Impaired cerebellar synapse maturation in waggler, a mutant mouse with a disrupted neuronal calcium channel γ subunit

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The *waggler*, a neurological mutant mouse with a disrupted putative neuronal Ca\(^{2+}\) channel γ subunit, exhibits a cerebellar granule cell-specific brain-derived neurotrophic factor deficit, severe ataxia, and impaired eyelink conditioning. Here, we show that multiple synapses of *waggler* cerebellar granule cells are arrested at an immature stage during development. Synaptic transmission is reduced at parallel fiber–Purkinje cell synapses. The Golgi cell–granule cell synaptic currents show immature kinetics associated with reduced γ-aminobutyric acid type A receptor α6 subunit expression in granule cells. In addition, the mossy fiber–granule cell synapses exhibit N-methyl-D-aspartate (NMDA) receptor-mediated excitatory postsynaptic currents (EPSCs), but not α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated EPSCs. Our results suggest that voltage-dependent Ca\(^{2+}\) channels are involved in synapse maturation. This defective synaptic transmission in the *waggler* cerebellum may account for their behavioral deficits.

The *waggler* (stg
\(^{emm}\)) is a recessive neurological mutation on chromosome 15 that arose spontaneously in the MRL/MpJ mouse strain. We have previously reported (1) that homozygous *wagglers* (later referred to as *wagglers*) exhibit normal cerebellar foliation and laminar structures. The cerebellar granule cells migrate completely into internal granule cell layer and survive normally in adult mice. However, *waggler* cerebellar granule cells do not exhibit developmental up-regulation of brain-derived neurotrophic factor (BDNF) expression, which normally occurs after granule cell maturation (2, 3). Behaviorally, *wagglers* fail to develop normal gait and motor coordination, and adult *wagglers* are severely impaired in cerebellum-dependent eyelink conditioning (1). It appears that the *waggler* mutation may disrupt the functional integrity of the cerebellar circuitry.

The Ca\(^{2+}\) channel γ subunit was discovered in skeletal muscle (4, 5), and was found to modulate calcium currents in a variety of ways (6, 7). Recently, the gene (Cacng2) that is mutated in *wagglers* was cloned and found to encode a putative neuronal calcium channel γ subunit—stargazin (8). Expression of stargazin in a cell line expressing neuronal α1 class A Ca\(^{2+}\) channels (including α1A/β2/δ) shifted steady-state inactivation toward hyperpolarization, as has also been shown for the muscle Ca\(^{2+}\) channel γ subunit (6). *Cacng2* is ubiquitously expressed in the brain, with cerebellar cortex among the regions of highest expression (8). The distribution of *Cacng2* mRNA signals in the cerebellum is consistent with granule cell layer demarcation. The *waggler* mutation results in a premature termination of the *Cacng2* transcript, leading to a substantially lower level of stargazin in the mutant mice (8). One potential effect of such reduction in stargazin is abnormal Ca\(^{2+}\) entry through voltage-dependent calcium channels (VDCCs) in *waggler* granule cells. Previous studies have shown that neuronal discharges (9) and subsequent activation of VDCCs (10) regulate the developmental transition of γ-aminobutyric acid-A (GABA\(_A\)) receptor subunit expressions, a landmark of granule cell maturation (11, 12). In addition, chronic depolarization-mediated Ca\(^{2+}\) entry also differentially regulates expression of N-methyl-D-aspartate (NMDA) receptors and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (13–16), which normally occurs during granule cell maturation (17–19). Thus, it is possible that the *waggler* mutation impairs cerebellar granule cell maturation and consequently causes the behavioral deficits. In the present study, we examined the maturation of cerebellar glutamatergic mossy fiber–granule cell synapses, GABAergic Golgi cell–granule cell synapses, and glutamatergic parallel fiber–Purkinje cell (pf-PC) synapses in *waggler* mutant mice.

