Connors (1991). Auditory thalamocortical slices were prepared as described by Cruikshank et al. (2002). Mice were killed by decapitation under deep halothane general anesthesia. Brains were removed and put into ice-cold cutting solution containing the following (in mM): 120 choline-Cl, 3 KCl, 1.25 NaH2PO4, 28 NaHCO3, 8 MgCl2, and 22 glucose, saturated with carbogen (95% O2 and 5% CO2). The tissue was cut into 450-μm-thick slices with a vibrotome (VT1000S; Leica, Nussloch, Germany). These slices were then incubated at 32°C for 30 min and room temperature for at least 30 min in artificial CSF (ACSF) containing the following (in mM): 125 NaCl, 25 NaHCO3, 25 glucose, 2.5 KCl, 1.25 NaH2PO4, 2 CaCl2, and 1 MgCl2, bubbled with carbogen.

To minimize artifactual effect of severing axons during the slicing procedure, we only used somatosensory thalamocortical slices in which thalamic stimulation evoked >100 pA EPSCs (10 mA intensity) in voltage-clamp mode or >1 mV EPSPs (50 μA intensity) in current-clamp mode.

Electrophysiological recordings. A whole-cell voltage-clamp recording was made from layer IV and V pyramidal neurons of the mouse barrel cortex, which were visually identified using an upright microscope equipped with a 60× water immersion objective (BX51WI; Olympus Optical, Tokyo, Japan) and an infrared differential interference contrast (DIC) video system (C2400–79H; Hamamatsu Photonics, Hamamatsu, Japan). Patch pipettes were made from borosilicate capillaries (2.0 mm outer diameter and 1.0 mm inner diameter; Hilgenberg, Malsfeld, Germany). EPSCs and IPSCs were recorded with an EPC9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany). The access resistance for recording was <15 MΩ and compensated by 50–70%. Capacitance of recorded cells was not significantly different between wt and tg mice (61.3 ± 7.9 pF for wt, n = 12; 55.6 ± 9.5 pF for tg, n = 11; p > 0.05). Cells were rejected if access resistance increased above 15 MΩ. Biocytin at 0.5% (Sigma, St. Louis, MO) was included in internal solutions for morphological characterization of recorded neurons. Stimulation and data acquisition were performed using the PULSE program (version 8.54; HEKA Elektronik). The current signals were filtered at 3 kHz and digitized at 20 kHz. All electrophysiological recordings were performed at a bath temperature of 32°C.

Thalamocortical responses. Thalamocortical EPSCs and IPSCs were evoked by electrical stimulation (10 mA, 200 μs), using a stimulus isolator (ISO-FLEX; A.M.P.I., Jerusalem, Israel) and stainless steel bipolar microelectrodes (A-M Systems, Carlsborg, WA) placed in the ventrobasal thalamic nucleus or the internal capsule (IC) (see Fig. 1A). Patch pipettes (3–6 MΩ) were filled with an internal solution containing the following (in mM): 140 Cs-CH3SO3, 5 KCl, 10 EGTA, 10 HEPES, 3 Mg-ATP, and 0.4 Na-GTP, adjusted to pH 7.4 with CsOH. QX-314 [N-(2,6-dimethylphenylcarbamoylmethyl)trimethylammonium bromide] (final concentration of 5 mM) was added to prevent Na+ spike generation. t-2-Amino-5-phosphonopentanoic acid (APV) (100 μM) was added to the external solution to block NMDA receptor-mediated currents, which otherwise often masked IPSCs. EPSCs were recorded at a holding potential (Vh) of −60 mV, whereas IPSCs were recorded at Vh of 0 mV. To obtain reversal potentials, AMPA receptor-mediated currents were recorded in response to pressure application of 5 mM AMPA (5–15 psi, 5–10 ms square pulse generated by PV830; World Precision Instruments, Sarasota, FL) in the presence of 1 μM tetrodotoxin (TTX) and 10 μM cyclothiazide, whereas GABA_t receptor-mediated currents were recorded by local stimulation in the presence of 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 100 μM APV. The reversal potential was determined by fitting the current–voltage plot by a linear regression (r ≥ 0.95). The reversal potentials (Vrev) of AMPA receptor-mediated or GABA_t receptor-mediated currents were +6.0 ± 1.4 mV (n = 4) and −64.4 ± 2.9 mV (n = 3), respectively. The conductance (G) was determined using the equation G = I/(Vh − Vrev), where I is EPSC or IPSC amplitude. Although excitatory and inhibitory thalamocortical responses were recorded in layer V pyramidal cells, we did not include the layer V thalamocortical responses, because the probability of the connection was low (Gil and Amitai, 1996).

