

The weaver mutation of GIRK2 results in a loss of inwardly rectifying K⁺ current in cerebellar granule cells

(patch-clamp/somatostatin/cell culture/glutamate)

D. JAMES SURMEIER*[†], PAUL G. MERMELSTEIN[†], AND DAN GOLDOWITZ

Department of Anatomy and Neurobiology, College of Medicine, University of Tennessee, Memphis, TN 38163

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ABSTRACT The weaver mutation in mice results in a severe ataxia that is attributable to the degeneration of cerebellar granule cells and dopaminergic neurons in the substantia nigra. Recent genetic studies indicate that the *GIRK2* gene is altered in weaver. This gene codes for a G-protein-activated, inwardly rectifying K⁺ channel protein (8). The mutation results in a single amino acid substitution (glycine → serine) in the pore-forming H5 region of the channel. The functional consequences of this mutation appear to depend upon the co-expression of other GIRK subunits—leading to either a gain or loss of function. Here, we show that G-protein-activated inwardly rectifying K⁺ currents are significantly reduced in cerebellar granule cells from animals carrying the mutant allele. The reduction is most pronounced in homozygous neurons. These findings suggest that the death of neurons in weaver is attributable to the loss of GIRK2-mediated currents, not to the expression of a nonspecific cation current.

The weaver mutation in mice results in the degeneration of cerebellar granule cells and dopaminergic neurons in the substantia nigra (1–6). These neurological deficits result in severe ataxia and tremor. Recent genetic studies indicate that it is the *GIRK2* gene that is altered in weaver (7). This gene codes for a G-protein-activated, inwardly rectifying K⁺ channel protein that tends to stabilize the membrane potential near the potassium equilibrium potential (8, 11–13). The mutation results in a single amino acid substitution (glycine → serine) in the pore-forming H5 region of the channel (7).

Recently published studies (9, 10) using *Xenopus* oocytes to express the mutant protein (*GIRK2_{wv}*) suggest that the functional consequences of the substitution depend upon the expression of other GIRK subunits thought to assemble with GIRK2 to form tetrameric channels (14). In the absence of other GIRK subunits, *GIRK2_{wv}* subunits give rise to nonspecific cation channels, as predicted from work with Shaker channels (15). On the other hand, when GIRK1 and *GIRK2_{wv}* subunits are co-expressed, basal and evoked currents are significantly smaller than seen with co-expression of wild-type subunits. The consequences, therefore, of the weaver mutation appear to be critically dependent upon how the expression of GIRK genes is coordinated and how subunits are assembled into channels.

In weaver mice, cerebellar granule cells fail to differentiate and die in the first 3 postnatal weeks (3, 5). It is unclear whether this results from the loss of GIRK-mediated K⁺ currents or the gain of a nonspecific cation current. Here, we show that G-protein-activated, inwardly rectifying currents are significantly reduced in cerebellar granule cells from animals carrying the mutant allele. The reduction is most pronounced in homozygous mutant (*wv/wv*) neurons. These findings sug-

gest that the death of neurons in weaver is attributable to the loss of GIRK2-mediated currents. The loss of this K⁺ current may promote cell death by allowing ionotropic inward currents activated by glutamate at the beginning of neural migration to produce excessive depolarization.

MATERIALS AND METHODS

Cell Culture/Histological Methods. Cerebellar cultures were obtained from P6–7 mice born to heterozygous weaver mice taken from the on-site, inbred weaver colony (derived from the original stock of weaver mice that was maintained on a C57BL/6 background at Children's Hospital in Boston) (16). Cultures were derived from cerebella taken from littermates produced by a *wv/+* × *wv/+* mating. Cerebella were judged as *wv/wv*, *wv/+*, or *+/+* based upon size and foliation pattern and were cut into five to six pieces. One midline slice was fixed in Bouin's solution, embedded in paraffin, cut into 6- μ m sections, and stained with cresyl violet to confirm genotype. A cell suspension was prepared from the remaining tissue as described previously (17, 18). Neurons were resuspended in Barrett's media (19) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM glutamine, 1 mM kynurenic acid, 5 μ g/ml insulin, 50 μ g/ml streptomycin, 50 units/ml penicillin, and 25 μ g/ml gentamycin (Sigma). Cells were plated at a density of approximately 750 cells/mm² in poly-L-lysine coated, plastic Petri dishes (Nunc, Inc., Naperville, IL) and maintained in humid, 37°C, 5% CO₂ environment. After plating, granule cells cease mitosis and enter the postmitotic state (obviating the impact of variation in their developmental state at the time of culturing). Two to three days after plating, cells were studied electrophysiologically.

