

Research report

Involvement of non-NMDA receptors in the rescue of weaver cerebellar granule neurons and sensitivity to ethanol of cerebellar AMPA receptors in oocytes

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Abstract

The cellular mechanism responsible for the death of cerebellar granule neurons in the weaver mutant mouse is still being intensely investigated. To determine if α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors are involved in producing the weaver phenotype or are altered by the weaver gene, we used (1) reverse transcription and polymerase chain reaction (RT-PCR) to detect transcripts of glutamate receptors (GluR1–4) from wild-type and mutant cerebella; (2) immunocytochemistry to establish the types of glutamate receptors present in granule neurons cultured from normal and homozygous weaver postnatal day 5–6 (P5–6) cerebella; (3) 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a blocker of glutamate (AMPA/Kainate/NMDA) receptors, and 6,7-dinitroquinoxaline-2,3-dione (NBQX), a blocker of AMPA and kainate receptors, to assess the number of neurons and the number of neurons with long neurites in cultures of homozygous weaver granule neurons; (4) two-electrode voltage clamp recordings to study AMPA glutamate receptor expression in *Xenopus* oocytes after injection of mRNA isolated from cerebella of normal and weaver P5–6, postnatal day 10 (P10) and postnatal day 23 (P23) mice; and (5) ethanol, which at low 1–10 mM concentrations had been shown previously to rescue homozygous weaver granule neurons in culture [Liesi et al., *J. Neurosci. Res.* 48 (1997) 571–579], to examine its effect on modulation of AMPA receptors expressed from mRNA. By RT-PCR, the mRNA coding for AMPA receptor subunits GluR1–4 were detected from $+/+$ and wv/wv cerebella, and by immunocytochemistry, GluR1, GluR2/3 and GluR4 were observed to be expressed in cultured $+/+$ and wv/wv granule cells. CNQX at 10 μ M or NBQX at 10 μ M significantly increased the number of surviving neurons and the number with long neurites as compared to wv/wv controls. In addition, CNQX was significantly more effective than NBQX. In oocytes injected with mRNA from P10 normal or weaver cerebella, the amplitudes of the responses to kainate were about equal. In contrast, the amplitudes of the kainate-activated currents in oocytes injected with weaver P23 mRNA were about twice as large as the currents observed in oocytes injected with mRNA from normal P23 cerebella, and both were larger than kainate-activated currents observed after injection of P10 normal and weaver mRNA. Kainate-activated AMPA receptor currents in oocytes injected with mRNA from P10 and P23 normal and homozygous weaver cerebella were inhibited by ethanol. There were no significant differences in the inhibition produced by ethanol on currents from P10 or P23 normal and wv/wv mRNA. Thus, P23 weaver cerebellar mRNA expressed more kainate-activated current in oocytes than P23 normal cerebellar mRNA; both normal and weaver cerebellar granule neurons express mRNA coding for functional AMPA receptors that are susceptible to ethanol inhibition. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

In homozygous weaver (wv/wv) mice, a neurological mutation is thought to cause a failure of cerebellar granule cell migration and extensive granule cell death in early postnatal life with severe ataxia as the phenotype [30,31,35]. Identification of a single base pair substitution in the gene encoding for the inwardly rectifying K⁺ channel, GIRK2, as responsible for the weaver phenotype mutation [29,34] explained an abnormal channel function. However, the extensive granule cell death could not be attributed only to the abnormal GIRK2 receptor channel function. Because of the known involvement of glutamate receptor activation in neuronal cell death [2,3], the coupling of GIRK2 channel defect to cerebellar granule cell death by glutamatergic neurons has been suggested in the weaver phenotype [36]. Some studies have shown that the weaver granule neurons express altered NMDA receptor [20,22]. Other studies have proposed that the weaver granule cell defect can be rescued by blocking NMDA receptor function [16] and that substances such as ethanol, verapamil and BAPTA can rescue weaver granule neurons and restore normal neurite outgrowth [23]. We therefore studied AMPA and NMDA receptor channels in *Xenopus* oocytes expressing total RNA isolated from normal and weaver cerebella at postnatal days 5–6, 10 and 23. In addition, we examined whether blocking glutamate receptors with CNQX or NBQX in cultured P5–6 vermal weaver granule neurons would affect the number of surviving granule neurons and the length of their processes. Finally, we investigated the effect of ethanol on AMPA receptor currents expressed in oocytes from total RNA isolated from normal and weaver cerebella.

2. Materials and methods

2.1. Animals

Heterozygous (+/wv) mice carrying the weaver mutation were originally obtained from the Jackson Laboratories (Bar Harbor, ME, USA) and bred by heterozygous matings at the National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health or at the University of Helsinki. The mice were free of MHV, Sendai and other common mouse pathogens. They were fed with a defined AIN-93 diet containing 4000 IU/kg of A vitamin (Harlan Teklad, Madison, WI, USA). The homozygous weaver (wv/wv) and normal control (+/+) littermates were used for experiments at days 4–23 after birth.

2.2. Cell cultures

Neuronal cultures of the vermal granule neurons from P5 to 6 cerebella of the weaver mice and their +/+ littermates were prepared using a previous method [26].

Cells were plated at low density (10⁵ cells) on 25-mm glass coverslips covalently coupled with laminin-1 [20], and cultured in a serum-free RPMI 1640 culture medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 200 μM glutamine and penicillin–streptomycin. The cover slips were pretreated with 10 μg/ml bovine serum albumin (Sigma–Aldrich, St. Louis, MO, USA) for 30 min to saturate free binding sites on the laminin-coated cover slips. The culture medium was changed after 1–2 h and the neurons cultured for 24–48 h in 5% CO₂/95% air at 37°C and fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for quantitation or immunocytochemistry. The granule neurons were identified by L1-antigen immunoreactivity as previously described [22].

