

Rescue of Cerebellar Granule Cells from Death in *weaver NR1* Double Mutants

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The *weaver* mutation results in the extensive death of midline cerebellar granule cells. The mutation consists of a single base pair substitution of the gene encoding the G-protein-activated inwardly rectifying potassium channel protein, GIRK2. The functional consequences of this mutation are still in dispute. In this study we demonstrate the *in vivo* and *in vitro* rescue of

weaver granule cells when NR1 NMDA subunits are eliminated in *weaver NR1* double mutants. This rescue of *weaver* granule cells provides evidence that *wvGIRK2* alone is not sufficient to cause granule cell death.

Key words: *weaver*; *GIRK2*; granule cell development; cerebellum; NMDA receptor; cell death

The external granular layer (EGL) of the cerebellum is the site of genesis of the most abundant neuron in the adult CNS, the granule cell. During the first 3 weeks of postnatal development in the mouse, the cells of the EGL undergo a well characterized temporospatial pattern of granule cell proliferation, migration, and differentiation that is dependent on both the appropriate expression and function of intrinsic and extrinsic factors (Hatten and Heintz, 1995). Genetic mutations that disrupt this orderly developmental progression result in aberrant cerebellar histogenesis (Goldowitz and Hamre, 1998).

This is nowhere more apparent than in the *weaver* neurological mutant. In this mutation, cerebellar cytoarchitectonics are disrupted severely, attributable primarily to the vast depletion of cerebellar granule cells. In homozygous *weaver* (*wv/wv*) mice, granule cell precursors proliferate normally in the EGL, exit the cell cycle, and die just before their migration from the EGL (Smeyne and Goldowitz, 1989).

Recently, the *weaver* mutation was identified as a single base pair substitution in the gene encoding a G-protein-activated inwardly rectifying potassium channel protein, GIRK2 (Patil et al., 1995). GIRKs are believed to play a role in controlling cell membrane excitability by opposing deviations from the K^+ equilibrium potential (Wickman and Clapham, 1995; Slesinger et al., 1996; Ehrenguber et al., 1997). The functional consequences of this mutation are in dispute. Results from whole-cell recordings of *wv/wv* and wild-type cerebellar granule cells have differed, suggesting that the *weaver* mutant phenotype is attributable either to a gain-of-channel function (*wvGIRK2* channels lose their selectivity for K^+ and become constitutively active) (Kofuji et al.,

1996) or to a loss of GIRK2-mediated currents (Surmeier et al., 1996; Lauritzen et al., 1997). In either case, the *weaver* mutation must be reconciled with the selective cell death that occurs in the *weaver* mutant cerebellum. GIRK2 has been shown to be expressed in all cells of the EGL (Kofuji et al., 1996; Slesinger et al., 1996; Chen et al., 1997; Wei et al., 1997), but only the *wv/wv* midline premigratory EGL cells experience massive degeneration (Herrup and Trenkner, 1987; Smeyne and Goldowitz, 1990). These findings mandate the involvement of another factor in addition to the mutated GIRK2 protein in causing granule cell death.

It is known that the activation of NMDA receptors coupled to an increase in Ca^{2+} influx through N-type Ca^{2+} channels triggers granule cell migration from the premigratory zone of the cerebellar EGL (Komuro and Rakic, 1993). We hypothesized that cerebellar granule cell death in *wv/wv* is attributable to the loss of GIRK2 currents that normally would moderate the NMDA-based depolarizing currents (Surmeier et al., 1996). To test this hypothesis, we generated *wv/wv* mice carrying the NMDA receptor 1 subunit (NR1) null mutation to see whether granule cell precursors could be rescued from death. The NR1 subunit is required for NMDA receptor function, and targeted disruption of the *NR1* gene has been shown to abolish NMDA responses (Forrest et al., 1994). In this study we provide evidence that *wvGIRK2* alone is not enough to cause granule cell death in *wv/wv*, by demonstrating that death is prevented when NMDA receptor function is blocked in *wv/wv NR1* double-mutant granule cells.

MATERIALS AND METHODS

Animals. The *weaver NR1* colony was established from matings between heterozygous *weaver* female mice from our 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, Boston, MA) (Sidman et al., 1965) and *NR1*^{-/+} mutant males obtained from the laboratory of Tom Curran (St. Jude Children's Research Hospital, Memphis, TN). These *NR1*^{-/+} mutants were produced from 129/Sv embryonic stem cells injected into C57BL/6 blastocysts. Founder mice were mated to C57BL/6 mice, and the NR1 colony was maintained by brother × sister matings (Forrest et al., 1994). All animals used in this study were generated from matings between female *wv/+ NR1*^{-/+} or *wv/wv NR1*^{-/+} mice and male *wv/+ NR1*^{-/+} mice.

Received Feb. 2, 1999; revised June 14, 1999; accepted June 30, 1999.

This research was supported by a UT-Memphis Center for Neuroscience Fellowship (P.J.) and the Department of Anatomy and Neurobiology and the University of Tennessee College of Medicine (D.G.). This work also was supported by National Institute of Neurological Diseases and Stroke Grant NS 36496 (D.J.S.). We thank Dr. Tom Curran for providing us with the *NR1* null mouse, Dr. Paul Mermelstein for designing Figure 5, Dr. Lily Jan for the GIRK2N antibody, Dr. Michisuke Yuzaki for help in establishing P0 cerebellar cultures, and Richard Carl Cushing for technical assistance.