Electrophysiological Methods. Whole-cell recordings were performed at room temperature using standard techniques (20, 21). Electrodes were pulled from Corning 7052 glass and fire-polished just before use. Electrode resistances were approximately 6 M Ω in bath. After formation of a G Ω seal, the membrane was ruptured and series resistance compensated (70–80%). Recordings were taken from granule neurons, identified by morphological criteria and having whole-cell capacitances between 1.5 and 4.5 pF. The intracellular recording solution contained 70 mM K₂SO₄ or 140 mM potassium gluconate, 5 mM Hepes, 3 mM MgCl₂, 5 mM EGTA, 12 mM phosphocreatine, 2 mM Na₂ATP, 0.4 mM Na₃GTP γ S or Na₃GTP, 0.01 mM spermine, 0.1 mM leupeptin (pH 7.25) adjusted with either H₃SO₄ or KOH, 265–275 mOsm/liter (adjusted with glucose). The extracellular recording solution contained 10 mM Hepes, 1 mM EDTA, 0.001 mM tetrodotoxin, and one of the following salts: 70 mM K₂SO₄ or 140 mM potassium gluconate, 20 mM K₂SO₄ + 50 mM Na₂SO₄, 70 mM Na₂SO₄, 70 mM Rb₂SO₄. To determine Ba²⁺ sensitivity, 70

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*To whom reprint requests should be addressed. e-mail: jsurm@nb.utmem.edu.

[†]D.J.S. and P.G.M. contributed equally to this work.

mM K_2SO_4 was replaced with 140 mM potassium glucamine, and EDTA was omitted. The pH for all solutions was 7.4 (adjusted with KOH except for the 70 mM Na_2SO_4 solution for which NaOH was used). Osmolarity was 295–305 mOsm/liter (adjusted with sucrose). All chemicals were obtained from Sigma except H_3SO_4 (Fluka, Ronkonoma, NY) and $Na_3GTP\gamma S$ (Boehringer Mannheim). Somatostatin and trans-(1*S*,3*R*)-1-amino-1,3-cyclopentanedicarboxylic acid (trans-ACPD) (RBI, Natick, MA) were stored as concentrated, frozen stocks and diluted immediately prior to use. Drugs were applied with a sewer pipe system as described previously (21). The currents evoked by somatostatin were determined by subtracting control records from those seen in the presence of agonist. Similar computations were made to determine Ba^{2+} -sensitive currents.

Statistical analyses were performed with StatView (Abacus Concepts, Berkeley, CA) running on a Macintosh workstation. Box plots were used for graphic presentation of the data because of the small sample sizes (22). The box plot represents the distribution as a box with the median as a central line and the hinges as the edges of the box (the hinges divide the upper and lower halves of the distributions in half). The inner fences (shown as a line originating from the edges of the box) run to the limits of the distribution excluding outliers (defined as points that are more than 1.5 times the interquartile range beyond the interquartiles); outliers are shown as asterisks or circles.

RESULTS

Granule cells in cerebellar cultures were readily identified by their neuronal morphology, small size, and sparse neuritic arborization (18). The properties of GIRK-mediated currents in these cells were studied using whole cell voltage clamp techniques in an ionic environment designed to minimize currents through voltage-dependent Na^+ and Ca^{2+} channels. To increase the voltage range over which GIRK-mediated currents could be examined, neurons were studied in isotonic K^+ solutions. In wild-type (+/+) cerebellar granule cells, somatostatin (500 nM) reversibly evoked an inwardly rectifying current (Fig. 1*A*) ($n = 7$). Isolation of the somatostatin-evoked current by subtraction (Fig. 1*B*) showed that the current reversed polarity near the K^+ reversal potential (E_K), as expected for a GIRK-mediated current (9–13). The addition of Ba^{2+} (500 μM), a GIRK-channel blocker, nearly eliminated the modulation by somatostatin (Fig. 1*B*). The metabotropic receptor agonist, trans-ACPD (10 μM), also evoked an in-

wardly-rectifying current with similar properties ($n = 3$, data not shown).