2.3. AMPA receptor subunits identified by RT-PCR

For reverse transcription followed by polymerase chain reaction (RT-PCR), total RNA was isolated from the whole cerebellum (*n*=2) or vermis (*n*=1) of P4–6 control and wv/wv mice, and reverse transcribed with oligo-dT and M-MLV reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD, USA) as described previously [21]. For each sample, a control for genomic DNA contamination was included in which the reverse transcriptase was omitted. A portion (200 ng) of each sample of cDNA was amplified by PCR for 30 cycles of 30 s at 92°C, 90 s at 55°C and 60 s at 72°C using the following primer sets: β-actin forward 5'-ACA GCT GAG AGG GAA ATC GTG-3' and reverse 5'-CTA GGA GCC AGG GCA GTA ATC T-3' (expected product 362 bp), GluR1 forward 5'-CAG CGG AGG AAG TGG CAG TG-3' and reverse 5'-GAG GCG GTG TTT CAT TTC TTT GTT-3' (222 bp), GluR2 forward 5'-CAA GGC AAG GCT GTC AAT-3' and reverse 5'-GAT ATC GGA TGC CTC TCA CC-3' (219 bp), GluR3 forward 5'-AAC TCA CAA AGA ACA CCC AAA ACT-3' and reverse 5'-TCA TGC CCG ACA CCA AGG AG-3' (216 bp), and GluR4 forward 5'-AGC AGG CGT CTT CTA CAT TC-3' and reverse 5'-CAC GGC CGT TTT CTC CCA CAC T-3' (176 and 289 bp). The smaller 176 bp GluR4 PCR product, which is missing the 3' end of exon 16 with its stop codon, actually codes for GluR4 with a long C-terminus [40]. The larger 289 bp GluR4 PCR product contains all of exon 16 with its stop codon, and thus codes for the short C-terminal form of the protein [12,40]. PCR products were visualized on 2% agarose gels containing ethidium bromide.

2.4. AMPA receptor immunocytochemistry

Immunoreactivity of wv/wv and +/+ mouse cerebellar granule neurons for AMPA receptor subunits was detected using specific polyclonal antibodies (Chemicon International, Inc., Temecula, CA, USA) against the receptors. Immunocytochemistry was performed as described [22].

To obtain cell-surface labeling, freshly prepared 2% paraformaldehyde was used to fix cells for 15 min. After fixation, the cells were immediately incubated with antibodies and mounted in PBS/glycerol. This protocol allowed cell surface detection of receptor protein.

2.5. Expression of cerebellar mRNA in oocytes

Mouse cerebellar mRNA was isolated from the whole cerebellum of P5–6, P10 and P23 normal and weaver mice using the 5 Prime \Rightarrow 3 Prime commercial mRNA isolation kit (Boulder, CO). These ages were selected for their different stages of granule cell development. At P5–6 there are many granule cells in the external germinal layer and few in the internal granule cell layer. At P10 the external germinal layer is past the peak of granule cell production and numerous granule cells are accumulating in the granule cell layer. Finally, at P23 the external germinal layer is no longer present and all surviving granule neurons have migrated to the internal granule cell layer. The RNA was concentrated to 1–2 μ g/ml and injected into *Xenopus* oocytes at a concentration of 15–20 ng/nl using a microinjector (PV 800 pneumatic picopump; World Precision Instruments, Sarasota, FL, USA).

2.6. Electrophysiology

Oocytes were excised from mature female *Xenopus laevis* frogs (*Xenopus* I, Ann Arbor, MI, USA), and manually separated into clusters of five to ten oocytes. Clusters were placed in a calcium-free modified Barth's solution (MBS) containing (in mM): 88 NaCl, 1 KCl, 2.4 NaHCO₃, 0.8 MgSO₄ and 10 HEPES (pH 7.5). The follicular cell layer was removed by incubating oocyte clusters in two changes of 2 mg/ml MBS solution of collagenase A (Boehringer Mannheim, Indianapolis, IN) with shaking for 1 h each. Healthy stage V and VI oocytes were incubated for 3–7 days at 19–21°C in MBS supplemented with 2 mM sodium pyruvate, 10 000 μ g/l penicillin, 10 mg/l streptomycin, 50 mg/l gentamycin, 0.5 mM theophylline, and 0.9 mM CaCl₂ after injection.

After detecting receptor expression (3–7 days), oocytes were placed in a recording chamber (~100 μ l solution volume) and constantly superfused with MBS (containing 0.9 mM CaCl₂) at a rate of 2.5 ml/min. Whole-cell currents were measured in oocytes, using a standard two-electrode voltage-clamp amplifier (Gene-clamp 500 amplifier, Axon Instruments, Foster City, CA), at a holding potential of –70 mV. Currents were activated by a solution containing 10–400 μ M kainic acid (Research Biochemicals Int., Natick, MA) for AMPA channel detection and 100 μ M NMDA (Sigma–Aldrich, St. Louis, MO, USA) coapplied with 10 μ M glycine (Research Biochemicals International, Natick, MA, USA), for NMDA channel detection. Receptor currents were recorded on a Gould TA11 thermal recorder (Gould Inc., Valley View, OH, USA). All experiments were performed at room tempera-

ture, with agonist and drugs dissolved in MBS and applied by gravity flow-through macropipettes (external diameter: ~1.0 mm) placed ~1 mm from oocyte. When 2 mM barium chloride (BaCl₂) was substituted for calcium chloride (CaCl₂) in the MBS solution, the only change in current recordings was a decrease in magnitude of kainate-activated current in both control and drug applied oocytes (data not shown). Drugs were either coapplied with agonist or preapplied for an indicated time period, with a washout time of about 5 min allowed between successive agonist and/or agonist plus drug applications. The glutamate receptor blocker, CNQX, was co-applied at 10 μ M with 200 μ M kainate.