Local responses. IPSCs and EPSCs were recorded at a holding potential of −60 mV in response to local electrical stimulation (0.1–10 mA, 200 μs) using a bipolar tungsten microelectrode with an interelectrode distance of 100 μm. The stimulation electrode was usually placed at −100 μm from the soma of the recorded neuron. The resistance of patch pipettes was 3–6 MΩ when filled with intracellular solution. The internal solution for IPSCs contained the following (in mM): 140 CsCl, 9 NaCl, 1 EGTA, 10 HEPES, and 2 Mg-ATP, adjusted to pH 7.3 with CsOH. The internal solution for EPSCs contained the following (in mM): 115 K-glutamate, 20 KCl, 1 EGTA, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, and 10 phosphocreatine, adjusted to pH 7.4 with KOH. QX-314 (final concentration of 5 mM) was added to prevent Na+ spike generation. IPSCs and EPSCs were recorded in the presence of 10 μM CNQX plus 100 μM APV and 10 μM (∼)-bicuculline methochloride (bicuculline), respectively. Bicuculline blockade (10 μM) did not lead to epileptic activities, presumably because there remained some inhibitory synaptic activities in thick slice preparations (450 μM) preventing abnormal discharges.

Miniature IPSCs. For recording miniature IPSCs (mIPSCs), slices from P21–P27 wt and tg mice were bathed in ACSF containing 1 μM TTX, 10 μM CNQX, and 100 μM APV. The internal solution was the same as used for IPSC measurement. The holding potential was −60 mV. Currents were filtered at 3 kHz and digitized at 20 kHz. Miniature IPSCs were measured periodically. Offline data analyses were performed with an IgorPro program (WaveMetrics, Lake Oswego, OR). Data were pooled from seven neurons (400 consecutive events each, n = 2800).

Current-clamp recording. Current-clamp recordings were obtained in bridge mode of an Axoclamp 2B amplifier (Molecular Devices, Palo Alto, CA). Patch pipettes (3–6 MΩ) were filled with an internal solution containing the following (in mM): 135 K-CH3SO3, 6 NaCl, 0.2 EGTA, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, and 10 phosphocreatine, adjusted to pH 7.3 with KOH. The series resistances were compensated with the bridge balance. Only cells that had resting membrane potentials more hyperpolarized than −50 mV and showed overshooting action potentials were accepted for this study. Electrical signals were filtered at 3 kHz and then digitized at 10 kHz using the ITC-16 interface (InstruTech, Port Washington, NY). Thalamocortical postsynaptic potentials (PSPs) in layer IV pyramidal cells were evoked by electrical stimulation (0.05–0.4 mA, 200 μs) using a bipolar tungsten microelectrode with an interstimulus distance of 250 μm placed in the internal capsule. In recordings of the thalamocortical PSPs, the membrane potential of layer IV pyramidal cells was set to −60 mV by current injection. The reversal potential of chloride currents, estimated by Nernst equation, is −81.6 mV under the condition of our current-clamp recordings.

Histological procedure. For immunohistochemistry, wt and tg mice aged P21–P23 were anesthetized with 75 mg/kg pentobarbital and per-
fused intracardially with 4% paraformaldehyde and 0.2% picric acid in 0.1 M sodium phosphate buffer (PB) for 12 min. Brains were removed and fixed overnight at 4°C and subsequently incubated in PB containing 10, 20, and 30% sucrose for overnight each. The tissue was sliced at 40 μm along the coronal section by a microtome (SM2000R; Leica). Tissue slices were washed in 0.05 M Tris-buffered saline (TBS) before incubation in 1% H2O2 for 1 h. Slices were washed three times in TBS and incubated with a rabbit polyclonal antibody against glutamic acid decarboxylase (GAD) (1:5000; Chemicon, Temecula, CA) in TBS containing 10% normal goat serum and 2% bovine serum albumin for two overnights at 4°C. The tissues were washed three times in TBS and incubated 3 h at room temperature in biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) using a 1:200 final dilution including normal goat serum and TBS. After rinses, the tissues were incubated with avidin–biotin–peroxidase complex (ABC Elite kit; Vector Laboratories) in TBS for 30 min. After washing in TBS, the tissues were reacted with DAB (0.05%) and H2O2 (0.002%) in TBS. They were then postfixed for 30 min in 1% OsO4 in PB, dehydrated in graded ethanol, and flat embedded on glass slides in Epon. Immunostaining clearly stained the somata of GABAergic neurons, sparing their nuclei. The number of the stained cells was measured in at least 1-mm-wide vertical strips in layer IV barrel cortex (typically 300 μm height × 1000 μm width). To ascertain the barrel cortex, alternate sections were stained for Nissl and GAD.

Data analyses and statistics. Data analyses were performed using Igor-Pro. The values of IPSC and EPSC peak amplitudes were obtained from averages of 5–10 consecutive traces. All values are given as means ± SEM. Statistical comparison between wt and tg mice was performed by t test (*p < 0.05, **p < 0.01) or by two-way repeated-measures ANOVA with post hoc Bonferroni’s test. Distributions of mIPSC amplitude and interevent interval (IEI) were compared between wt and tg mice using Kolmogorov–Smirnov test. In all cases, significance was set at p < 0.05. Statistical analyses were performed using SPSS (SPSS, Chicago, IL).

Chemicals. Phosphocreatine, QX-314, CNQX, APV, and picrotoxin were obtained from Sigma. TTX was obtained from Sankyo (Tokyo, Japan). Bicuculline was obtained from Tocris Cookson (Avonmouth, UK). Peptide toxins ω-agonatox IVA (ω-Aga-IVA) and ω-conotoxin GVIα (ω-CTx-GVIα) were obtained from Peptide Institute (Osaka, Japan). All other chemicals were from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemical Industries (Osaka, Japan). ω-Aga-IVA and ω-CTx-GVIα were coapplied with 1 mg/ml cytochrome c from horse heart (Nacalai Tesque) to prevent unspecific binding of the peptide toxins.