When +/+ granule cells were dialyzed with GTP γS to activate G proteins directly, the currents evoked by voltage ramps were similar to those seen in the presence of somatostatin (Fig. 2*A*). The application of Ba^{2+} (500 μM) significantly reduced inward current at negative membrane potentials. Isolation of the Ba^{2+} -sensitive current by subtraction (Fig. 2*B*) yielded a current that was very similar to that evoked by somatostatin (or trans-ACPD). In the absence of GTP γS or somatostatin to activate G-proteins, there was very little Ba^{2+} -sensitive current in +/+ neurons, suggesting that G-protein-independent inward rectifiers (IRKs) were not expressed in significant numbers (Fig. 2*C*). In the presence of GTP γS , shifting E_K by lowering the extracellular K^+ concentration, shifted the reversal potential of currents to values close to those predicted from the Nernst equation for a K^+ -selective channel population (median $P_{Na}/P_K = 0.07$, $n = 5$) (Fig. 2*D*). Thus, wild-type (+/+) cerebellar granule cells exhibited G-protein-activated, inwardly rectifying, K^+ -selective currents with properties very similar to those expected from channels containing GIRK2 subunits.

There were no obvious differences in the appearance of individual granule cell somata derived from +/+, $wv/+$, or wv/wv weaver mice 48–72 h after culturing in the presence of a broad spectrum glutamate receptor antagonist (kynurenic acid) (Fig. 3*A*). Furthermore, the voltage-dependent K^+ currents evoked by depolarization from negative holding potentials in low external K^+ solutions (5 mM) were similar to those described previously in granule cells (23, 24). Peak current densities evoked by depolarizing voltage ramps were not significantly different in +/+, $wv/+$, and wv/wv neurons ($P > 0.05$, analysis of variance). The normality of these outward currents argues that neurons carrying the mutant allele were not physiologically compromised at the time of study.

In contrast to the similarities in the voltage-activated K^+ currents, neurons carrying the weaver mutant allele expressed very little inwardly rectifying K^+ current that could be activated by receptor stimulation or by direct activation of G-proteins. As shown in Fig. 3*B*, somatostatin (500 nM) evoked very little inward rectification in granule cells from wv/wv weaver mice, in contrast to +/+ neurons (inset is a box plot summary). To determine whether this deficit reflected a disorder in receptor coupling or G-protein activation, neurons were dialyzed with GTP γS . In contrast to +/+ neurons, wv/wv and $wv/+$ neurons possessed very little Ba^{2+} -sensitive inward rectification in this recording configuration (Fig. 3*C*). A sum-

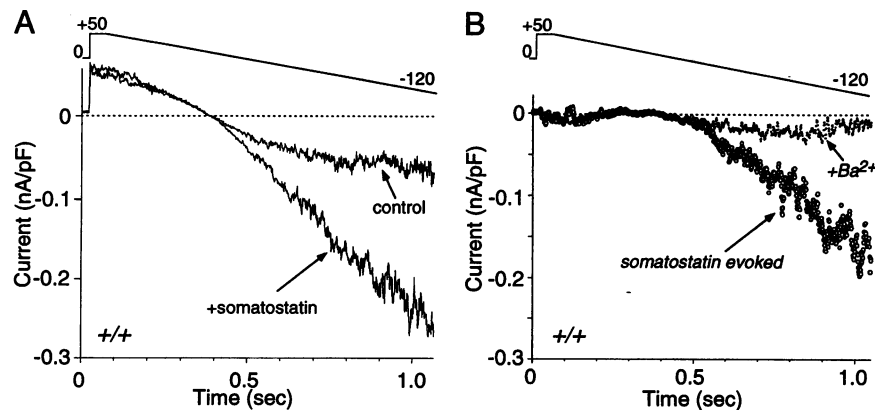


FIG. 1. Somatostatin evokes an inwardly rectifying K^+ current in +/+ granule cells. (*A*) In isotonic potassium solutions, a small, nonrectifying current is evoked by a voltage ramp from +50 mV to -120 mV from a holding potential of 0 mV. The addition of somatostatin (500 nM) enhanced the current at negative membrane potentials. (*B*) Subtracted records obtained in the presence and absence of somatostatin. Note that the current strongly rectifies and has a reversal potential near 0 mV, the K^+ equilibrium potential. Also shown are the currents evoked by somatostatin in the presence of Ba^{2+} (500 μM). At -100 mV, somatostatin increased current on average by 54.3 ± 16.9 pA/pF (mean \pm SEM, $n = 7$). Granule cells had been maintained in culture 48–72 h prior to recording.