2.7. Data analysis

Concentration–response relations were fitted with the Hill equation, $y = ((E_{\max} - E_{\min}) / (1 + (x/EC_{50})^{-n})) + E_{\min}$. In the equation, x represents concentration and y is the amplitude of the response. E_{\min} and E_{\max} are the minimal and maximal responses. The EC_{50} is the half-maximal excitatory concentration and n is the slope factor. IC_{50} is the half-maximal inhibitory concentration. In cases when E_{\min} did not significantly differ from zero, E_{\min} was constrained to zero. Data are expressed as mean \pm standard error (S.E.M.) and were tested for statistical significance by using either Allfit [5] for comparing EC_{50} values of concentration–response curves or one-way analysis of variance (ANOVA) for comparing peak amplitudes of concentration–response curves and percent inhibition of kainate-evoked currents by ethanol. Multiple comparisons were made using the Tukey or the Student–Newman–Keuls test.

3. Results

3.1. AMPA glutamate receptor subunit mRNA detected by RT-PCR

As exemplified in Fig. 1, all four GluR subunits were present in +/+ and wv/wv total vermal RNA. Although the primer set used to detect GluR4 mRNA will amplify products coding for both the long (176 bp fragment) and short (289 bp fragment) C-terminal forms of GluR4, only the sequence coding for the long form was detected here. Thus, GluR1–3 and GluR4 long were found in total RNA isolated from P4 to 6 +/+ and wv/wv cerebellar vermis.

3.2. Immunolabeling of AMPA receptor subunits in cerebellar granule neurons

Granule neurons in culture for 1 day on a laminin substrate were fixed and labeled on their surfaces with antibodies to AMPA receptor subunits. From the labeling intensity seen in Fig. 2, the level of receptor expression in both +/+ and wv/wv granule neurons were approximate-

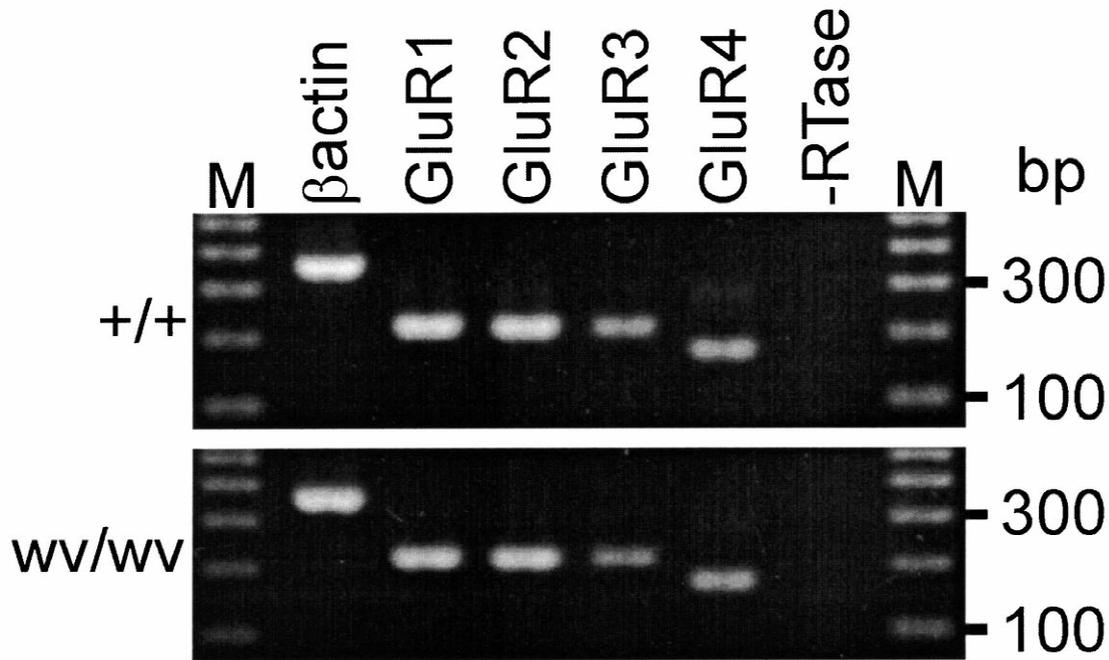


Fig. 1. Detection of mRNA coding for AMPA receptor subunits (GluR1–4) by RT-PCR. P4–6 cerebellar mRNA from $+/+$ and wv/wv mice contained each of the four GluR subunits. Although the primer set for GluR4 detects both the long (expected band size 176 bp) and short (expected band size 289 bp) C-terminal forms of GluR4, only the mRNA coding for the long form was detected here. A primer set to rat β -actin was used as a positive control (expected band size 362 bp) and to detect genomic DNA contamination ($-RTase$ lane, expected band size 450 bp). Contamination by genomic DNA was undetectable. M indicates the DNA size markers (100 bp ladder).

ly equal for the GluR1, GluR2/3 and GluR4 subunits. However, the level of receptor expression between AMPA subunits showed distinct variation, with the strongest labeling intensity obtained with the antibody recognizing both the GluR2 and 3 subunits (Fig. 2). For all three antibodies, granule neurons were labeled uniformly on cell bodies, axons and growth cones. Hence, cultured granule neurons from the cerebellum of normal and weaver mice expressed AMPA receptor subunits GluR1, GluR2 and/or 3 and GluR4.

3.3. Survival and differentiation of weaver vermal granule neurons in culture

P5–6 vermal cultures of the homozygous weaver granule neurons were examined for survival and process outgrowth with and without rescuing agents (Fig. 3). Neuronal survival was assessed by determining the number of neuronal cell bodies and neuronal differentiation was assayed by counting the number of cells with neurites longer than 10 times the soma diameter. Treatment of neurons with 10 μ M CNQX (a blocker of all glutamate receptors) or 10 μ M NBQX (a non-NMDA receptor blocker) significantly increased neuronal survival (open columns in Fig. 3) and the number of weaver neurons with long neurites (closed columns in Fig. 3). Thus, AMPA or kainate or both receptors appear to be involved in differentiation and survival of homozygous weaver granule neurons.