Results
Thalamocortical responses in adult wt and tg mice
In the somatosensory cortex, layer IV is the main gateway for afferent fibers originating in the respective thalamic relay nuclei (for review, see Douglas and Martin, 2004). The interplay between the cerebral cortex and the thalamus can be investigated using rodent thalamocortical brain slice preparations, which are unique in including both the ventrobasal nucleus of the thalamus and the somatosensory (barrel) cortex with the functional connectivity between them (Agmon and Connors, 1991). There are two major types of morphologically different excitatory neurons in layer IV: spiny stellate cells and pyramidal cells (Simons and Woolsey, 1984). Because it was technically easier to identify pyramidal cells in the slice preparation under the DIC video microscopy, we focused on layer IV pyramidal cells in this study so that a relatively homogenous neuronal population could be studied. Voltage-clamp recordings were made from layer IV pyramidal cells of the barrel cortex in the whole-cell patch-clamp configuration (Fig. 1A). Most of the recorded neurons were examined morphologically by including biocytin in the pipette and staining after electrophysiological recordings. EPSCs and IPSCs evoked by thalamic stimulation were measured in the presence of the NMDA receptor antagonist APV (100 μM) and compared between wt and epileptic tg mice (P21–P30) (Fig. 1B1).

When the holding potential was −60 mV, prominent EPSCs were observed in layer IV pyramidal cells of wt and tg mice (Fig. 1B1, top traces). At the holding potential of 0 mV, EPSCs became very small and there appeared large outward currents in wt, whereas the current amplitude was much smaller in tg mice (Fig. 1B1).
The outward currents were GABA$_A$ receptor-mediated IPSCs, because they were completely blocked by the GABA$_A$ receptor antagonist picrotoxin (50 μM) (Fig. 1B2). Both EPSCs and IPSCs were completely abolished by the non-NMDA receptor antagonist CNQX (10 μM) (Fig. 1B2). Because the synaptic delay between the stimulation and the onset of EPSCs was short (2.1 ± 0.12 ms; n = 12), EPSCs were mainly monosynaptic responses. In contrast, the onset of IPSCs always followed the onset of EPSCs, with the mean delay between the onsets of EPSCs and IPSCs of 1.3 ± 0.08 ms (n = 12) (Fig. 1B3). Together with the CNQX sensitivity of IPSCs, these observations suggested that IPSCs were mediated by a disynaptic response, i.e., by inhibitory interneurons excited by excitatory thalamocortical inputs, forming a circuit of feedforward inhibition. The observation that layer IV pyramidal neurons receive strong feedforward inhibition was consistent with the previous reports (Agmon and Connors, 1992; Porter et al., 2001).

Figure 1C shows the relationship between the conductance values for EPSCs and IPSCs obtained from individual cells, in which the excitatory conductance ranged from 1 to ~8 mS in both wt and tg mice. The inhibitory conductance in wt became larger with increasing excitatory conductance, although the conductance values were quite variable. In contrast, the inhibitory conductance in tg mice was very small, even with a large excitatory conductance. The ratio of inhibitory conductance to excitatory conductance was significantly reduced in tg mice (2.34 ± 0.60 for wt, n = 12; 0.46 ± 0.06 for tg, n = 11; **p < 0.01, t test). These results demonstrated that feedforward IPSCs disynaptically evoked by thalamic stimulation were disproportionately decreased in layer IV pyramidal cells of tg mice.

**Locally evoked IPSCs and EPSCs**

Thalamocortical responses recorded in layer IV pyramidal cells were composed of at least two components of monosynaptic EPSCs and disynaptic IPSCs. The lags of the synaptic onsets between two types of responses were short. Pharmacological blockade of EPSCs also abolished IPSCs. These facts made it difficult to evaluate synaptic currents quantitatively, especially IPSCs. To circumvent this problem, we measured IPSCs and EPSCs evoked by local stimulation within the somatosensory cortex. The stimulating electrode was placed at ~100 μm from the soma of the recorded pyramidal cells. IPSCs were recorded in the presence of CNQX (10 μM) and APV (100 μM), and EPSCs were recorded in the presence of bicuculline (10 μM). For measuring IPSCs, a pipette solution with a high chloride concentration was used so that IPSCs were observed as inward currents at a holding potential of ~60 mV. IPSCs were blocked by bicuculline in both wt and tg mice (data not shown).