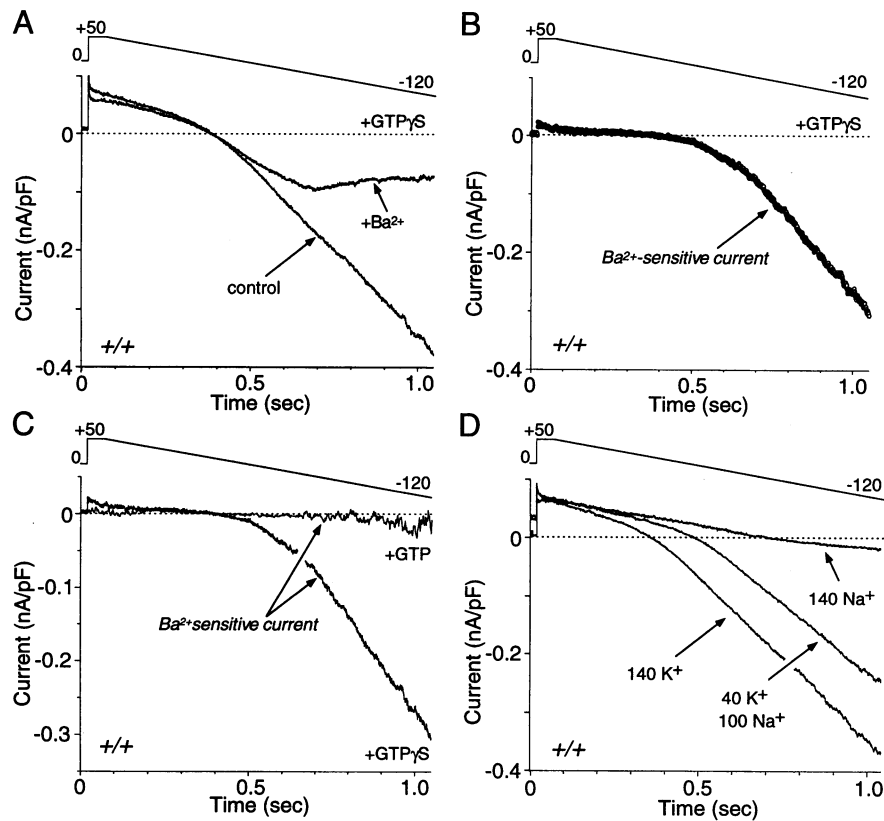


FIG. 2. Direct G-protein activation in $+/+$ granule cells also increases the inwardly rectifying currents that appear to be attributable to GIRK1 and GIRK2 subunits. (A) Direct activation of G-proteins by dialysis with the nonhydrolyzable GTP analog, GTP γ S, resulted in currents similar to those seen in the presence of somatostatin. The application of Ba $^{2+}$ (500 μ M) blocked a portion of the current at negative membrane potentials. (B) Isolation of the Ba $^{2+}$ -sensitive current by subtraction of the records in A revealed a strongly rectifying current similar to that evoked by somatostatin. (C) The Ba $^{2+}$ -sensitive inward rectifier was not observed when neurons were dialyzed with GTP, rather than GTP γ S. (D) With dialysis of GTP γ S, shifts in the reversal potential with substitution of extracellular Na $^{+}$ for K $^{+}$ reflected a K $^{+}$ -specific current. Using the biionic equation, the median P_{Na}/P_K was 0.07 ($n = 5$). The biophysical and pharmacological characteristics of the inward potassium currents described in this figure and in Fig. 1 are consistent with a GIRK2-containing channel.

mary of the results from $+/+$ ($n = 15$), $wv/+$ ($n = 14$), and wv/wv ($n = 13$) neurons is shown in Fig. 3D. It is clear from this comparison that there was a correlation between expression of the mutant allele and cellular phenotype. The absence of evocable current argues that either GIRK wv channels were not capable of being activated by G proteins or conducted current poorly when activated. This finding also argues against the presence of constitutively active, functional GIRK wv channels (9, 10).