3.4. Two-electrode voltage clamp recordings of AMPA and NMDA receptors in oocytes

The expression of NMDAR2B ($\epsilon 2$) was shown previously to be reduced in weaver as compared to normal cerebellar granule neurons *in vitro* and *in vivo* [20], and functional NMDA and AMPA receptors were undetectable in weaver granule neurons *in vitro* [22]. Since normal and weaver granule neurons in culture exhibited immunolabeling for AMPA receptor subunits, we hypothesized that perhaps weaver mRNA injected into *Xenopus* oocytes would express detectable functional AMPA receptors. Thus, we used two-electrode voltage clamp to study the expression of AMPA and NMDA receptors after injection of mRNA purified from $+/+$ and wv/wv cerebella obtained at postnatal days 5–6, 10 and 23. In the presence of kainate, AMPA receptors desensitize slowly compared to kainate receptors [28]. Accordingly, AMPA receptors were selectively studied by kainate and blocked by CNQX at 10 μ M, and NMDA receptors were detected in the presence of NMDA and the co-agonist glycine.

Kainate-activated currents were detectable in oocytes injected with mRNA from $+/+$ and wv/wv cerebella at P5–6, P10 and P23. The amplitudes of the kainate-activated currents were correlated with the age of the mice with older animals producing larger amplitude currents compared to younger mice. At P5–6 or P10, the amplitudes of the kainate-activated currents were similar for $+/+$ (P5–6 13 ± 3 nA, $n = 5$; P10 KA (Fig. 4 left panel)

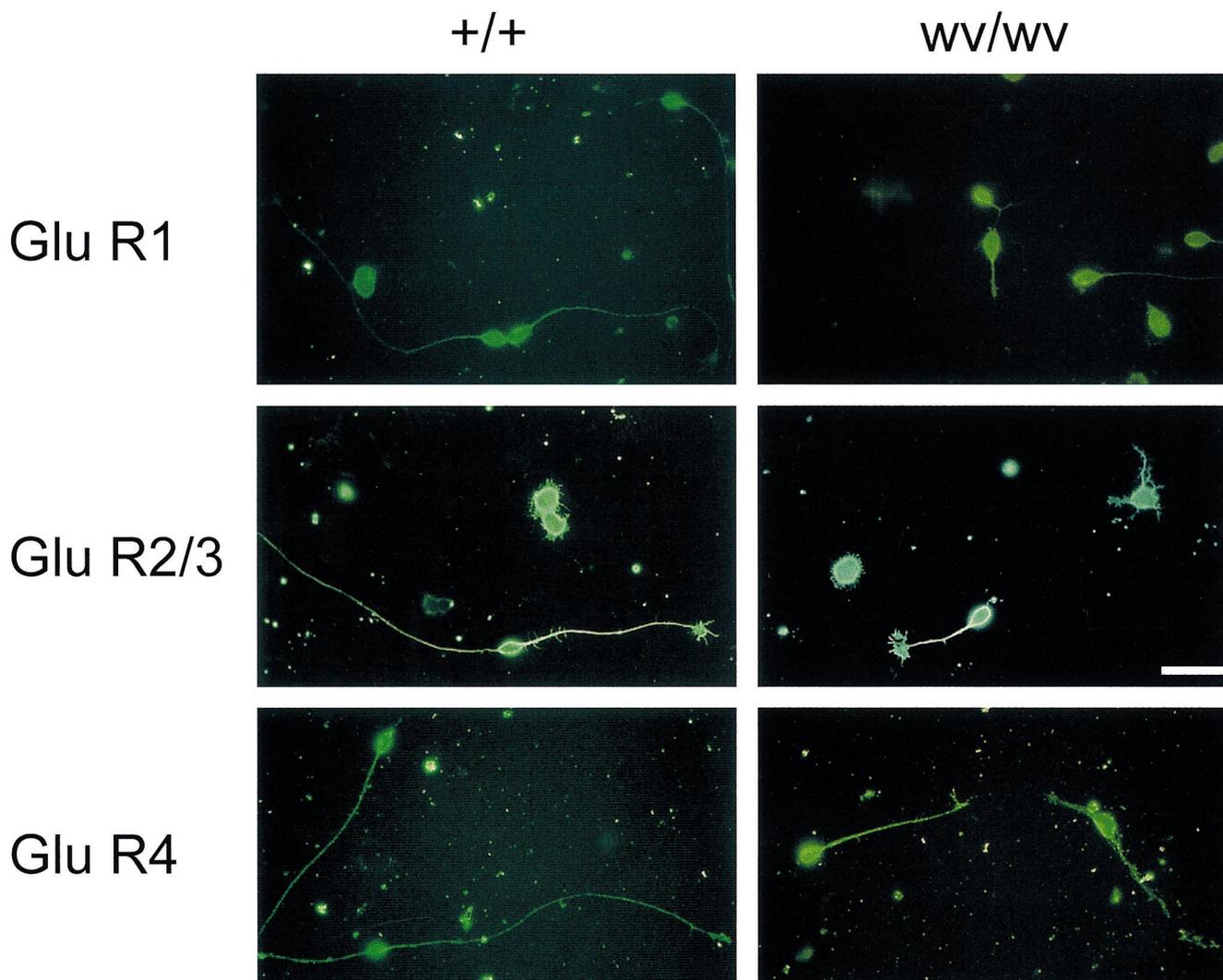


Fig. 2. Immunocytochemical localization of GluR1, GluR2/3 and GluR4 in $+/+$ and wv/wv granule neurons on a laminin substratum 24 h in vitro. Both normal and weaver granule neurons expressed at least GluR1, GluR2 and/or 3 and GluR4. The level of expression between the normal and weaver granule neurons was approximately at the same extent, although levels of expression between the different AMPA receptors showed distinct variations. The strongest labeling intensity was obtained with the antibody recognizing both the GluR2 and 3 subunits. The scale bar in panel GluR2/3 wv/wv equals 20 μm and applies to all panels.