Materials and Methods

Animals. Heterozygous *waggler* mice (on C57BL/6 genetic background) were originally obtained from The Jackson Laboratory and were used for breeding in a University of Southern California vivarium. Homozygous mutant mice were identified by the atactic gait. Heterozygous and homozygous wild-type littermates were used in the experiments as controls.

Histology. Adult mutant and wild-type mice at postnatal day 28 (P28) were deeply anesthetized with halothane and transcardially perfused with 0.9% saline and 10% buffered formalin. The brains were removed and postfixed in 10% formalin for at least 24 hr. For general histological examination, the brain was sliced with a sliding microtome. Coronal and parasagittal sections (40 μm) were mounted onto gelatin-coated slides, stained with cresyl violet, and observed with a light microscope. For Golgi staining, the cerebellum was cut into 2-mm\(^3\) cubes, and further fixed with 0.33% OsO\(_4\) and 2.6% K\(_2\)Cr\(_2\)O\(_7\) for 6 days. Then, the brain tissues were stained with 0.75% AgNO\(_3\) for 20 hr. After being rinsed with ethanol, the tissues were sliced (100 μm) with a Vibratome, and the sections treated with xylene for 1 hr, mounted with Permount, and observed with a light microscope. To assess the cell morphology, 20 granule cells with clearly identified somas, dendritic branches, and glomeruli were sampled for each phenotype. Two-dimensional images were used to measure the somatic and dendritic sizes. The long diameter was defined as the largest distance between two points on the somatic periphery. The short diameter was defined as the longest diameter perpendicular to the long axis. The dendritic length was measured from somatic periphery to the glomerulus. Granule cell count was performed as described previously (20). Briefly, fixed tissues were dehydrated, infiltrated with propylene oxide, embedded in Epon (which was polymerized overnight at 67°C), and cut into semithin sections (3 μm). The sections were deplasticized in NaOH/ethanol solution and stained with he-
matoxylin and eosin. Numbers of granule cells were counted in 6500-μm² rectangular areas located in lobules 6 and 8. Cells on the left and bottom edges were counted, and those on top and right edges were not.

**In Situ Hybridization.** The mutant and wild-type (P28–P35) brain sections (16 μm, fresh frozen) were mounted on the same slides and processed identically for optimal comparison. The sections were fixed with 4% paraformaldehyde in 0.1 M PBS and pretreated with 0.25% acetic anhydride and 0.1 M triethanolamine. Then, the tissue sections were incubated overnight at 50°C with 35S-labeled oligonucleotide probes [50% formamide, 10% dextran sulfate, 300 mM NaCl, 0.5 mg/ml yeast RNA, 10 μM DTT, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA, 1 mM EDTA, and 10 mM Tris-HCl (pH 8.0)]. After hybridization, the sections were rinsed, dried, and autoradiographed. Probe sequences for pam GABA A receptor α6 mRNAs (21), flip and flop forms of GluR1–3 (22) and GluR4 (23) were adopted from previous studies.

**Electrophysiology.** Cerebellar sagittal slices (400 μm) were prepared following standard procedures and incubated in artificial cerebrospinal fluid (ACSF; 124 mM NaCl/3 mM KCl/1.25 mM KH2PO4/3 mM CaCl2/1 mM MgCl2/26 mM NaHCO3/10 mM glucose, buffered with 5% CO2/95% O2) for at least 30 min. Incubations and all subsequent experiments were performed at room temperature. Cerebellar pf-PC excitatory postsynaptic potentials (EPSPs) were recorded with sharp electrodes (80–120 MΩ, filled with 2 M potassium-acetate) in the presence of picrotoxin (40 μM). Purkinje cells were current-clamped at a resting membrane potential of −80 mV. To study paired-pulse facilitation, biphasic electrical stimulation of the outer molecular layer was adjusted to elicit EPSPs with amplitudes of 5–7 mV. A series of paired-pulse stimuli with variable interpulse intervals was applied at a frequency of 0.1 Hz. To examine input–output relations of the pf-PC EPSPs, cell membrane potentials were current-clamped at −80 to −90 mV, and a series of stimuli (5–50 μA, 5 μA steps) was applied at 0.1 Hz. Serial resistance and input resistance were frequently monitored throughout the experiment.