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Determination of genotype. DNA was isolated from the ends of tails, and *wv* and *NRI* genotypes were determined by two separate PCR reactions. *NRI* genotypes were identified by a protocol, developed by Douglas Forrest at the former Institute of Molecular Biology at Roche (Nutley, NJ), which uses a common antisense primer (5'-CCA GCC TGC ACA CTT TAG GTC ACA TTG-3'), a sense primer for the wild-type *NRI* allele (5'-CCA ACG CCA TAC AGA TGG CCC TGT-3'), and a sense primer for the null allele (5'-GTG CCA GCG GGG CTG CTA AAG-3'). The reaction was performed in a total vol of 50 μ l and included an initial denaturation at 94°C/4 min plus 35 cycles consisting of denaturation at 94°C/30 sec, annealing at 59°C/30 sec, and extension at 72°C/90 sec. The wild-type allele yielded a 950 bp band, and the null allele yielded a 500 bp band.

weaver genotypes were determined by a restriction site-generating PCR protocol developed by Patricia Ehrhard (Roche, Nutley, NJ). This protocol used a 3' wild-type primer (5'-CAT GAA GGC GTT GAC AAT GGA-3') and a 5' mismatched primer (5'-CCA TAG AGA CAG AAA CCA CGA TC-3'), which creates a *PvuI* restriction site in the wild-type allele. PCR reactions were performed in a total vol of 20 μ l and included an initial denaturation at 94°C/3 min plus 30 cycles each consisting of denaturation at 94°C/30 sec, annealing at 55°C/45 sec, and extension at 72°C/60 sec. The PCR product was digested with *PvuI* restriction enzyme, and 8 μ l samples of the digested PCR product were subjected to electrophoresis; the bands were visualized with ethidium bromide. The wild-type allele yielded a 101 bp band, and the *wv* allele yielded a 119 bp band.

Histology and morphometric analysis. Postnatal day 0 (P0) and adult mice were anesthetized with Avertin and perfused with PBS, pH 7.35, followed by a 3:1 95% ethanol/acetic acid fixative. Brains were removed and post-fixed for 24 hr. After post-fixation, the brains were bisected sagittally, dehydrated, and embedded in paraffin. A series of 50 sagittal sections, cut at 6 μ m, were mounted on slides; every third slide was stained with cresyl violet.

For each P0 animal of the *wv/wv NRI*^{+/+} ($n = 5$), *wv/wv NRI*^{-/+} ($n = 6$), *wv/wv NRI*^{-/-} ($n = 9$), *+/+ NRI*^{+/+} ($n = 3$), *+/+ NRI*^{-/+} ($n = 3$), and *+/+ NRI*^{-/-} ($n = 3$) genotypes, three nonconsecutive sections of the midline cerebellum were analyzed and averaged. The most posterior 300 μ m of the external granule cell layer was measured along the pial surface, and three features within this area were quantified by (1) total number of cells, (2) total number of mitotic figures, and (3) total number of pyknotic cells. Pyknotic cells were defined as densely heterochromatic figures in which cellular details were no longer visible. Mitotic figures were differentiated from pyknotic cells on the basis of the appearance of defined heterochromatic structure and phase of cell division. For each animal the percentage of total cells that were either pyknotic or mitotic was calculated. To determine if there were significant differences among the genotypes, we performed an ANOVA. TUNEL staining was performed as another indicator of cell death, according to the manufacturer's instructions (Apoptag kit, Oncor, Gaithersburg, MD).

For each adult animal of *wv/wv NRI*^{-/+} ($n = 3$) and *wv/wv NRI*^{+/+} ($n = 3$) genotypes, three nonconsecutive sections of the midline cerebellum were analyzed and averaged. The total number of granule cells in lobules 2/3, 8 and 10 was counted. Granule cells were identified easily by their size, shape, and characteristic heterochromatin structure. To determine if there were significant differences between the genotypes, we performed an unpaired Student's *t* test.

GIRK2 immunohistochemistry. Tissue sections were prepared as described above. Slides were rinsed in PBS containing 0.3% Triton X-100 (PBS/T), blocked with 1% hydrogen peroxide in PBS/T, washed with PBS/T, and then blocked in 2% dry milk in PBS/T. Slides were rinsed in PBS/T and overlaid with rabbit polyclonal GIRK2 antibody (1:250) directed against the N terminus (Liao et al., 1996) in PBS/T with 5% normal goat serum (NGS). On control slides the primary antibody was omitted. Slides were rinsed with PBS/T and then overlaid with biotinylated goat anti-rabbit IgG (1:200) (Vector Laboratories, Burlingame, CA) in PBS/T with 5% NGS. Slides were rinsed with PBS/T, and the sections were developed with Vectastain ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine (DAB), yielding a brown precipitate. After being rinsed, the slides were dehydrated and coverslipped.

Cell culture. Cerebellar granule cell cultures were prepared from P0 cerebella. Mice were decapitated, and cerebella were removed and carefully cut into four pieces in calcium- and magnesium-free buffer solution (CMF) containing HBSS and HEPES, pH 7.35 (Sigma, St. Louis, MO). After being rinsed with CMF, cerebellar pieces were placed in CMF containing 0.25% trypsin for 10 min at room temperature with slight

rotary agitation. The tissue was rinsed three times in CMF to remove the trypsin and then placed in trituration medium [Neurobasal medium supplemented with B27, 0.5 mM L-glutamine (NBM; Life Technologies, Gaithersburg, MD), and 0.05% deoxyribonuclease and 0.25% glucose]. Trituration of tissue was done on ice by using successively smaller bore, fire-polished sterile Pasteur pipettes (ten times each with pipettes of 1, 0.5, and 0.1 mm internal diameter). Then the entire cell suspension was sedimented by centrifugation (600 rpm) for 8 min at 4°C. The pellet was resuspended in culture medium (NBM supplemented with B27, 0.5 mM L-glutamine, 10 pg/ml GDNF, and 100 μ g/ml of penicillin and streptomycin). Cell viability was assessed by using the trypan blue dye exclusion method. Cells were plated on round sheets of Aclar (Pro Plastics) coated with poly-L-lysine at a density of 2×10^5 cells/well. After 8 d *in vitro*, some cultures were fixed with 4% paraformaldehyde/0.3% glutaraldehyde, and immunohistochemical analysis was performed with rabbit polyclonal antibody against glutamate, according to the manufacturer's instructions (Incstar, Stillwater, MN).