44 ± 4 nA, $n=5$) and wv/wv (P5–6 14 ± 5 nA, $n=5$; P10 KA (Fig. 4 right panel) 40 ± 4 nA, $n=5$) cerebellar mRNA. Interestingly, mRNA from P23 wv/wv cerebella produced kainate-activated currents (P23 KA right panel, 150 ± 10 nA, $n=6$) of nearly twice the magnitude of the $+/+$ mRNA current (P23 KA left panel, 85 ± 5 nA, $n=6$); as shown in Fig. 5, this result was statistically significant and was observed for kainate concentrations from 100 to 400 μM ($n=6$, $P \leq 0.01$, ANOVA). CNQX at 10 μM completely blocked currents activated by 200 μM kainate in oocytes expressing P23 $+/+$ ($n=5$) and P23 wv/wv ($n=5$) mRNA. By comparison, responses to 100 μM NMDA plus 10 μM glycine, which were inhibited selectively by 100 μM APV, were of similar amplitude in oocytes injected with cerebellar mRNA from $+/+$ and

wv/wv mice at P5–6 ($+/+$ 10 ± 5 nA, $n=8$ and wv/wv 12 ± 5 nA, $n=8$), P10 ($+/+$ 35 ± 5 nA, $n=8$ and wv/wv 31 ± 4 nA, $n=8$) or P23 ($+/+$ 70 ± 3 nA, $n=8$ and wv/wv 60 ± 5 nA, $n=8$; second and fourth set of traces in Fig. 4). Thus, P23 responses to NMDA/glycine were significantly larger than those at P10, and P10 responses were significantly greater than at P5–6 ($P < 0.05$, ANOVA).

3.5. Ethanol inhibition of $+/+$ and wv/wv AMPA receptor currents expressed in oocytes

As shown in Fig. 6, 100 mM ethanol inhibited AMPA receptor currents activated by 200 μM kainate in oocytes expressing P10 or P23 $+/+$ and wv/wv mouse mRNA.

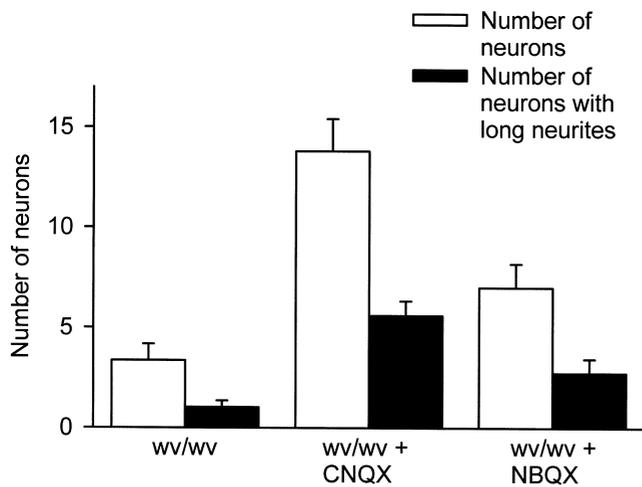


Fig. 3. Quantitation of neuronal survival (open columns) and outgrowth of long neurites ($>100 \mu\text{m}$; closed columns) in vermal cultures of the homozygous weaver granule neurons wv/wv in the presence or absence of test reagents. The cultures were initiated from the vermis of P5–6 cerebella and maintained for 24 h. The error bars indicate S.E.M. The statistical analysis of the results was performed using one-way analysis of variance (ANOVA). The individual groups were compared using Student–Newman–Keuls multiple comparisons test. The results indicate that the numbers of cell bodies (open columns) in CNQX- ($P < 0.001$) and NBQX- ($P < 0.05$) treated cultures were significantly higher than those in the untreated weaver cultures. CNQX was significantly more effective than NBQX at increasing the numbers of surviving neurons ($P < 0.001$). Neurite outgrowth of the untreated control weaver neurons (closed columns) was significantly impaired as compared to the CNQX- ($P < 0.001$) and NBQX- ($P < 0.05$) treated weaver neurons. CNQX was significantly more effective than NBQX at increasing the numbers of neurons with long neurites ($P < 0.001$).

There was no significant difference in the sensitivities of AMPA receptor currents to 100 mM ethanol in oocytes injected with P10 or P23 $+/+$ or wv/wv mRNA. As illustrated in Fig. 7A and B, the percent inhibition by ethanol of AMPA receptor currents increased as the concentration of ethanol was increased.

4. Discussion

In this study, we presented evidence for the presence of AMPA receptors in normal and weaver cerebellar granule neurons. Both normal and weaver granule neurons expressed GluR1, GluR2 and/or 3 and GluR4 as shown by immunolabeling of cultured granule neurons. Our RT-PCR results indicated that mRNA coding for GluR1–4 was present, but only the long C-terminal form of GluR4 was detected. The presence of mRNA for GluR3 suggested that GluR3 protein is likely to be present in granule neurons. Expression of both GluR2 and 3 in cultured granule neurons may explain the greater intensity of antibody labeling compared to GluR1 and GluR4 labeling. In contrast to the long forms of GluR2 and 4, the short forms are known to interact with binding proteins, such as PICK1

(Protein Interacting with C Kinase type 1), which associates with protein kinase C α and contains a receptor binding protein motif, the PDZ domain, for localizing AMPA receptors to subcellular sites [6]. However, only the mRNA coding for the long form of GluR4 was detectable in both $+/+$ and wv/wv cerebella, corroborating the absence of AMPA receptor clustering observed by immunolabeling, as the staining was evenly distributed over the surfaces of both $+/+$ and wv/wv granule neurons.