When expressed in *Xenopus* oocytes, homomeric GIRK2 wv channels lack cation selectivity—channels readily pass either K $^{+}$ or Na $^{+}$ (9, 10). To determine whether homomeric GIRK wv channels were a significant complement of the channel population in wv/wv granule cells, GIRK-mediated currents were measured first in an isotonic K $^{+}$ bath solution (as described above) and then in a solution containing only Na $^{+}$. In $+/+$ granule neurons, as expected, replacement of extracellular K $^{+}$ with Na $^{+}$ dramatically reduced Ba $^{2+}$ -sensitive inward currents (Fig. 3E). In wv/wv granule cells, replacement of extracellular K $^{+}$ with Na $^{+}$ also reduced GIRK currents (Fig. 3F). To quantitatively estimate selectivity, the peak current at -100 mV in the presence of Na $^{+}$ (I_{Na}) and in the presence of K $^{+}$ (I_K) were measured. The ratio I_{Na}/I_K was then computed. For K $^{+}$ selective channels, this ratio should be near 0, whereas for nonselective channels it should be near 1. Inset in Fig. 3F is a box plot summary of the ratios computed in $+/+$ ($n = 5$) and wv/wv neurons ($n = 5$). Although the dispersion of the selectivity ratios was greater in wv/wv neurons, the median ratios were very similar in the two groups. We also did not see any significant difference in holding or "leak" currents be-

tween $+/+$ and wv/wv neurons. In high Na $^{+}$ external solutions, the median holding current at 0 mV was 40.7 pA/pF in $+/+$ neurons (mean = 49.8 ± 11.3 , $n = 12$) and 21.4 pA/pF in wv/wv neurons (27.9 ± 5.3 , $n = 11$). At -100 mV, the median holding current was 12.2 pA/pF in $+/+$ neurons (33.8 ± 10.5 , $n = 17$) and 14.5 pA/pF in wv/wv neurons (17.6 ± 5.17 , $n = 12$). In neither case were the differences between $+/+$ and wv/wv neurons statistically significant ($P > 0.05$, paired t test). These results argue that the principal defect in wv/wv granule cells is not a loss in selectivity or the addition of a cationic leak current, but a reduction in evocable K $^{+}$ current.

DISCUSSION

Our results provide the first demonstration that cerebellar granule cells express a robust, G-protein-activated, inwardly rectifying K $^{+}$ current. The biophysical and pharmacological properties of the current are consistent with those arising from channels formed from GIRK1 and GIRK2 subunits (25–27). This inference is also consistent with the work of Slesinger *et al.* (9) and others (10, 28) showing that cerebellar granule cells express both GIRK1 and GIRK2 subunits.

More importantly, this conclusion is also in agreement with the functional changes observed in neurons carrying the weaver mutant allele. In wv/wv neurons, there was a significant loss of inwardly rectifying current attributable to G-protein coupled channels. The current remaining in these neurons was further reduced by substitution of Na $^{+}$ for K $^{+}$ in the bathing solution, arguing that functioning GIRK channels retained