Local stimulation easily evoked large IPSCs in wt mice at P21–P30 (1079 ± 227 pA; n = 10, 1.0 mA stimulation) (Fig. 2A). In contrast, the IPSC amplitudes evoked by local stimulation were drastically reduced in tg mice at P21–P30 (145 ± 33 pA; n = 10, 1.0 mA stimulation). Statistical test using two-way repeated-measures ANOVA revealed a significant effect of the genotype ($F_{(1,18)} = 12.288; p < 0.004$) on IPSC amplitude. Additionally, there was a statistically significant interaction between stimulus intensity and genotype ($F_{(4,72)} = 7.484; p < 0.001$). Post hoc analyses using the Bonferroni’s test revealed that the IPSC amplitudes were significantly smaller in tg than wt mice at stimulus intensities ≥0.8 mA [*p < 0.05, **p < 0.01 (Fig. 2A)]. Conversely, the EPSC amplitudes evoked by local stimulation increased with increment of stimulus intensity. Although EPSC amplitude appeared smaller in tg than wt mice (554 ± 96 pA, n = 10 in wt; 368 ± 88 pA, n = 10 in tg; 1.0 mA stimulation), two-way repeated-measures ANOVA failed to show a significant effect of the genotype ($F_{(1,18)} = 3.907; p > 0.06$) (Fig. 2B).

To see whether a similar synaptic impairment was observed in other primary sensory cortical areas, we also examined the primary auditory cortex. We recorded IPSCs of pyramidal cells evoked by local stimulation as used in the somatosensory cortex. In both wt ($n = 10$) and tg ($n = 10$) mice, locally evoked IPSC amplitudes increased with increments of stimulus intensity. Two-way repeated-measures ANOVA revealed no significant effect of the genotype on layer IV IPSC amplitudes in the primary auditory cortex ($F_{(1,18)} = 0.249; p > 0.6$) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

**Miniature IPSCs in layer IV pyramidal neurons**

The responses evoked by local stimulation demonstrated that inhibitory synaptic transmission onto layer IV pyramidal neurons in the somatosensory cortex was drastically impaired in epileptic tg mice. The site of the impairment could be presynaptic or postsynaptic. One possibility was that neurotransmitter release from presynaptic terminals was not functional in tg mice because of the mutation of the Ca$_{2.1}$ channel, although, in a normal condition, local stimulation should evoke neurotransmitter release at a high probability from the synaptic terminals of the
reduced evoked IPSCs in layer IV pyramidal cells of wt mice. However, a statistical test of the cumulative probability distributions (Fig. 3B, C) showed a significant shift toward longer IEIs (Fig. 5A) on layer V IPSC amplitudes. Two-way repeated-measures ANOVA revealed no significant effect of the genotype ($F_{1,18} = 0.236; p > 0.6$) (Fig. 5A) on layer V IPSC amplitudes.

Conversely, the EPSC amplitude was reduced in layer V pyramidal cells of $tg$ mice (392 pA; $n = 5; p < 0.05$) in wt mice, and 34 pA; $n = 5; p < 0.01$) in $tg$ mice. These results suggest that, although there are slight alterations on the presynaptic (prolonged IEIs) or postsynaptic (reduced mIPSC amplitude) side, these changes could not account for the greatly reduced evoked IPSCs in layer IV pyramidal cells of $tg$ mice.

**Number of inhibitory neurons**

Another possibility to cause the markedly reduced IPSCs in layer IV was that the number of inhibitory interneurons was reduced in layer IV of $tg$ mice. To test this possibility, distribution of inhibitory neurons was analyzed by the immunohistochemical method using polyclonal antibody against GAD (McLaughlin et al., 1975).

There was no obvious difference in the distribution of GAD-immunoreactive cells in the barrel cortex between wt and $tg$ mice (Fig. 4). The density of GAD-immunoreactive cells in layer IV showed no statistical difference between wt mice (184 ± 26 cells/mm²; $n = 5$) and $tg$ mice (192 ± 24 cells/mm²; $n = 5; p > 0.05$, $t$ test). Cell bodies of large, presumable pyramidal neurons, in layer IV in both wt and $tg$ mice were similarly surrounded by immunoreactive GABAergic terminals (Fig. 4, insets, arrows). Furthermore, $tg$ mice grow without epilepsy until 3 weeks of age, suggesting that inhibitory neurons and their connections develop normally (see below). Putting all these results together, we concluded that the impaired IPSCs in $tg$ mice was caused by functional impairments of neurotransmitter release from the presynaptic nerve terminals.

**Layer-specific impairment of IPSCs**

Thalamocortical afferents form synapses not only with neuronal elements in layer IV including pyramidal cells (White and Hersch, 1981) but also with corticothalamic pyramidal cells, which are located in lower layer V and upper layer VI and project to the ventrobasal complex of the thalamus (White and Hersch, 1982; White and Keller, 1987). To test whether the impairment of IPSCs was dependent on the laminar position in the somatosensory cortex, locally evoked IPSCs and EPSCs were recorded in layer V pyramidal cells. Different from the layer IV neurons, the reduction in IPSC amplitude was not observed in layer V pyramidal cells in $tg$ compared with that of wt mice. IPSC amplitudes with 1.0 mA stimulation were 999 ± 389 pA ($n = 10$) in wt and 1179 ± 312 pA ($n = 10$) in $tg$ mice. Two-way repeated-measures ANOVA revealed no significant effect of the genotype ($F_{1,18} = 0.236; p > 0.6$) (Fig. 5A) on layer V IPSC amplitudes.