As shown previously, verapamil — a calcium channel blocker — rescued weaver granule neurons from cell death [20,22]; CNQX and NBQX were also able to rescue weaver granule neurons. In addition, both CNQX and NBQX were able to increase the number of granule neurons with long processes. As these cultures were from the vermis — the region of the cerebellum most affected by the weaver mutation — these results suggest that non-NMDA receptors may have an influence on survival and process outgrowth perhaps by stimulation of Lyn protein tyrosine kinase mediated by GluR2 channels, a process that does not require channel activity [15].

AMPA receptors were recorded by two-electrode voltage clamp in oocytes after injection of mRNA isolated from the cerebella of $+/+$ and wv/wv mice. At P5–6 or P10, the kainate-activated currents were similar in peak amplitude. However, at P23 the wv/wv mRNA produced kainate-activated currents that were approximately twice the amplitude of the currents recorded from P23 $+/+$ mRNA. The EC_{50} values of 111 (wv/wv) and 100 μM ($+/+$) were similar and within the range reported previously (98 μM [38] and 130 μM [27]). Finally, both $+/+$ and wv/wv mRNA produced AMPA receptor currents that were inhibited by ethanol at concentrations ranging from 10 to 300 mM.

The involvement of NMDA receptors in the death of weaver granule cells was proposed based on rescue of cultured granule neurons by NMDA receptor channel blockers, such as MK-801 [18,19,37]. However, the high concentrations of MK-801 used also partially blocked L-type calcium channels [11,22]. Indeed, low concentrations of MK-801, which inhibit NMDA receptors selectively or of another NMDA receptor antagonist — D(-)-2-amino-5-phosphonopentanoic acid (D-AP5), were ineffective at rescuing weaver granule neurons [22]. Blockade of calcium channels by verapamil [22], chelation of Ca^{2+} by BAPTA-AM [23] or exposure to low concentrations of ethanol [23] have all been shown to rescue weaver cerebellar granule neurons from cell death. The reduced subunit expression and function of the NMDA receptor in weaver neurons [20] may result from elevated levels of intracellular calcium [4,14,39] or an increased sensitivity to calcium [23]. In other experiments, crossing NMDA receptor 1 subunit knock-out mice with mice containing the GIRK2 mutation was shown to rescue cerebellar granule cells in vivo and in vitro [16] presumably by decreasing calcium entry through NMDA receptors; and

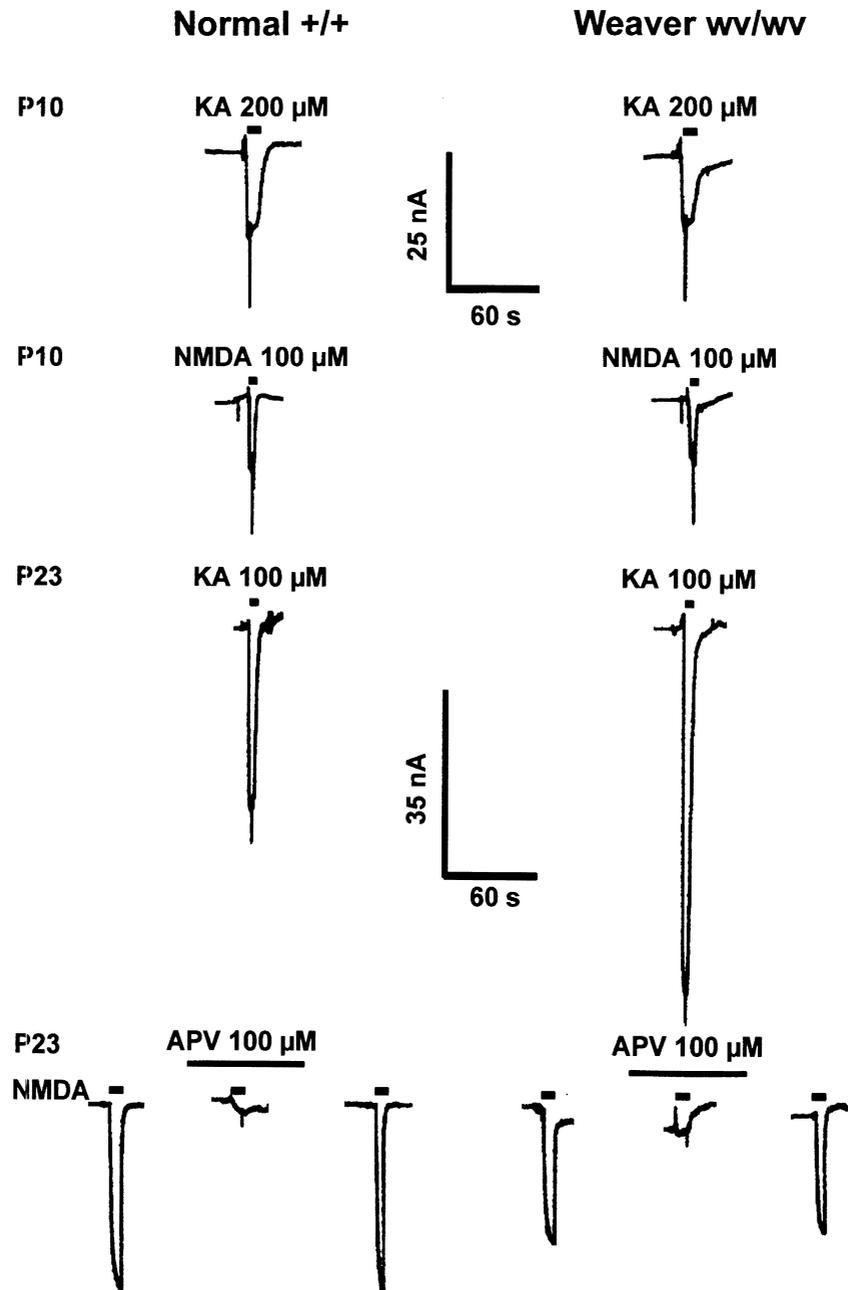


Fig. 4. Kainate- and NMDA-activated currents elicited from *Xenopus* oocytes injected with normal (+/+) and weaver (wv/wv) cerebellar mRNA. Currents were activated by 200 μ M kainate (for AMPA receptor) or 100 μ M NMDA and 10 μ M glycine (for NMDA receptor) in oocytes expressing postnatal day 23 (P23) or P10 +/+ or wv/wv mRNA. The bars above current records represent the time duration of agonist (kainate or NMDA) and antagonist (APV) application while the vertical and horizontal bars indicate magnitude and duration of their application.