RESULTS

Generation and behavior of double-mutant mice

To determine the effects of the *NRI* null mutation on *weaver* granule cell survival, we crossed *weaver* mice with *NRI*^{-/+} mice to generate *wv/+ NRI*^{-/+} male and female mice. These mice were intercrossed to produce homozygous mutant offspring. *wv/wv NRI*^{-/+} were viable and survived until adulthood, whereas *wv/wv NRI*^{-/-} offspring died within the first 24 hr after birth. This neonatal lethality is the same as that reported by Forrest et al. (1994) in *NRI*^{-/-} mice that were wild-type at the *weaver* locus. Adult and neonatal mice were genotyped by a PCR-based method that allowed for the determination of both *weaver* and *NRI* null alleles (see Materials and Methods).

By the second postnatal week, when the first signs of ataxia are evident in *wv/wv* mice, nonataxic littermates were culled to ensure the maximum viability of *wv/wv* young. It was immediately apparent that among the ataxic mice there were two phenotypically distinct groups. One group paralleled the well characterized *wv/wv* behavioral phenotype, including gait instability, outward splaying of hindlimbs, tremor, curled posture, and severe ataxia (Sidman et al., 1965). The behavioral phenotype of the second group was markedly different. These mice had a more normal posture and leg stance and attenuated tremor and ataxia. Furthermore, these mice had an overall increased viability and better reproductive success and were better at caring for their young. On genotyping, the healthier animals were invariably *wv/wv NRI*^{-/+}, whereas the more ataxic animals were *wv/wv NRI*^{+/+}.

Analysis of the adult *wv NRI* cerebellum

Histological analysis of the adult *wv/wv NRI* cerebellum was performed to determine whether the improved behavioral phenotype reflected an improved cerebellar phenotype. Overall, *wv/wv NRI*^{+/+} cerebella appeared similar to those of the inbred *wv/wv* cerebella. In these cerebella there is a near-total loss of midline granule cells and a diminutive cerebellum (Fig. 1A,C). In contrast, the cerebella of *wv/wv NRI*^{-/+} mice appeared larger, and there was an increased survival of granule cells (Fig. 1B,D). To quantitate this, we made counts of the total number of granule cells in regions of the midline cerebellum (lobules 2/3, 8, and 10) between *wv/wv NRI*^{-/+} and *wv/wv NRI*^{+/+} mice. There was a significant difference in the total number of granule cells between the two groups (unpaired *t* test, $p < 0.05$), with more than twice as many granule cells found in *wv/wv NRI*^{-/+} cerebella (Fig. 1E).

Analysis of the P0 *wv NRI* cerebellum

It was expected, given the partial survival of granule cells in the adult *wv/wv NRI*^{-/+} cerebellum, that an even more profound

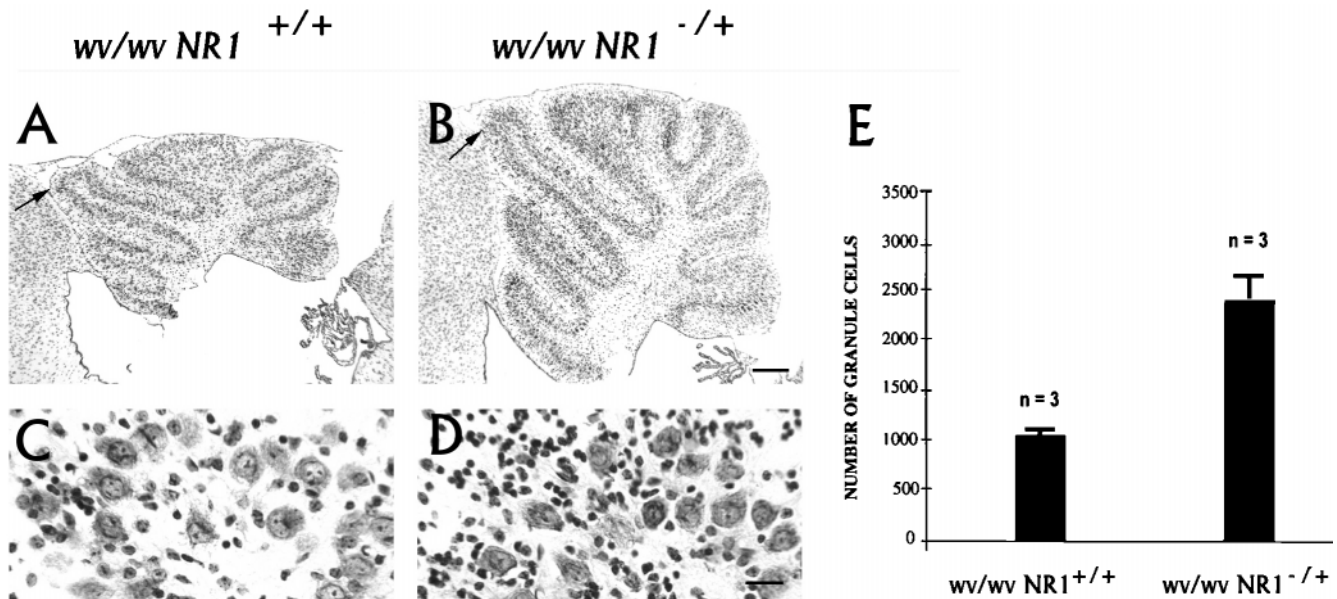


Figure 1. Analysis of adult *wv/wv NR1* cerebella. *A, B*, Sagittal sections of P60 midline cerebella (posterior is to the right). *wv/wv NR1*^{+/+} cerebellum (*A*) is smaller than *wv/wv NR1*^{-/+} cerebellum (*B*). *C, D*, High magnification of lobule IV (arrows in *A* and *B*), demonstrating the increased numbers of granule cells in *wv/wv NR1*^{-/+} (*D*) as compared with *wv/wv NR1*^{+/+} (*C*). Scale bars: *A, B*, 200 μ m; *C, D*, 20 μ m. *E*, Total number of granule cells in lobules 2/3, 8, and 10 of adult *wv/wv NR1* cerebella, showing significantly more granule cells in the *wv/wv NR1*^{-/+} cerebellum ($p < 0.05$). Error bars represent \pm SEM.