Conversely, the EPSC amplitude was reduced in layer V pyramidal cells of $tg$ mice (932 pA; $n = 10$; 10 mA stimulation) compared with that of wt mice (615 ± 38 pA; $n = 10$; 10 mA stimulation). Two-way repeated-measures ANOVA showed a significant effect of the genotype ($F_{1,18} = 6.100; p < 0.03$) (Fig. 5B).
the synaptic transmission was studied by comparing nonepileptic change. To examine this prediction, the developmental effect on epileptogenesis, the IPSCs may show a related developmental (Noebels, 1984). If the impairment of IPSCs contributes to the between wt and layer IV and layer V pyramidal cells were not significantly differ-

\[ \text{wt and } \text{tg} \] m\text{ices} \]

Homozygous \text{tg} m\text{ice}s develop absence seizures at \text{tg} weeks of age (Noebels, 1984). If the impairment of IPSCs contributes to the epileptogenesis, the IPSCs may show a related developmental change. To examine this, the developmental effect on the synaptic transmission was studied by comparing nonepileptic young (P14–P16) and epileptic adult (P21–P30) \text{tg} m\text{ice}s.

In younger mice, the amplitudes of locally evoked IPSCs in layer IV and layer V pyramidal cells were not significantly different between wt and \text{tg} m\text{ice}s [layer IV, \( F_{(1,18)} = 0.351, p >0.5 \) (Fig. 6A); layer V, \( F_{(1,18)} = 1.973, p >0.1 \) (Fig. 6B)]. Moreover, the evoked EPSC amplitudes in layer IV and layer V were not significantly different in P14–P16 m\text{ice}s [layer IV, \( F_{(1,18)} = 0.653, p >0.4 \) (Fig. 6C); layer V, \( F_{(1,18)} = 0.579, p >0.4 \) (Fig. 6D)]. The IPSC and EPSC amplitudes evoked by maximum stimulation of P14–P16 and P21–P30 m\text{ice}s were summarized in Figure 6E. In \text{tg} m\text{ice}s, the EPSC amplitudes were smaller, but the reduction was not as remarkable as layer IV IPSC reduction at P21–P30.

During the development period from P14–P16 to P21–P30, the IPSC amplitudes of \text{tg} m\text{ice}s dramatically diminished in the layer IV pyramidal cells (Fig. 6E). The IPSC amplitudes in layer V did not show such a developmental change (Fig. 6E). These results indicated that the impairment of IPSCs in layer IV was well correlated with the onset of absence seizure.

Developmental switching of \( \text{Ca}^{2+} \) channel subtypes

The previous studies demonstrated that \( \text{Ca}^{2+} \) channels involved in neurotransmitter release switch developmentally from the \( \text{Ca}_{2,2} \ dread ) to \( \text{Ca}_{2,1} \) (P/Q-type) \( \text{Ca}^{2+} \) channels at various mammalian fast synapses (Iwasaki et al., 2000). Although the contribution of the \( \text{Ca}_{2,2} \) channel to EPSCs in layer IV pyramidal cells of the visual cortex was reported not to change developmentally (Iwasaki et al., 2000), developmental changes in \( \text{Ca}^{2+} \) channel subtypes of cortical IPSCs were not well investigated. Because the developmental switch usually occurs at \text{tg} weeks of age, it was expected that the switching from \( \text{Ca}_{2,2} \) to \( \text{Ca}_{2,1} \) \( \text{Ca}^{2+} \) channel might lead to the impairment of IPSCs in \text{tg} m\text{ice}s.

Developmental changes in sensitivity of IPSCs to \( \text{Ca}_{2,1} \) channel-selective blocker \( \text{oA-gA-IVa} \) \((200 \text{ nM})\) and the \( \text{Ca}_{2,2} \) channel-selective blocker \( \text{oC-tx-GVIA} \) \((3 \text{ \mu M})\) were studied in layer IV pyramidal cells of \text{wt} m\text{ice}s. At P14–P15, application of \( \text{oA-gA-IVa} \) only weakly blocked IPSCs (Fig. 7A, left). Subsequent coapplication of \( \text{oC-tx-GVIA} \) almost completely blocked the remaining IPSCs. Conversely, at P21–P22, the IPSCs were more strongly reduced by application of \( \text{oA-gA-IVa} \) (Fig. 7B, left). The \( \text{oA-gA-IVa} \)-sensitive fraction of IPSCs was increased from 20% \pm 4% at P14–P15 \((n = 7)\) to 45% \pm 3% at P21–P22 \((n = 7)\) (**p < 0.01, t test). In \text{tg} m\text{ice}s, however, the developmental change in sensitivity of IPSCs to \( \text{oA-gA-IVa} \) was not as evident as in wt \((25% \pm 4% \text{ at P}14–P15, n = 3; 18% \pm 7% \text{ at P}21–P22, n = 3; p = 0.43)\) (Fig. 7A, B, right). These results demonstrated that, whereas the \( \text{Ca}_{2,1} \) channel plays a minor role in young mice, it plays a predominant role in \text{wt} adult mice but not in \text{tg} adult mice for IPSCs in layer IV pyramidal cells of the somatosensory cortex.