Granule cell membrane currents were recorded with whole-cell configuration, and the “blind” techniques (24) were used to form tight seals. The impedance of the patch pipettes ranges from 3 to 6 MΩ when filled with internal solutions [whole-cell solution for recording GABA A receptor currents: 140 mM CsCl/2 mM MgCl2/5 mM EGTA/10 mM Hepes/4 mM Na2ATP/0.3 mM Na3GTP (pH 7.3); for recording AMPA receptor or NMDA receptor currents: 108 mM CsMeSO4/4.5 mM MgCl2/9 mM EGTA/4 mM MgATP/0.3 mM Na3GTP/24 mM Hepes (pH 7.3)]. Granule cells were identified by their typical low whole-cell capacitance (<5 pF) and high input resistance (>1 GΩ) (17). To record evoked EPSCs at the mf–granule cell synapses, picrotoxin (40 μM) was included in ACSF. Biphasic stimulation (0.1 Hz) was delivered through a coaxial electrode placed on nearby white matter. AMPA receptor EPSCs were recorded in the presence of β-2-amino-5-phosphonovaleric acid (100 μM) with Vm at −80 mV. NMDA receptor EPSCs were measured with 6-cyano-7-nitroquinoxaline (CNQX; 10 μM) in ACSF and Vm = +60 mV. To record AMPA-activated whole-cell currents, picrotoxin (40 μM) was included to block spontaneous GABA A receptor currents, and tetrodotoxin (0.5 μM) to prevent trans-synaptic activation of granule cell synapses. AMPA (500 μM) was applied [20 psi, 1 s (1 psi = 6.9 kPa)] through a picospritzer (General Valve, Fairfield, NJ) into the perfusion ACSF in the close vicinity of the recorded granule cells. PCLAMP6 programs (Axon Instruments, Foster City, CA) were used for data acquisition and the analysis of evoked responses. To study GABA A receptor-mediated Golgi cell–granule cell synaptic currents, kynurenic acid (2 mM) was applied extracellularly to eliminate glutamatergic synaptic currents. Spontaneous GABA A synaptic currents were continuously recorded (Vm = −80 mV) for 1 min. Spontaneous GABA A receptor-mediated events were identified with custom-made programs and were later visually verified by experimenters. Decay time constant of the GABA A receptor-mediated synaptic currents was calculated by using the average of all events in the 1-min recording period. All error bars in the figures indicate SEM.

**BDNF Treatment.** Brain slices (both wagglers and WT, P17, prepared as described above) were incubated in ACSF containing 100 ng/ml BDNF (human recombinant; Sigma) at room tem-

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**Fig. 1.** Cerebellar histology and granule cell morphology. (A) Midsagittal sections of the cerebellum stained with cresyl violet. The wagglers cerebellum exhibits laminar structure. (B) Cerebellar granule cell density. (C) Golgi staining of cerebellar granule cells. wagglers granule cells show characteristic morphology. (Scale bar: 10 μm.) (D) Granule cell sizes. The short somal axis and dendritic length are significantly reduced in wagglers (*, P < 0.05).
temperature for 12 hr (25). A set of WT slices were also incubated under the same condition, except that no BDNF was used to assess the effects of BDNF on wild-type granule cells. After incubation, slices were transferred to a submission recording chamber and recorded as described before. During the recordings, slices were perfused with normal ACSF without BDNF.

Results

Normal Cerebellar Architecture, but Reduced Cerebellar Granule Cell Sizes in wagglers. The adult wagglers cerebellum exhibits normal foliation (Fig. 1A) and laminar structure. The thickness of each layer is comparable between mutants and age-matched wild types. In adult wagglers, all granule cells finish migration and are located in the internal granule cell layer. We examined granule cell density in the central vermal areas of lobules 6 and 8, and found a slight, but nonsignificant, increase in granule cell density in wagglers (Fig. 1B; n = 12 for each group). Adult mutant granule cells exhibited claw-like telodendria (Fig. 1C), characteristic of differentiated granule cells. Quantification of granule cell sizes indicated that mutant granule cells exhibited smaller short somatic diameters (Fig. 1D; n = 20 for each group), suggesting an irregular shape of the soma. Dendritic length was also reduced in wagglers granule cells (Fig. 1D; n = 70 for WT, and n = 58 for wag). These results indicate that wagglers external germinal cells proliferate, migrate into internal granule cell layer, and differentiate into granule cells, which survive with normal gross morphology.