effect on granule cell survival should be seen in the *wv/wv NR1*^{-/-} cerebellum. In *wv/wv* cerebella the first evidence of granule cell death is at P0 (Smeyne and Goldowitz, 1989). At this time cell death is restricted to the deep aspect of the EGL in the most posterior lobules of the cerebellum. Because the *NR1* null mutants survive birth (but die during the first 24 hr postnatal), we were able to determine the effects of the *NR1* homozygous deletion on *weaver* granule cell death at the P0 time point. Qualitative comparisons of the midline cerebellar EGL were made among *wv/wv NR1*^{+/+}, *wv/wv NR1*^{-/+}, and *wv/wv NR1*^{-/-} mice. There were no noticeable differences in the overall size and shape of the cerebellum among mice of the three genotypes (Fig. 2*A–C*). Additionally, there were no obvious genotypic differences in the total numbers of cells or mitotic figures within the EGL. However, there were marked differences in the presence of pyknotic cells in the deep aspect of the EGL among cerebella of the three genotypes (Fig. 2*D–F*). Prominent cell death was evident in the posterior lobules of the *wv/wv NR1*^{+/+} EGL (Fig. 2*D*), mimicking the phenotype of the inbred P0 *weaver* cerebellum (Smeyne and Goldowitz, 1989). In the *wv/wv NR1*^{-/-} cerebellum there was notably less cell death in this region (Fig. 2*E*). Many of the pyknotic cells in the EGL of all genotypes were also TUNEL-positive (data not shown), as previously demonstrated for the *weaver* cerebellum (Wullner et al., 1995; Migheli et al., 1997; Harrison and Roffler-Tarlov, 1998).

The total number of cells in the most posterior lobules of the EGL was counted, and the percentages of mitotic figures and pyknotic cells were calculated for each of the three genotypes. There were no significant differences in the percentage of mitotic figures (Table 1). However, there were highly significant differences (ANOVA, $p < 0.01$) in the percentage of pyknotic cells among the three genotypes, with the highest percentage of pyknotic cells found in *wv/wv NR1*^{+/+} mice and a dramatic decrease in the percentage of pyknotic cells in *wv/wv NR1*^{-/-} mice (Table 1). Interestingly, there was an intermediate amount of pyknotic

cells in *wv/wv NR1*^{-/+} mice, indicating a gene dosage effect. What made these differences even more compelling is that in all but one of the *wv/wv NR1*^{-/-} pups that were included in the cerebellar analysis there were varying degrees of neural cell death in other regions of the brain, especially the colliculi and cortex. This is most likely attributable to the declining state of the *NR1*^{-/-} animals. Despite this fact, *wv/wv NR1*^{-/-} cerebella exhibited less cell death in the EGL.

In the postnatal developing cerebellum the granule cells undergo the process of naturally occurring cell death (Wood et al., 1993). It is possible that the *NR1* null mutation somehow delays or inhibits this process, resulting in the apparent dearth of cell death in the *wv/wv NR1*^{-/-} EGL. To control for this possibility, we examined the effects of the *NR1* null mutation on the non-*weaver* cerebellum. We quantitatively analyzed cell death and proliferation in the EGL of *+/+ NR1*^{+/+}, *+/+ NR1*^{-/+}, and *+/+ NR1*^{-/-} mice. There were no significant differences in either the percentage of pyknotic or percentage of mitotic figures among mice of the three genotypes (Table 1). However, it is notable that there was a significant difference (unpaired *t* test, $p < 0.05$) in the percentage of pyknotic cells between *+/+ NR1*^{-/-} and *wv/wv NR1*^{-/-} cerebella. This indicates that, although NMDA receptor activation is the principal means of effecting cell death in *wv/wv*, there are other as yet to be identified mechanisms that likely are responsible for a component of cell death in *wv/wv* granule cells.

To rule out the possibility that the *NR1* null mutation interferes with the expression of GIRK2, we assessed GIRK2 immunostaining in P0 cerebella. We compared the expression of GIRK2 in *+/+ NR1*^{+/+}, *+/+ NR1*^{-/+}, *wv/wv NR1*^{+/+}, and *wv/wv NR1*^{-/-} brains. GIRK2 immunoreactivity was present throughout the EGL and in some Purkinje cells and nerve fibers in the future cerebellar white matter. The location of immunoreactivity was the same in all of the genotypes (Fig. 3*A–H*). GIRK2 immunoreactivity was observed throughout the EGL, both anterior to posterior and medial to lateral, which is consistent with previous