transplantation of wv/wv granule neurons into normal cerebellum rescued the weaver cells and allowed their differentiation [13] suggesting that cell–cell interactions can negate the effects of the weaver mutation. Clearly, the mechanisms controlling the migration and survival of weaver granule neurons need to be studied further. In particular, as the present oocyte data indicate that functional NMDA receptors were recovered by injecting the oocytes with total RNA from P10 and P23 weaver cerebella, although the weaver cerebellar granule neurons

had no functional NMDA receptors on a laminin-1 substratum [22]. It is possible that the NMDA receptors cannot function in the weaver granule neurons due to impaired cytoskeletal links. In oocytes, the cytoskeletal links could form normally and expression would be allowed.

The neurotransmitter, glutamate, participates in excitatory neurotransmission [17,33]. Perhaps the increased AMPA receptor activity observed after injection of P23 wv/wv mRNA is a compensatory effect resulting from the

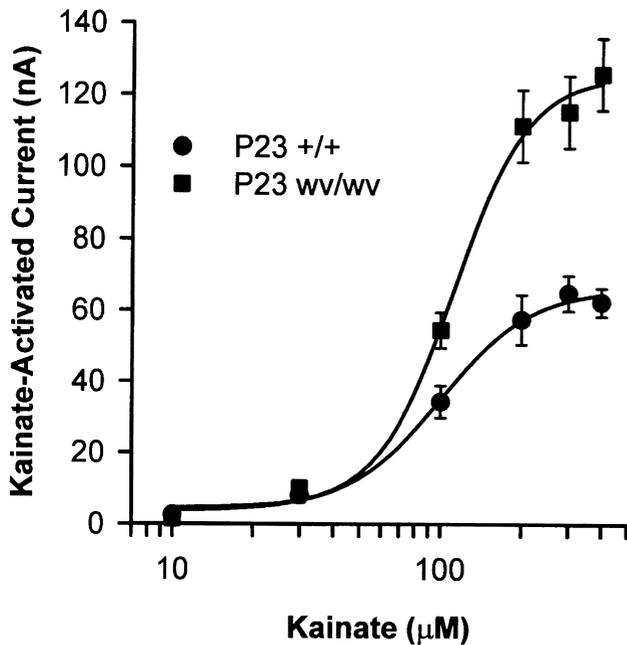


Fig. 5. Kainate-activated currents from cultured normal postnatal day 23 (P23 +/+) and P23 wv/wv cerebellar mRNA. Peak amplitudes of kainate-activated currents were plotted against increasing kainate concentrations to measure agonist sensitivity of normal and weaver cells. Each point is the mean \pm S.E. of six to 12 oocytes. Error bars not visible are smaller than the symbols. The curves were fitted with the Hill equation and then compared statistically using Allfit [5]. The EC_{50} values of 111 μ M for P23 wv/wv and 100 μ M for P23 +/+ were not significantly different ($P > 0.05$).

increased synthesis of AMPA receptors in the lateral part of the cerebellum, which is less affected by the weaver mutation. Alternatively, it may be due to reduced expression of functional NMDA receptors in the weaver neurons. There were no differences in AMPA receptor activity observed for P10 normal and weaver cerebellar mRNA, which may partially result from using mRNA from the whole cerebellum. Evidence for non-NMDA receptor activity was demonstrated by the significant increase in survival and neurite outgrowth observed for weaver cells in culture, when NBQX — a non-NMDA receptor channel blocker — or CNQX — a blocker of NMDA and non-NMDA glutamate receptors — was added to the culture medium. NMDA receptor expression and function is reduced or absent in cerebellar granule neurons in the weaver mutant mouse; however, rescued weaver cerebellar granule neurons begin to express NMDA receptors indicating that NMDA receptors may play no significant role in the death of weaver granule neurons [20,22]. As normal cerebellar granule neurons express functional NMDA receptors in vivo [10,32], and NMDA receptor antagonists are known to inhibit the late stages of neuronal migration in the rat cerebellum in vitro [19], it is possible that NMDA receptor function is down regulated as a protective measure to reduce the calcium load of the cells [20]. A role