Altered pf-PC Synaptic Transmission in wagglers. During early postnatal development, as migrating granule cells pass the molecular layer, they extend axon collaterals as parallel fibers, which form synapses with expanding Purkinje cell dendrites. This process occurs during the second and third postnatal weeks (26). We studied the developmental changes in pf-PC EPSPs by examining paired-pulse facilitation (PPF), a phenomenon generally believed to be due to presynaptic mechanisms (27, 28). The pf-PC synaptic responses are mediated by AMPA receptor in wagglers, as well as in wild types, and were completely blocked by 10 μM CNQX (data not shown). Compared with adult (older than postnatal 28th day, >P28) wild-type synapses, developing (P17) wild-type pf-PC synapse exhibited higher PPF of EPSP amplitudes over the range of 100- to 300-ms interpulse interval (Fig. 2A), suggesting low transmitter release probabilities (29). Raising the extracellular [Ca2+] /[Mg2+] ratio from 3 to 6 ([Ca2+] from 3 to 4.2 mM, and [Mg2+] from 1 to 0.7 mM), which increases transmitter release probability, reduced the degree of PPF at developing synapses to that of adult synapses. Interestingly, PPF of the adult mutant synapses was significantly greater than that of adult wild-type synapses, and resembled that of developing synapses. Raising extracellular [Ca2+] /[Mg2+] ratio reduced PPF of adult mutant synapses, as it did for P17 wild-type synapses. These results suggest that transmitter release probability is low at wagglers granule cell terminals, possibly because of impaired synapse maturation. We also compared input–output relations in adult wild-type and mutant pf-PC synapses. The pf-PC EPSPs exhibited smaller slopes of input–output function and lower maximal responses in mutants than in wild types (Fig. 2B).

Slow Golgi Cell–Granule Cell GABA A Synaptic Currents and Reduced GABA A Receptor α6 Subunit Expression in wagglers. Cerebellar granule cells express different combinations of GABA A receptor at Golgi cell–granule cell synapses during development (12, 30, 31). For example, the α6 subunit of the GABA A receptors is expressed only in mature cerebellar granule cells and is considered a molecular marker of maturity (12, 32). In addition to changes in receptor subunit expression, GABA A receptor-mediated Golgi cell–granule cell synaptic currents also undergo a developmental transition from slow-decaying to fast-decaying currents (33), which may be because of the switch in GABA A receptor subunit composition (32, 33). We compared whole-cell spontaneous GABA A currents from wagglers and wild types. Excitatory current was blocked by adding kynurenic acid (2 mM) in the perfusion bath. The remaining spontaneous currents were completely blocked by the GABA A receptor antagonist picrotoxin (data not shown). Adult wild-type granule cells (n = 10) exhibited typical fast GABA A currents (Fig. 3A–C). In contrast, the responses recorded from adult (P35) wagglers granule cells (n = 6) were much slower, had an increased amount of slow component, and resembled responses from immature (P17) wild-type granule cells (n = 9). In situ hybridization revealed that GABA A receptor α6 subunit expression was reduced by 20% in adult (P28) wagglers granule cells as compared with adult wild types (OD: WT, 8718 ± 182, n = 13; wag, 6942 ± 182, n = 13; P < 0.001. See Fig. 3D for examples). A similar reduction in GABA A receptor α6 subunit protein level was previously described in stargazer granule cells (34). Thus, the granule cells in the adult wagglers cerebellum are not fully mature.