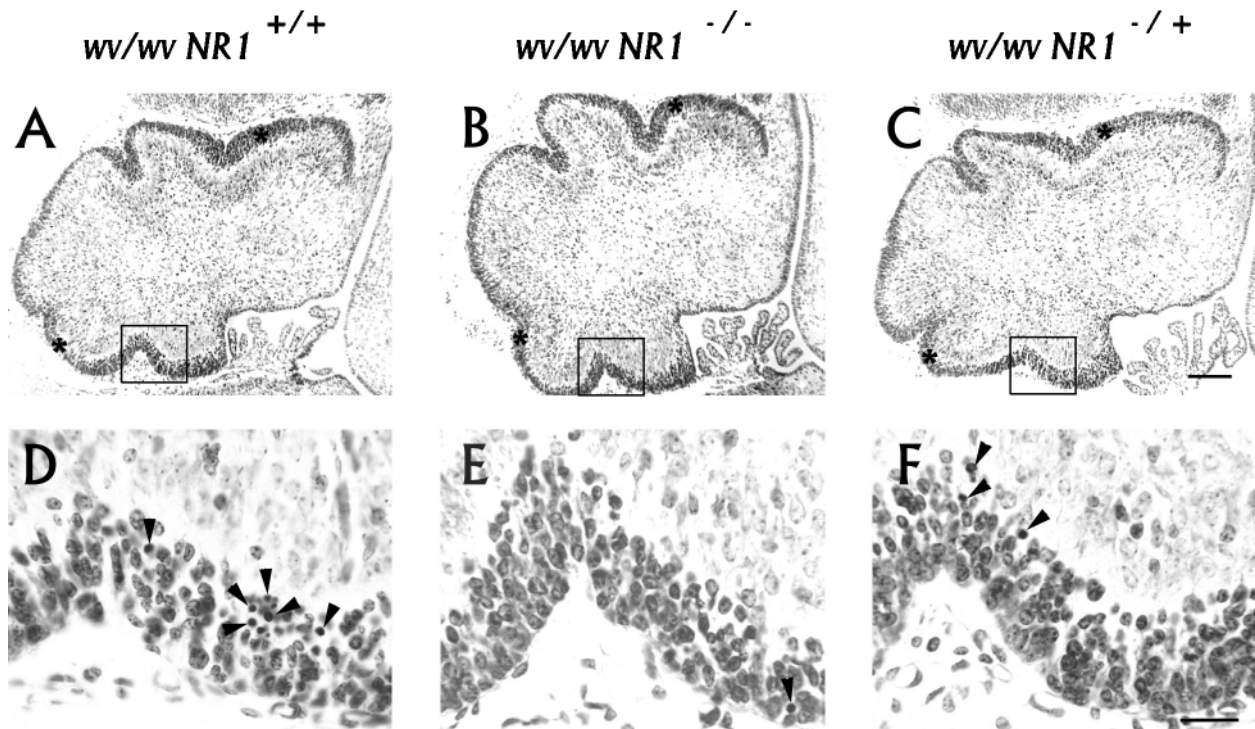


Figure 2. Analysis of neonatal *wv/wv* NR1 cerebella. *A–C*, Sagittal sections of P0 cerebella demonstrating no differences in the size and shape of the midline cerebella among the three genotypes (posterior is down). *D–F*, High magnification of posterior EGL marked by the box in *A–C*. There are many pyknotic figures (arrowheads) in the inner aspect of the EGL in *wv/wv* NR1^{+/+} (*D*), demonstrating the well documented cell death that is abundant in the *wv/wv* cerebellum. In contrast, there are very few, if any, pyknotic cells in the *wv/wv* NR1^{-/-} cerebellum (*E*). An intermediate amount of cell death is seen in the *wv/wv* NR1^{-/+} cerebellum (*F*). Scale bars: *A–C*, 100 μ m; *D–F*, 20 μ m.

Table 1. Mean percentage of pyknotic nuclei and mitotic figures in the posterior lobules of P0 cerebellar EGL

Genotype	<i>n</i>	Pyknotic (%)	Mitotic (%)
<i>wv/wv</i> NR1 ^{+/+}	5	3.72 \pm 0.56 ^a	0.62 \pm 0.15
<i>wv/wv</i> NR1 ^{-/-}	9	1.17 \pm 0.16 ^b	0.26 \pm 0.10
<i>wv/wv</i> NR1 ^{-/+}	6	1.90 \pm 0.21	0.42 \pm 0.17
+/+ NR1 ^{+/+}	3	0.21 \pm 0.14	0.73 \pm 0.39
+/+ NR1 ^{-/-}	3	0.39 \pm 0.23	1.14 \pm 0.13
+/+ NR1 ^{-/+}	3	0.69 \pm 0.30	1.09 \pm 0.01

Values are mean \pm SEM.

^aSignificant difference ($p < 0.00005$) among *wv/wv* NR1 genotypes.

^bSignificant difference ($p < 0.05$) between *wv/wv* NR1^{-/-} and +/+ NR1^{-/+} genotypes.

reports (Kofuji et al., 1996; Slesinger et al., 1996). GIRK2 immunoreactivity was found to be more intense in non-*weaver* cerebella, as was demonstrated from studies of the adult *weaver* brain (Liao et al., 1996). However, there were no detectable differences in GIRK2 immunoreactivity attributable to the NR1 null mutation in any of the genotypes that were examined.

In vitro analysis of *wv* NR1 granule cells

It is conceivable the NR1 null mutation affects *wv/wv* granule cell development to delay the onset of *weaver*-induced cell death. We addressed this issue by establishing cerebellar cultures to examine the effects of NR1^{-/-} on *wv/wv* granule cell survival beyond P0. The morphological and physiological maturation of NR1^{-/-} granule cells *in vitro* has been shown to be normal (Forrest et al., 1994). Dissociated *wv/wv* NR1^{+/+} and *wv/wv* NR1^{-/-} granule cells were cultured and assessed every 24 hr for features as

previously characterized for wild-type granule cells in culture (Trenkner and Sidman, 1977). These features include reaggregation within the first 24 hr of plating, neurite extension, the formation of cables between the reagggregates, and cell migration along the cables. The behavior of *wv/wv* NR1^{+/+} granule cells in culture was comparable to that of *wv/wv* granule cells derived from the original stock of *weaver* mice (Trenkner et al., 1978; Willinger et al., 1981). *wv/wv* NR1^{+/+} granule cells reaggregated within the first day of culture; however, the reagggregates were much smaller than what are seen in wild-type cultures, and there was also considerable neuronal degeneration. Compared with wild-type cultures, there was very little cable formation and no migrating neurons in *wv/wv* NR1^{+/+} cultures. After 8 d *in vitro* all *wv/wv* NR1^{+/+} granule cells were dead or degenerating (Fig. 4A).

After the first 24 hr in culture *wv/wv* NR1^{-/-} granule cells also reaggregated; however, there were few degenerating neurons. The reagggregates were also much larger, with increased cable formation and numerous migrating granule cells as compared with *wv/wv* NR1^{+/+} cultures. After 8 d *in vitro*, *wv/wv* NR1^{-/-} cultures had numerous clusters of small round cells (Fig. 4B), identified as granule cells by their glutamate immunoreactivity (data not shown). In all measures *wv/wv* NR1^{-/-} cultures were not readily distinguishable from wild-type cultures.