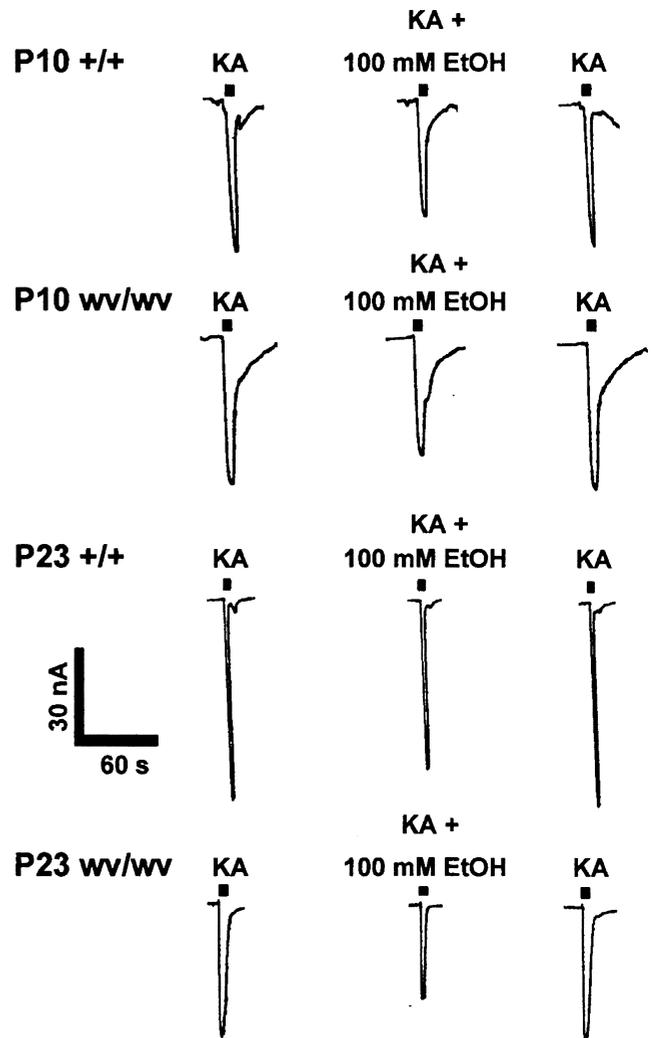


Fig. 6. Ethanol inhibition of normal +/+ and weaver wv/wv AMPA receptor currents. Current traces of ethanol inhibition of kainate-activated currents in *Xenopus* oocytes injected with P10 +/+ and wv/wv or P23 +/+ and wv/wv mRNA. Each set of traces show the effect of 100 mM ethanol on currents activated by 200 μ M kainate in oocytes. Only the P23 wv/wv currents were more sensitive to ethanol inhibition than the P23 +/+ currents. The horizontal bars above the traces represent the time duration of kainate and/or ethanol application, while the calibration bars to the left of the traces represent the magnitude and duration.

for the direct involvement of non-NMDA receptors in the death of weaver granule neurons was shown by their rescue by NBQX.

Previous studies have shown a low sensitivity to ethanol inhibition of AMPA receptor currents from cultured rat cortical [24] and cerebellar neurons [8]. In addition, ethanol is known to inhibit the AMPA receptor non-competitively at a location different from the agonist-binding site [1,7,9]. In this study, AMPA receptor currents expressed from mouse cerebellar mRNA also had a low sensitivity to ethanol inhibition, with a maximal inhibition by 300 mM ethanol of 45%. The effects of ethanol on

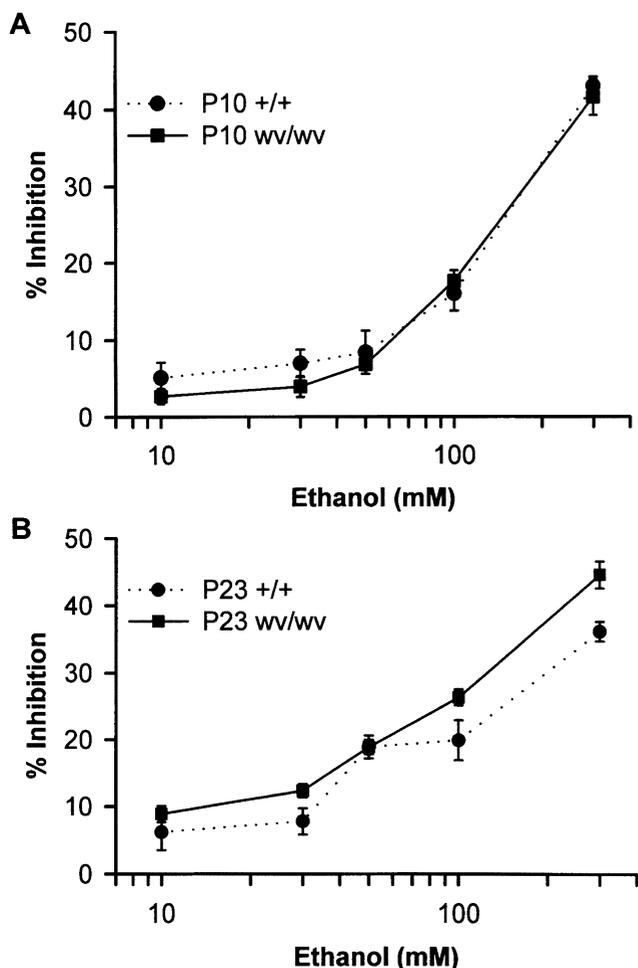


Fig. 7. Percent inhibition of AMPA receptor currents by increasing concentrations of ethanol. (A). Sensitivities to ethanol inhibition of the AMPA receptor responses from P10 +/+ (●) and P10 wv/wv (■) cerebellar mRNA were not significantly different at all ethanol concentrations (Tukey multiple comparisons test). (B). Sensitivities of the P23 +/+ (●) and wv/wv (■) kainate-activated currents to ethanol inhibition were not significantly different at all ethanol concentrations (Tukey multiple comparisons test). Dotted lines in A and B connect each +/+ (●) data point and continuous lines join each wv/wv (■) point.

survival and differentiation of normal and weaver granule neurons in culture are opposite. In normal granule neurons, process outgrowth in the presence of ethanol was inhibited in a concentration-dependent manner with an IC_{50} of 28 mM. In addition, 50 mM ethanol significantly increased cell death by apoptosis. Weaver granule neurons, on the other hand, were protected from cell death by low concentrations of ethanol (1–10 mM, [23]), presumably by inhibiting L-type calcium channels [25]. We found that ethanol inhibited kainate-activated currents in oocytes expressing normal or weaver mRNA in a concentration-dependent manner. However, ethanol at 10 mM only inhibited kainate-activated currents from 2 to 8% suggesting that AMPA receptors are not involved in the rescue of weaver granule neurons by ethanol.

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