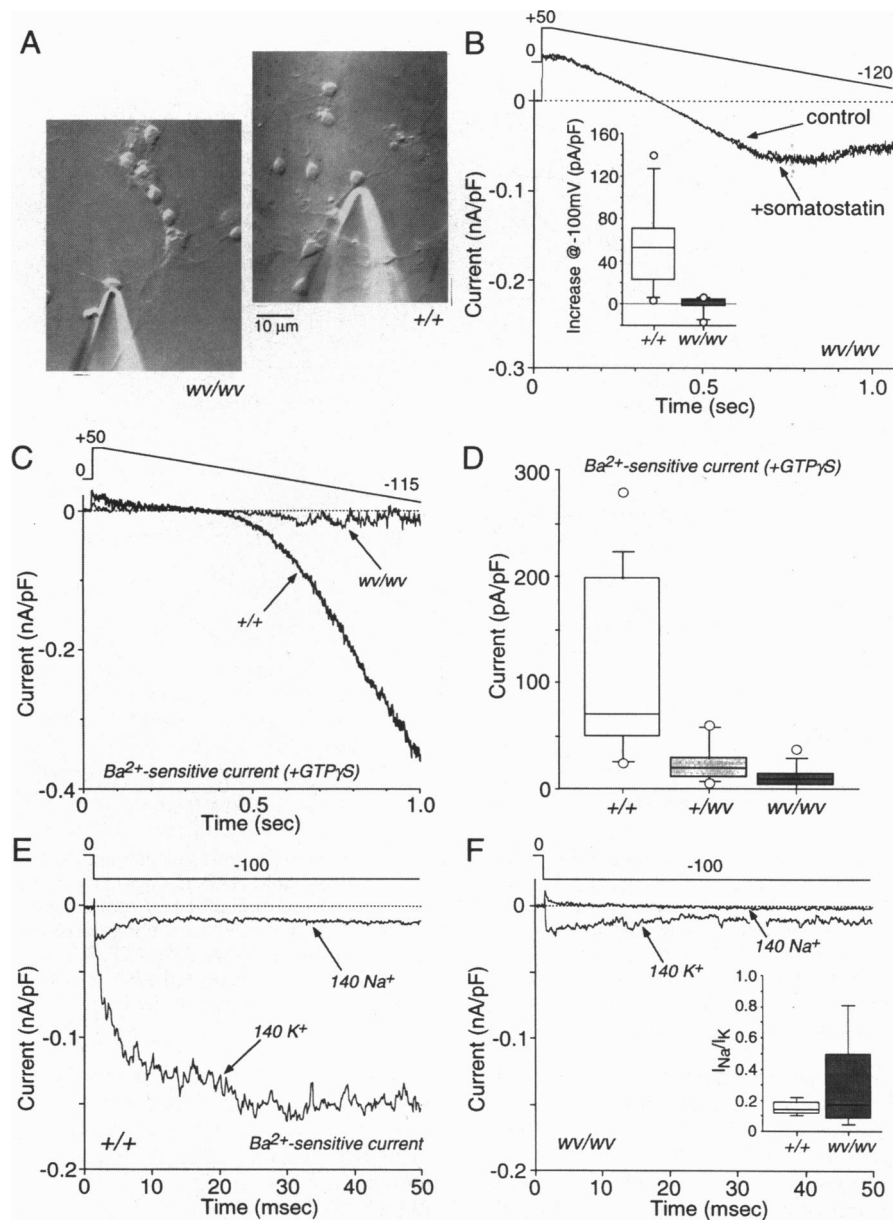


FIG. 3. Somatostatin and GTP γ S dialysis do not evoke inwardly rectifying currents in *wv/wv* granule cells. (*A*) Photomicrographs of *+/+* and *wv/wv* cerebellar cultures. No differences were observed in cell size or morphology between genotypes. Outward potassium currents also appeared unaffected by the mutation during the time of recording. (*B*) In *wv/wv* granule cells, somatostatin did not evoke inwardly rectifying current. At -100 mV, there was a -0.3 ± 3.4 pA/pF decrease in current with somatostatin ($n = 6$). The trajectory of the current records in response to the voltage ramp reflects the presence of currents that deactivate or inactivate at hyperpolarized membrane potentials; these currents were similar in *+/+* and *wv/wv* neurons. (*Inset*) Box-plot summary of the change in current between *+/+* and *wv/wv* cells. See *Materials and Methods* for a description of the plot. The median increase for *+/+* neurons was 51.5 pA/pF; *wv/wv* 2.9 pA/pF. Somatostatin produced a significant increase in current in *+/+* granule cells, compared with *wv/wv* granule cells ($P < 0.02$, unpaired *t* test). (*C*) Activation of G-proteins with intracellular dialysis of GTP γ S also failed to evoke a Ba $^{2+}$ -sensitive current in *wv/wv* neurons. (*D*) Box-plot summary of the Ba $^{2+}$ -sensitive current at -100 mV in neurons dialyzed with GTP γ S. The median Ba $^{2+}$ -sensitive current in *+/+* granule cells was 70.6 pA/pF ($n = 15$); *wv/+*: 19.2 pA/pF ($n = 14$); and *wv/wv*: 9.0 pA/pF ($n = 13$). The differences between groups was significant ($P < 0.0001$, analysis of variance). (*E*) Reduction of the Ba $^{2+}$ -sensitive current in a wild-type neuron with substitution of extracellular K $^{+}$ with Na $^{+}$. (*F*) In weaver neurons, substitution of K $^{+}$ with Na $^{+}$ also produced a reduction in the Ba $^{2+}$ -sensitive current, although results were more variable. (*Inset*) Box-plot summary of the ratio of Ba $^{2+}$ -sensitive current in 140 mM extracellular Na $^{+}$ and K $^{+}$. The median ratio for *+/+* neurons was 0.14 ($n = 5$); in *wv/wv* neurons it was 0.17 ($n = 5$). The differences between groups were not significant ($P > 0.05$, unpaired *t* test).