DISCUSSION

The present study demonstrates for the first time the *in vivo* rescue of granule cells in the *wv/wv* cerebellum. Analysis of *wv/wv* NR1 double mutants shows that *wv/wv* granule cells are rescued from cell death when NMDA receptor function is blocked and also shows that there is a gene dosage effect. This rescue is not

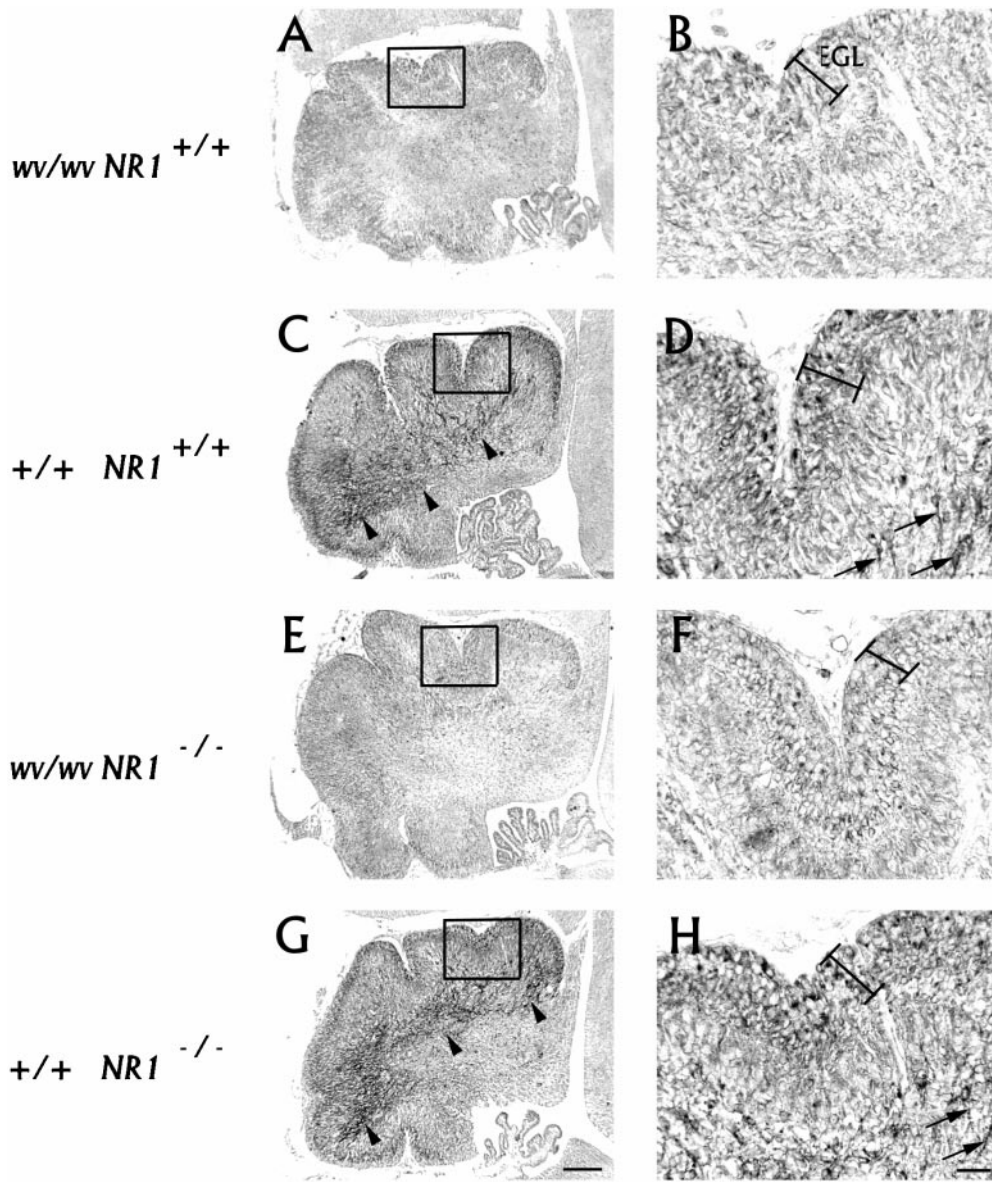


Figure 3. Expression of GIRK2 in P0 cerebella. *A, C, E, G*, GIRK2 immunoreactivity is seen throughout the EGL in all genotypes that were examined (posterior is down). However, immunoreactivity is much lower in *wv/wv* cerebella (*A, E*), as compared with non *wv/wv* cerebella (*C, G*), in which there is more intense immunoreactivity in the EGL and in what are likely Purkinje cells and their axons (arrowheads). This Purkinje cell staining appears to be transitory because only a few cells in the Purkinje cell plate are immunopositive, whereas many migrating Purkinje cells (arrows) and their axons are intensely immunoreactive. There are no noticeable differences in immunoreactivity because of the *NR1* null mutation (compare *C* with *G*). *B, D, F, H*, High magnification of anterior EGL demarcated in *A, C, E*, and *G*, demonstrating GIRK2 immunoreactivity throughout the anterior EGL (bracketed bar), which is void of cell death in the P0 *wv/wv* cerebellum. Scale bars: *A, C, E, G*, 100 μm ; *B, D, F, H*, 20 μm .

likely attributable to genetic background introduced via the matings necessitated to produce the double mutants, because *wv/wv NR1*^{+/+} mice exhibit the same level of cell death as the inbred *wv/wv* strain. However, this does not address the remote possibility that a locus tightly linked to the insertional event that created the *NR1* knock-out is responsible for the effects we see. It is also very unlikely that the *NR1* null mutation alters *wv/wv* granule cell development before the onset of death, because adult *wv/wv NR1*^{-/-} mice show enhanced granule cell survival. Furthermore, unlike P0 *wv/wv NR1*^{+/+} granule cells in culture, *wv/wv NR1*^{-/-} granule cells differentiate and survive in a manner analogous to wild-type granule cells.