Absence of AMPA Receptor Currents at wagglers mf-Granule Cell Synapses. A major feature of central synapse maturation is the transition from silent NMDA receptor–only glutamatergic synapses to functional NMDA–AMPA receptor synapses (35–38). The glutamatergic mf-granule cell synapses undergo a similar
process during early postnatal development, in which NMDA receptor-mediated responses decrease and AMPA receptor-mediated responses increase (17). We compared AMPA receptor responses of wild-type and mutant mf-granule cell synapses. In adult wild types, AMPA receptor-mediated fast synaptic currents were prominent in every granule cell recorded (n = 8, see Fig. 4 A and C). In contrast, 12 of 19 cells recorded from developing (P17) wild-type granule cells showed small or no AMPA receptor EPSCs (Fig. 4C). The presence of voltage-activated Na\(^+\) currents was used to confirm that the recorded cells were neurons. Surprisingly, the fast EPSCs were missing in both developing (n = 11) and adult waggler mutant granule cells (n = 11; Fig. 4 A and C), whereas all of the 22 cells showed voltage-activated Na\(^+\) currents. To determine whether the absence of AMPA receptor responses was because of the absence of functional postsynaptic AMPA receptors, we recorded granule cell responses to exogenous AMPA. Pressure-ejected AMPA (500 \(\mu\)M, 1 s, 20 psi) produced inward currents in wild-type granule cells (n = 5), but not in mutant granule cells (n = 5, see Fig. 4B for examples), suggesting that no functional AMPA receptors were present in granule cell membranes. The deficit does not seem to be a general property of all cerebellar synapses because both pf- and climbing fiber-PC synapses exhibit AMPA receptor-mediated responses as mentioned before (also see Fig. 2). To determine whether the absence of AMPA responses was because of a lack of AMPA receptor expression, mRNA levels of GluR1–4 (both flip and flop) splice variants in adult mutant and wild-type mice were studied with in situ hybridization. No difference in mRNA levels was found between the wild-type and waggler cerebellar granule cell layers for any of the eight probes. Fig. 4D presents the autoradiographs for GluR4 (flop) mRNA, which is highly expressed in cerebellar granule cells. Our results indicate that the development of functional AMPA receptors at mf-granule cell synapses is impaired in wagglers.

**NMDA Receptor Currents Are Present at waggler mf-Granule Cell Synapses.** The development of functional AMPA receptors appears to be NMDA receptor-dependent (35). To determine whether the absence of AMPA responses in mutant granule cells is because of impairment in NMDA receptor functions and whether mf transmitter release is impaired, we compared NMDA receptor-mediated synaptic responses in wild-type and waggler granule cells. In contrast to AMPA receptor-mediated responses, NMDA receptor-mediated responses were larger in developing waggler granule cells than in age-matched wild-type ones (Fig. 5C, waggler, n = 12; wild type, n = 16; P < 0.05) and exhibited normal Mg\(^{2+}\) blockade (Fig. 5A and B). In adults, NMDA receptor-mediated responses decreased in both wild-type and waggler granule cells (Fig. 5C, waggler, n = 10; wild type, n = 10; P > 0.1). Thus, glutamate release at waggler cerebellar mf terminals is not impaired, and the failed development of functional AMPA receptors in waggler granule cells is not caused by impairment in NMDA receptor functions. The enhanced NMDA receptor currents in P17 waggler granule cells may reflect delayed developmental down-regulation of NMDA receptor currents.

**Prolonged BDNF Treatment Fails to Restore AMPA Receptor Response in waggler Granule Cells.** To address the possibility that impaired cerebellar granule cell synapse maturation in waggler mice is caused by cerebellar BDNF deficit, we attempted to rescue mutant granule cell synapses with BDNF incubation. P17 cerebellar slices were used because BDNF expression normally starts at about this age (2) and because some wild-type granule cells at this stage are still undergoing synapse maturation. We incubated cerebellar slices with BDNF (100 ng/ml in ACSF) for 12 hr as described (25). In wild-type slices, BDNF incubation did not result in significant changes in mf-granule cell AMPA receptor EPSCs (n = 9), as compared with those recorded from slices.
incubated with ACSF for 12 hr ($n = 10$). This is consistent with results of previous studies showing that neither prolonged BDNF incubation (25) nor virus-mediated BDNF gene transfer (39) enhanced hippocampal CA1 excitatory synaptic transmission in wild types. Further, wagglers ($n = 15$) did not show any up-regulation of AMPA receptor EPSCs after 12 hr of incubation with BDNF, whereas the same cells exhibited NMDA receptor-mediated EPSCs when the membrane potentials were held at $+60 \text{ mV}$.