Thalamocortical PSPs in wt and \text{tg} m\text{ice}s

We finally examined how the IPSC impairment in \text{tg} m\text{ice}s affects excitability in layer IV pyramidal cells using current-clamp recording (Fig. 8). Mean resting membrane potentials of layer IV pyramidal cells were similar in wt mice \((-69.4 \pm 1.0 \text{ mV}; n = 16)\) and \text{tg} m\text{ice}s \((-69.6 \pm 0.8 \text{ mV}; n = 14; p >0.05)\). The intrinsic membrane properties were then examined using negative current injection \((-100 \text{ pA}, 500 \text{ ms duration})\). There were no significant differences between wt and \text{tg} m\text{ice}s in membrane input resistance \((157 \pm 5 \text{ MΩ}; n = 16 \text{ in wt}; 172 \pm 9 \text{ MΩ}; n = 14 \text{ in } \text{tg}; p >0.05)\) and membrane time constant \((13.9 \pm 0.8 \text{ ms}; n = 16 \text{ in wt}; 12.9 \pm 0.9 \text{ ms}; n = 14 \text{ in } \text{tg}; p >0.05)\) of layer IV pyramidal cells.

We then examined differences in thalamocortical PSPs in layer IV pyramidal cells of wt and \text{tg} m\text{ice}s (P21–P30) (Fig. 8A, B). Thalamic stimulation with weak stimulus intensity evoked just EPSPs in layer IV pyramidal cells of both wt and \text{tg} m\text{ice}s \((50 \mu \text{A})\) in Fig. 8A, which was reported previously in wt mice (Beierlein et al., 2003). In wt mice, stronger thalamic stimulation evoked transient \((-1 \text{ ms})\) depolarization, followed by long-lasting hyperpolarization in layer IV pyramidal cells \((400 \mu \text{A})\) in Fig. 8A, left, B, top) because of recruitment of thalamocortical feedforward IPSCs (Agnon and Connors, 1992; Porter et al., 2001). In contrast to wt mice, we found that \(400 \mu \text{A}\) stimulation evoked transient depolarization followed by small but long-lasting depolarization in layer IV pyramidal cells of \text{tg} m\text{ice}s (Fig. 8A, right, B, bottom).

The differences in thalamocortical PSPs were then quantified between wt and \text{tg} m\text{ice}s. The EPSP amplitudes with \(50 \mu \text{A}\) stimulation were not different \((4.11 \pm 0.90 \text{ mV}; n = 10 \text{ in wt}; 3.69 \pm 0.52 \text{ mV}; n = 10 \text{ in } \text{tg}; p >0.05, t \text{ test})\). Amplitudes of the early transient depolarization with \(400 \mu \text{A}\) stimulus intensity were also not different \((2.76 \pm 0.99 \text{ mV}; n = 10 \text{ in wt}; 2.50 \pm 0.43 \text{ mV}; n = 10 \text{ in } \text{tg}; p >0.05)\) (Fig. 8C, Peak). However, thalamic stimulation with \(400 \mu \text{A}\) intensity evoked long-lasting hyperpolarization in wt \((-1.91 \pm 0.84 \text{ mV} \text{ at 40 ms after stimulation}; n = 10)\) (Fig. 8C) but long-lasting depolarization in \text{tg} m\text{ice}s \((1.16 \pm 0.66 \text{ mV} \text{ at 40 ms after stimulation}; n = 10)\) (Fig. 8C). Two-way repeated-measures ANOVA revealed a remarkable difference in time
course profiles of the thalamocortical PSPs with 400 μA intensity ($F_{(1,18)} = 7.679, p < 0.02$) (Fig. 8C). Also, there was a significant difference in stimulus intensity profiles of the thalamocortical PSPs at 40 ms after stimulation, revealed by two-way repeated-measures ANOVA ($F_{(1,18)} = 5.515, p = 0.03$) (Fig. 8D). These results indicated that thalamic inputs preferentially induce long-lasting depolarization in layer IV pyramidal cells of tg mice.

**Discussion**

Absence epilepsy has long been speculated to arise from an aberration of the interplay between the cerebral cortex and the thalamus. In epileptic model animals, there are many reports to study intrinsic and synaptic defects in the thalamus (Caddick et al., 1999; Zhang et al., 2002) and in the cerebral cortex (Di Pasquale et al., 1997). However, there has been no report to examine directly the synaptic transmission between the thalamus and the cortex. In this study, we used thalamocortical slices of tg mice, a well-known mouse model of absence seizure, and revealed a significant impairment of feedforward inhibition from the thalamus to layer IV of the somatosensory cortex (Figs. 1, 8).

**Layer IV inhibitory synapses dysfunction in epileptic tg mice**

Our principal finding of this study was a significant reduction (87%) in the amplitude of locally evoked IPSCs, but not that of EPSCs, in layer IV pyramidal cells located in the somatosensory cortex of epileptic tg mice. Similar results were obtained with the thalamocortical responses.