One important implication of our results is that *wvGIRK2* alone is not sufficient to cause granule cell death. Our findings indicate that it is critical to pair the *wvGIRK2* mutation with a depolarizing influence to effect cell death. In the absence of such an influence in *wv/wv NR1* double-mutant granule cells, cell death is prevented. These conclusions are in line with a host of *in vitro* studies that demonstrate that *wv/wv* granule cells can be rescued by altering culture conditions to reduce cell membrane depolar-

ization. Altered conditions that saved *weaver* granule cells include the NMDA channel blockers APV (Trenkner, 1990) and MK-801 (Kofuji et al., 1996), the voltage-gated Na⁺ channel blocker QX-314 (Kofuji et al., 1996), the Ca²⁺ channel blocker verapamil (Liesi and Wright, 1996), the intracytoplasmic Ca²⁺ chelator BAPTA-AM (Liesi et al., 1997), and ethanol (Liesi et al., 1997), which causes a reduction in Ca²⁺ signaling in response to NMDA in cerebellar granule cells (Gruol and Parsons, 1996; Gruol et al., 1998). Our current results, along with these studies, indicate a critical role for epistatic factors that influence cellular depolarization in effecting *wv/wv* granule cell death.

It is axiomatic that the presence of a mutated *wvGIRK2* channel is also requisite for cell death in the *weaver* mutant mouse. In granule cells GIRK channels are heteromeric tetramers (Liao et al., 1996). In the absence of GIRK2 the other GIRKs should form functional channels. The analysis of GIRK2 null mutant mice supports such a conclusion. In *girk2* knock-out mice there is no apparent cerebellar granule cell death (Signorini et al., 1997). Therefore, the GIRK2 null mutant may not adequately portray the normal contributions of this protein to granule cell

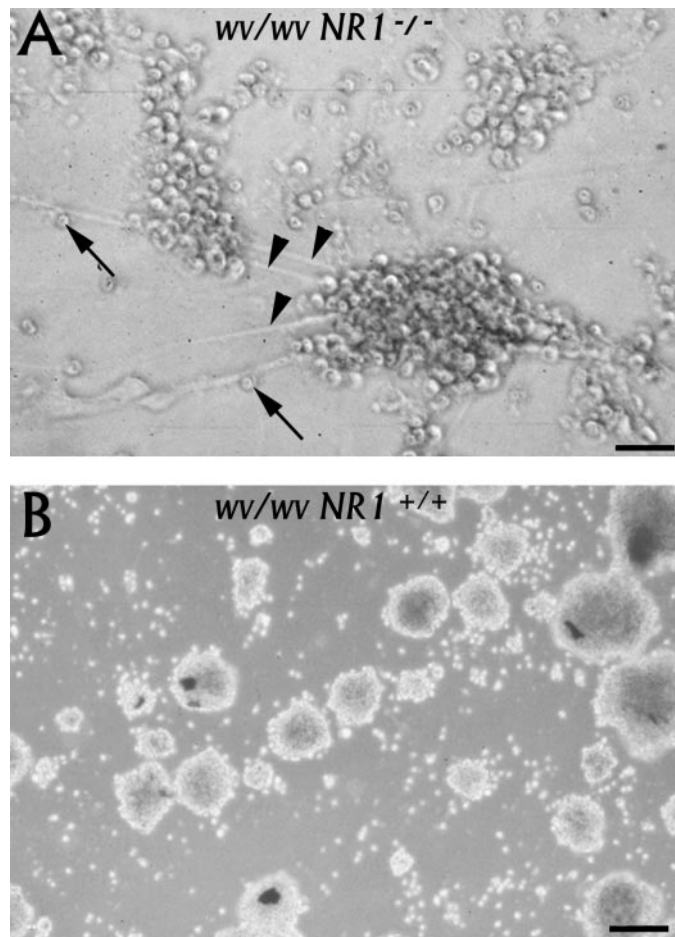


Figure 4. Neonatal *wv/wv NR1* granule cell cultures. P0 granule cell cultures after 8 d *in vitro*, demonstrating granule cell aggregates with numerous cables (arrowheads) and migrating granule cells (arrows) in *wv/wv NR1*^{-/-} cultures (A), whereas *wv/wv NR1*^{+/+} cultures (B) contain only detached clusters of nonviable cells. Scale bars: A, 100 μ m; B, 30 μ m.

development because of compensatory mechanisms that may be activated when the gene is missing. Conversely, the loss of a gene (as in the *GIRK2* knock-out) is not necessarily equivalent to an altered gene product (as in the *weaver* mutation).

An additional point of discussion is that the gain-of-function hypothesis put forward by Kofuji and coworkers (1996) is difficult to sustain. In their gain-of-function hypothesis, the cause of cell death in *wv/wv* is attributable solely to new functions of the *wvGIRK* channel. These new functions are a loss of ionic selectivity and constitutive activity allowing for the passage of sodium and calcium ions and the resulting lethal depolarization of the cell. The ability to block cell death with various pharmacological agents (QX314, MK-801, and verapamil) was interpreted as a direct effect on the mutant *GIRK* channel. However, this may not have been the case. In the present paper we show that the abolition of the NMDA receptors in *wv/wv* granule cells, a perturbation that has no known effect on *GIRK* function, mainly blocks cell death. Although this does not rule out that the mutation in *wvGIRK2* results in a gain-of-channel function or a loss of *GIRK2* currents, it does indicate that *wvGIRK2* alone is not sufficient to cause granule cell death.

A further problem with the gain-of-function hypothesis is its failure to address adequately the selective cell death seen in the

wv/wv brain. One of the continuing puzzles of the *weaver* story is the selective vulnerability of certain neurons (e.g., cerebellar granule cells, dopaminergic cells of the substantia nigra), whereas other *wvGIRK2*-positive neuronal populations, such as the hippocampal granule cells and thalamic neurons, apparently are unscathed. This selective vulnerability also is seen within a single population of neurons. *GIRK2* has been shown to be expressed in all cells of the EGL (Kofuji et al., 1996; Slesinger et al., 1996; Chen et al., 1997; Wei et al., 1997), but only the *wv/wv* midline premigratory EGL cells experience massive degeneration (Herup and Trenkner, 1987; Smeyne and Goldowitz, 1990). For the gain-of-function hypothesis to be valid, all unaffected *wvGIRK2*-expressing cells would require a compensatory mechanism to be in place to oppose the constitutive Na⁺ influx. In the loss-of-function hypothesis, cell death would occur only in cells in which the normal functioning *GIRK2* currents are critical to oppose depolarizing influences.