**Discussion**

In the present study, we show deficits in multiple types of granule cell synapses in adult wagglers. Although the abnormalities found in the wagglers may seem to be diverse, all the deficits represent features of immature synapses. Because the lack of stargazin may increase $\text{Ca}^{2+}$ influx at presynaptic terminals, it is also possible that the observed deficits in wagglers are because of altered $\text{Ca}^{2+}$ channels at presynaptic terminals. However, our results suggest that normal presynaptic $\text{Ca}$ entry cannot fully account for the deficits.

First, excitatory neurotransmitter release is decreased at pf-PC synapses, instead of increased as predicted by enhanced $\text{Ca}^{2+}$ channel inactivation (8). Second, the differential effects of the wagglers on AMPA receptor- and NMDA receptor-mediated synaptic responses suggest postsynaptic mechanisms. Third, developmental transition of the kinetics of $\text{GABA}_A$ receptor-mediated synaptic responses is generally believed to be mediated by postsynaptic mechanisms, as mentioned before.

The impaired granule cell synapse maturation may be caused by several factors including a granule cell-specific BDNF deficit and/or altered $\text{Ca}^{2+}$ channel properties. Although BDNF has been shown to be involved in cerebellar development (40), our results indicate that normal granule cell BDNF expression is not essential for granule cell migration, differentiation, survival, or cerebellar foliation. The failure to rescue AMPA receptor synaptic responses with prolonged BDNF incubation in wagglers also suggests that BDNF deficit is not a direct cause of the deficiency in the mutant glutamatergic synapses. Alternatively, the lack of BDNF may be because of developmental arrest of the granule cells.

Activation of silent synapses may be a mechanism underlying long-term synaptic potentiation and maturation of glutamatergic synapses (35, 38, 41). AMPA receptor insertion and clustering in postsynaptic membranes have been considered as potential mechanisms for modulation of AMPA receptor responses (42–44). However, the intracellular signals that guide the proper targeting of AMPA receptors are yet to be discovered. Stargazin is highly expressed in cerebellar granule cells. Losing normal stargazin in wagglers would lead to reduced steady-state inactivation of $\text{Ca}^{2+}$ currents in cerebellar granule cells and enhanced
Ca\textsuperscript{2+} entry through VDCCs (8). L-type VDCCs are mainly located at the base of the major dendrites (45) and initiate Ca\textsuperscript{2+} signaling spatially distinct from that mediated by NMDA receptor (46–48). It has been shown that regulation of gene expression by intracellular Ca\textsuperscript{2+} depends on the spatial compartmentation of Ca\textsuperscript{2+} signaling (49, 50). In waggles, the anticipated increase in nonsynaptic Ca\textsuperscript{2+} entry through deficient VDCC may disrupt the local Ca\textsuperscript{2+} signaling through synaptic NMDA receptors. The failure of synapse maturation in mutant granule cells suggests that such a signal may be one of the intracellular messengers that target AMPA receptors to postsynaptic membranes. This hypothesis is consistent with previous findings that NMDA receptor-independent long-term potentiation is generally presynaptic and does not involve modulation of postsynaptic AMPA receptors (51–54) and that activation of VDCCs often leads to long-term depression (55, 56). Similarly, depolarization and consequent activation of VDCC by blocking GABA\textsubscript{A} receptors down-regulate surface AMPA receptors, AMPA receptor EPSCs and AMPA receptor miniature EPSCs (57, 58).

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