To identify the mechanism of the IPSC amplitude reduction, we made several experiments and obtained the following results. (1) GAD-immunoreactive inhibitory neurons were not decreased in number in layer IV of tg mice, and GAD-immunoreactive nerve terminals were well observed around presumable pyramidal cells. (2) mIPSCs could be recorded in layer IV pyramidal cells of tg mice. Although the amplitude was smaller and interevent interval was prolonged in tg mice, these changes were relatively mild. These results suggested that the postsynaptic apparatus was, at least, functional. (3) IPSCs once normally developed in younger mice, but IPSCs became impaired in later developmental stages, when the inhibitory synaptic transmission to layer IV pyramidal cells became more dependent on the Ca$^{2+}$,2.1 channel. All of these results suggest that significant impairment of inhibitory synaptic transmission in layer IV pyramidal neurons was not caused by failure of wiring or synapse formation but was caused primarily by dysfunction of neurotransmitter release from presynaptic terminals. Our results, however, do not exclude involvement of secondary effects of the mutation in the pathogenesis of absence seizures, because imbalance of excitation and inhibition could lead indirectly to subtle developmental rearrangements in the number or placement of synaptic inputs.
assay showed severely attenuated releases of both glutamate and drites and axons (Porter et al., 2001). The inhibitory interneurons are morphologically heterogeneous in terms of their dendrons in layers IV that are activated by the thalamocortical projecting inputs from the thalamus, although they receive strong inputs (for review, see Douglas and Martin, 2004). A simplified organization of neocortical circuitry is considerably complicated (for review, see Douglas and Martin, 2004). A simplified

Types and functions of layer IV interneurons

The organization of neocortical circuitry is considerably complicated (for review, see Douglas and Martin, 2004). A simplified diagram shows that layer IV neurons are a main gateway receiving inputs from the thalamus, although they receive strong inputs from neurons in layer VI and other layers. Inhibitory interneurons in layers IV that are activated by the thalamocortical projections are morphologically heterogeneous in terms of their dendrites and axons (Porter et al., 2001). The inhibitory interneurons are often grouped into two types based on the patterns of the intrinsic firing, which are the fast-spiking (FS) cells and the low-threshold-spiking (LTS) cells (Gibson et al., 1999; Beierlein et al., 2000; Amitai et al., 2002). Inputs from thalamocortical relay cells are more selective for FS cells and frequently and strongly excite them (Agmon and Connors, 1991; Porter et al., 2001; Beierlein et al., 2002), whereas the thalamocortical inputs only rarely and weakly excite LTS cells (Gibson et al., 1999). Thus, FS neurons play a dominant role in feedforward inhibition of the thalamocortical projection, and this feedforward inhibition is defective in tg mice.

Development-dependent dysfunction of inhibitory synapses

Absence seizure in tg mice begins at ~3 weeks of age (Noebels and Sidman, 1979). Inhibitory synaptic transmission in layer IV is intact in nonepileptic youngtg mice but becomes impaired later. These findings imply a relationship between onset of absence epilepsy and impairment of inhibitory synaptic transmission. The Ca²⁺ channel involved in neurotransmitter release often switches developmentally from the CaV₂.2 (N-type) channel to the CaV₂.1 (P/Q-type) channel at various mammalian fast synapses (Iwasaki et al., 2000). In the present work, the sensitivity of inhibitory synaptic transmission to ω-Aga-IVA increased developmentally in layer IV, indicating the subtype switching. Together, we speculate that the neurological phenotype becomes overt at ~3 weeks through the subtype switching, which makes some groups of synapses more dependent on the CaV₂.1 channel. However, we do not think that subtype switching alone can explain such drastically impaired inhibitory synaptic transmission in the cortical layer IV. Whereas the CaV₂.1-dependent component of the inhibitory synaptic transmission was ~45% in adult wt mice, the IPSC amplitude in layer IV pyramidal cells was more severely reduced in tg mice (down to 13%). Other mechanisms, including various secondary effects, also must contribute to the drastic reduction.

Compensation of Ca²⁺ channel subtypes in tg mice

For the phenotypic effect of the tg mutation, two factors seem to be involved: predominance of the CaV₂.1 channel and compensation by other subtypes. If the CaV₂.1 channel is a trivial component, the mutation will not affect the function. If the compensation is adequate, mutational effects will not appear. Compensatory effects of other Ca²⁺ channel subtypes have been investigated in other synapses of tg mice. The hippocampal CA3–CA1 synapses are maintained because of compensatory upregulation of CaV₂.2 (Qian and Noebels, 2000). A similar situation is observed in the neuromuscular junction (Plomp et al., 2000). Conversely, cerebellar Purkinje cells lack an ability of compensation. Our preliminary observation showed a drastic reduction in IPSC amplitude measured in deep cerebellar nucleus neurons.

Layer-specific dysfunction of synaptic transmission in tg mice

Because the CaV₂.1 channel is the predominant Ca²⁺ channel type in the brain, it was anticipated that some parts of the cerebral cortex were impaired in tg mice. In fact, a cortical microdialysis assay showed severely attenuated releases of both glutamate and GABA evoked by high K⁺ solution (Ayata et al., 2000). In the present study, our analyses at a finer level revealed that synaptic dysfunctions are relatively confined to specific neuronal populations. The reduction in IPSCs was prominent in layer IV pyramidal cells of wt (left) and tg (right) mice, with increments of the stimulus intensity. Vertical expansion of the thalamocortical PSPs with the 400 μA stimulus intensity, which are identical to those in A. A. The early transient depolarization is indicated by Peak, whereas the late long-lasting hyperpolarization and depolarization are indicated by arrowheads. The membrane potentials before the stimulation were set to ~60 mV by current injection. Stimulus artifacts were truncated. C. Time course profiles of thalamocortical PSPs with 400 μA stimulus intensity in wt and tg mice. PSP values were calculated as membrane potentials at each time point after stimulation minus those just before stimulation. Peak amplitudes of the early transient depolarization (indicated by Peak) were searched from 2 to 7 ms after stimulation in individual preparations. The time course profiles were significantly different between wt and tg mice (p < 0.02, two-way repeated-measures ANOVA). *p < 0.05, **p < 0.01, Bonferroni’s test between genotype at each time point. D. Stimulus intensity profiles of thalamocortical PSPs at 40 ms after stimulation. The stimulus intensity profiles were significantly different between wt and tg mice (p < 0.03, two-way repeated-measures ANOVA). *p < 0.05, **p < 0.01, Bonferroni’s test between genotypes at each stimulus intensity.