The activation of *GIRKs* in other neurons of the CNS has been shown to function in controlling cell excitability by hyperpolarizing the cell membrane (Ehrengruber et al., 1997; Svoboda and Lupica, 1998). To understand fully how *GIRK2* functions in regulating cell excitability in the premigratory granule cells, it will be necessary to identify the mechanism of *GIRK2* activation. It is clear from recordings of cerebellar slice preparations that the activation of *GIRK* channels is regulated developmentally (Rossi et al., 1998). Premigratory granule cells of the EGL exhibit G-protein-dependent inward rectifier currents. In *wv/wv* premigratory granule cells, Rossi and coworkers (1998) observed no inwardly rectifying currents. However, in both *wv/wv* and wild-type granule cells in deeper positions, presumed postmigratory granule cells expressed a constitutively active inward rectifier current (Rossi et al., 1998). The molecular identity of the channels underlying this current is unknown at present. These developmental differences in electroresponsiveness of the granule cells may account for the conflicting results obtained from single-cell recordings of *wv/wv* granule cells.

In the developing cerebellum it appears that the activation of NMDA receptors on premigratory granule cells makes this population of neurons more vulnerable to the effects of the *weaver* mutation. During this period, premigratory granule cells transiently express functional NMDA receptors believed to be composed of NR2B and NR1 subunits, which are known to make this cell population more susceptible to excitotoxicity (Garthwaite and Garthwaite, 1986). NMDA receptors composed of both NR2B and NR1 subunits form high conductance channels (Hollmann and Heinemann, 1994). Ca²⁺ entry through these channels could be the trigger for *wv/wv* cell death. The spatiotemporal activation of NMDA receptors appears to coincide with *wv/wv* granule cell death. Although functional NMDA receptors are present on premigratory cells, their activation is tightly regulated by both the density of NMDA receptors and by glutamate uptake (Rossi and Slater, 1993; Farrant et al., 1994). Premigratory neurons display spontaneous NMDA receptor-channel activity that increases with postnatal age. When the uptake of glutamate by glutamate transporters is blocked with the application of L- α -amino adipate, the level of tonic channel activity is greatly enhanced (Rossi and Slater, 1993). Glutamate transporters have been localized to the Bergmann glial fibers (Rothstein et al., 1994). In fact, when these glial cells are ablated, there is massive granule cell death (Delaney et al., 1996). This death is linked to a loss of glutamate uptake and excitotoxicity that is specific to NMDA-type glutamate receptors, because granule cells are saved

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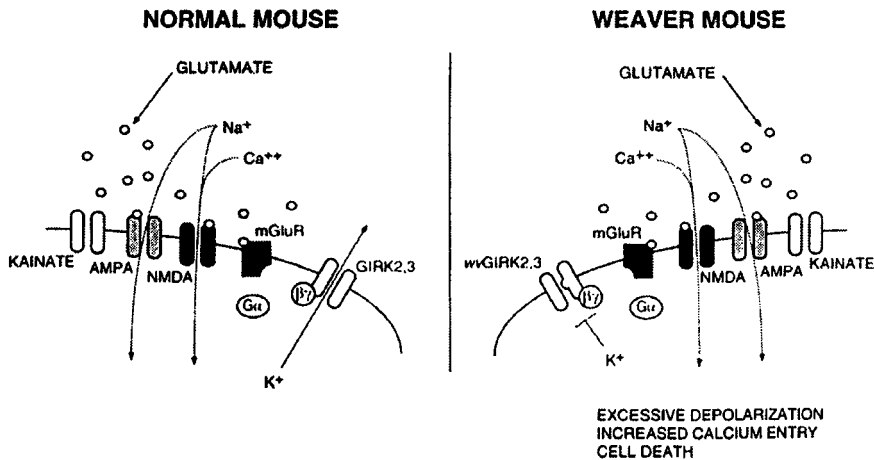


Figure 5. Proposed mechanism of *weaver* cerebellar granule cell death. In the normal mouse cerebellum postmitotic granule cells are exposed to high levels of extracellular glutamate that are believed to, via the activation of NMDA receptors, trigger neuronal migration. This extracellular glutamate also activates metabotropic glutamate receptors (*mGluR*), which leads to GIRK channel function and cellular hyperpolarization. In the *weaver* cerebellum the loss of GIRK currents results in excessive depolarization and granule cell death.

when these transgenic mice are injected with the NMDA receptor antagonist MK-801, whereas the non-NMDA receptor antagonist CNQX has no effect (Delaney et al., 1996). From these studies it is apparent that the activation of NMDA receptors in premigratory granule cells is important to the development of these cells. This well orchestrated activation of NMDA receptors on neurons at the EGL–molecular layer interface may account for the vulnerability of these neurons to cell death in the *weaver* cerebellum.

We propose that, in premigratory granule cells, NMDA receptors are activated in parallel with GIRK2-containing channels. Under normal conditions the activation of K⁺-selective GIRK2-containing channels counterbalances ionotropic glutamate receptor-mediated depolarization, preventing excessive glutamate-mediated depolarization (Fig. 5). Although cerebellar granule cells express several receptors that could couple to GIRK channels, metabotropic glutamate receptors are the most likely to play an important role in this context (Kinney and Slater, 1993). These receptors, by rapidly activating GIRK2-containing channels, may effectively regulate membrane potential and the Mg²⁺ block of NMDA receptors (Hollmann and Heinemann, 1994). In the absence of hyperpolarizing GIRK currents—as in the *weaver* mutant—ambient glutamate may depolarize more effectively the granule cells, leading to relief of the Mg²⁺ block of NMDA channels and activation of voltage-dependent Ca²⁺ channels, providing a fatal Ca²⁺ load. Prolonged Ca²⁺ influx via NMDA receptors has been shown to be a key event in neuronal excitotoxicity (Choi, 1992) and the death of *weaver* granule cells (Tucker et al., 1996) (Fig. 5). This proposal is consistent with either a loss-of-function hypothesis (see Surmeier et al., 1996) or a weaker version of the gain-of-function hypothesis in which the mutation in wvGIRK2 is not sufficient to cause cell death but requires an additional element, such as NMDA receptor activation.

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