some measure of ionic selectivity. Furthermore, we found no evidence for a constitutively active cation current in *wv/wv* neurons that would be reflected in a resting, leak current at either 0 or -100 mV. In *Xenopus* oocytes, co-expression of GIRK1 and GIRK2 $_{wv}$ also results in a significant reduction in inwardly rectifying current—in contrast to the changes seen with homomeric GIRK2 $_{wv}$ channels (9, 10). The magnitude of the currents seen in oocytes depended upon the relative

abundance of the two subunits, with substantially less current being seen with higher levels of GIRK1 expression. This finding taken together with our data suggests that GIRK1 must be expressed along with GIRK2 $_{wv}$ in weaver granule cells. If homomeric GIRK2 $_{wv}$ channels are present, they must contribute very little to the ionic behavior of granule cells. As a result, it is clear that the principal defect in *wv/wv* granule cells is not a loss in GIRK channel selectivity or the addition of a

constitutively active cation current, but the failure of receptor activated G-proteins to evoke a K⁺-selective current.

How might the loss of functional GIRK channels contribute to the death of granule cells in the developing cerebellum? One clue is that external granule layer granule cells do not exhibit signs of degeneration until they are postmitotic and in the premigratory region of the external granule layer (5, 29). At this point, granule cells are exposed to elevated levels of extracellular glutamate, resulting in activation of NMDA receptors (30, 31). Komoro and Rakic (32) have hypothesized that elevations in cytosolic Ca²⁺—subsequent to activation of N-methyl-D-aspartate glutamate receptors—actually triggers migration. Co-activation of metabotropic glutamate receptors potentiate these currents (33) and should activate GIRK2-containing channels (26, 27), providing a hyperpolarizing current to counterbalance the depolarizing, ionotropic glutamate currents. In *wv/wv* granule cells, the loss of functional GIRK2-mediated currents could lead to sustained depolarization, excessive Ca²⁺ entry, and death (34). In line with this hypothesis, block of NMDA channels rescues weaver mutant granule cells *in vitro* (35).

Several questions remain to be resolved. One is why some neurons that express GIRK2 die as a consequence of the weaver mutation, whereas others do not. For example, granule cells in the lateral cerebellar hemispheres are more resistant to the weaver mutation than midline neurons (3, 5, 36). We saw no evidence for variation in the intrinsic properties of granule cells that could account for this difference in vulnerability, suggesting that some other factor—like the level of extracellular glutamate—must be involved. Hippocampal granule cells also express high levels of GIRK2 protein yet appear unaffected by the weaver mutation (D.G. and R. Smeyne, unpublished). This variation in sensitivity strongly argues against the proposition that expression of GIRK2_{wv} alone is responsible for cell death. Rather some combination of intrinsic and extrinsic factors must determine cell fate.

A grasp of these factors may prove to be of value in understanding normally occurring degenerative processes. For example, Parkinson's disease is characterized by the progressive loss of dopaminergic neurons in the substantia nigra (37). In weaver mice, these same neurons are also lost progressively (4, 6, 38), suggesting that disruption of GIRK-channel function may play a part in the pathophysiology of parkinsonism.

Note Added in Proof. After submission of this manuscript, Kofuji *et al.* (39) reported that activation of G proteins induced significantly less GIRK-mediated current in weaver cerebellar granule cells than in wild-type neurons. This finding is similar to that reported here. They also reported that weaver granule cells express a significant, non-selective leak current that they attribute to GIRK channels containing mutant GIRK2 subunits. As described above, we found no evidence for such a current in our recordings from weaver granule cells.

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