Figure 8. Postsynaptic potentials in layer IV pyramidal cells in response to thalamic stimulation. A. Typical responses of the thalamocortical PSPs in layer IV pyramidal cells of wt (left) and tg (right) mice, with increments of the stimulus intensity. B. Vertical expansion of the thalamocortical PSPs with the 400 μA stimulus intensity, which are identical to those in A. A. The early transient depolarization is indicated by Peak, whereas the late long-lasting hyperpolarization and depolarization are indicated by arrowheads. The membrane potentials before the stimulation were set to ~60 mV by current injection. Stimulus artifacts were truncated. C. Time course profiles of thalamocortical PSPs with 400 μA stimulus intensity in wt and tg mice. PSP values were calculated as membrane potentials at each time point after stimulation minus those just before stimulation. Peak amplitudes of the early transient depolarization (indicated by Peak) were searched from 2 to 7 ms after stimulation in individual preparations. The time course profiles were significantly different between wt and tg mice (p < 0.02, two-way repeated-measures ANOVA). *p < 0.05, **p < 0.01, Bonferroni’s test between genotype at each time point. D. Stimulus intensity profiles of thalamocortical PSPs at 40 ms after stimulation. The stimulus intensity profiles were significantly different between wt and tg mice (p < 0.03, two-way repeated-measures ANOVA). *p < 0.05, **p < 0.01, Bonferroni’s test between genotypes at each stimulus intensity.
evoked by white matter stimulation (presumably Purkinje axons) (Matsushita and Imoto, 2002). In the present work, we do not know whether the compensatory upregulation of CaV2.2 occurred in layer IV inhibitory neurons, but it is evident that the upregulation, if any, was insufficient.

These lines of evidence imply that different neuronal types have various capacity of compensatory upregulation of other Ca2+ channel subtypes in response to the CaV2.1 channel impairment, and such a variation may underlie selective vulnerability of certain parts of neuronal networks. Molecular determinants of the compensatory upregulation will be an interesting subject for future studies.

Functional implications

Absence epilepsy is characterized by SWDs in wide cortical areas, whose underlying mechanism involves the thalamocortical circuit (for review, see Manning et al., 2003). To date, synaptic and intrinsic defects in the thalamocortical circuit have been extensively studied in tg mice. In the thalamus of tg mice, there are several studies focusing on the role in the generation of the SWDs of low-voltage-activated T-type Ca2+ currents in thalamic relay neurons (Zhang et al., 2002; Song et al., 2004). Defects of synaptic transmission were also reported in the thalamus of tg mice (Caddick et al., 1999). In the present study, we found a significant impairment of feedforward inhibitory connection from the thalamus to the input layer of the somatosensory cortex.

The thalamocortical feedforward inhibition is an important determinant of excitability to cortical layer IV neurons. As shown in Figure 8B, single thalamic stimulation induced transient (~1 ms) depolarization in layer IV pyramidal cells of wt mice, whereas it induced long-lasting depolarization in layer IV pyramidal cells of tg mice, because of the impairment of the feedforward inhibition. The longer depolarization in tg mice means that thalamic inputs tend to produce bursting activity in layer IV pyramidal cells. Thus, these results imply that synchronous bursts in thalamic relay cells, observed in vivo, may be readily transformed into synchronous bursts in layer IV pyramidal cells of tg mice, which could be partially reflected as SWDs in the EEG of the somatosensory cortex.

We found that IPSC impairment was observed in the somatosensory cortex but not in the primary auditory cortex (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Interestingly, a previous study reported that SWDs first originate in the somatosensory cortex and then rapidly spread to other regions of the cortex in the WAG/Rij rat model of absence seizures (Meeren et al., 2002).

In summary, we demonstrated that the effect of the tg mutation was variable depending on neuronal cell types and developmental stages, and that the feedforward inhibition was severely impaired in the somatosensory thalamocortical pathway. Because the tg phenotype is expected to arise from a complex pattern of altered transmitter release in the cerebral cortex, the thalamus, and other brain regions, further search for synaptic defects is necessary particularly in synaptic connections directly related to the thalamocortical interplay to understand the pathophysiologic of absence epilepsy.

References


Caddick SJ, Wang C, Fletcher CF, Jenkins NA, Copeland NG, Hosford DA (1999) Excitatory but not inhibitory synaptic transmission is reduced in lethargic (CaCn2β0) and tottering (CaCn2α1) mouse thalami. J Neurophysiol 81:2066–2074.


