Heteromeric channel formation and Ca^{2+}-free media reduce the toxic effect of the *weaver* K_{ir} 3.2 allele

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Abstract ***weaver*** **mice have a severe hypoplasia of the cerebellum with an almost complete loss of the midline granule cells. Recent genetic studies of *weaver* mice have identified a mutation resulting in an amino acid substitution (G156S) in the pore of the inwardly rectifying potassium channel subunit K_{ir} 3.2. When expressed in *Xenopus* oocytes the *weaver* mutation alters channel selectivity from a potassium-selective to a nonspecific cation-selective pore. In this study we confirm by cell-attached patch-clamp recording that the mutation produces a non-selective cation channel. We also demonstrate that the cell death induced by *weaver* expression may be prevented by elimination of calcium from the extracellular solution as well as by coexpression with the wild-type K_{ir} 3.2 allele, or other members of the K_{ir} 3.0 subfamily. These results suggest that the *weaver* defect in K_{ir} 3.2 may cause cerebellar cell death by cell swelling and calcium overload. Cells which express the *weaver* subunit, but which normally survive, may do so because of heteromeric subunit assembly with wild-type subunits of the K_{ir} 3.0 subfamily.**

Key words: *Weaver*; Inwardly-rectifying potassium channel; Cell death; Heteropolymerisation

1. Introduction

The principal phenotype of the *weaver* mouse (*wv/wv*) is an ataxic gait which results from a severe hypoplasia of the cerebellum, including an almost total loss of the internal granule layer (IGL) [1,2]. Although initially believed to be a defect in glial-guided neuronal migration, it has more recently been shown that *wv* granule cells die in the proliferative zone prior to migration. Heterozygous animals (wt/*wv*) do not demonstrate ataxia but develop a significantly smaller cerebellum than wild-type (wt/wt) animals.

The *weaver* gene has been localised and a point mutation, G953A, identified in the *weaver* gene which encodes a G-protein coupled inward rectifier potassium channel subunit, K_{ir} 3.2 [3–5]. This mutation results in a single amino acid substitution, G156S, in the putative pore region of the channel.

In situ hybridization studies of K_{ir} 3.2 mRNA expression in wild-type adult mice found that K_{ir} 3.2 mRNA is expressed in cerebellar granule cells and substantia nigra, areas affected in *weaver* mice. K_{ir} 3.2 mRNA was also detected in hippocampus, pontine nuclei, olfactory bulb, cerebral cortex, septum and amygdala, areas not obviously affected in *weaver* animals [6]. Therefore, mechanisms must exist among cerebellar granule cells and other cell types which distinguish the potentially debilitating effects of *weaver* expression.

Previous structure-function studies of cloned voltage-dependent potassium channels have shown that a conserved GYG sequence in the pore region endows the channel with potassium selectivity [7]. In agreement with this, recent studies have shown that when expressed in *Xenopus* oocytes the *wv* allele produces nonselective cation channels [8], suggesting that *wv/wv* cerebellar cells die because of collapse of the Na^+ and K^+ gradient leading to cell depolarisation.

In this study we confirm by cell-attached patch recording that the *wv* K_{ir} 3.2 allele produces a non-selective cation channel when expressed in *Xenopus* oocytes and that the higher current amplitudes observed at the macroscopic level are the likely result of increased channel activity. We also demonstrate that the cell death observed upon expression of high levels of the *wv* channel may be reduced by removal of calcium from the extracellular medium. Additionally, the toxic effect of the *wv* allele can be reduced by heteropolymerisation with all other members of the K_{ir} 3.0 family. This suggests that cell types which express the *wv* allele but survive, do so because of heteromeric channel assembly with wild-type K_{ir} 3.2 or other subunits of the K_{ir} 3.0 subfamily.

2. Materials and methods

2.1. Electrophysiology

2.1.1. Two-electrode voltage-clamp *Xenopus laevis* oocytes were prepared as previously described [14] and were maintained in ND96 unless otherwise stated (in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$ 10 HEPES (pH 7.4) and supplemented with 100 μg/ml gentamycin, 0.5 mM theophylline, and 5 mM pyruvate). Standard recording solution contained 90 mM KCl, 3 mM $MgCl_2$, 10 mM HEPES (pH 7.4) unless otherwise stated. Microelectrodes were filled with 3 M KCl and had resistances of 0.1–0.5 MΩ. Recordings were performed at 22°C, 24–36 h after injection. Currents were evoked by voltage commands from a holding potential of −5 mV, delivered in −10 mV increments from 40 mV to −100 mV, unless otherwise stated. Values for the average whole cell current were obtained by measuring the steady-state current at −100 mV. All data are presented as the mean ± S.E.M. for groups of at least six oocytes (actual numbers used in each group are indicated above the bars). For the oocyte viability studies the 'high K, low Cl depolarising solution' consisted of 9 mM NaCl, 27 mM KCl, 65 mM K-gluconate, 1 mM $MgCl_2$, 10 mM HEPES pH 7.4 (with KOH) with or without 1.8 mM $CaCl_2$.

2.1.2. Patch-clamp Oocytes were maintained in modified Barth's solution containing (in mM) 88 NaCl, 1 KCl, 1.7 $MgSO_4$, 0.47 $CaCl_2$, 2.4 $NaHCO_3$, 10 HEPES (pH 7.4), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 5 mM pyruvate. Currents were studied 1–5 days after injection. Single-channel currents were recorded from cell-attached membrane patches using an EPC-7 amplifier (List Electroniks). Patch electrodes had resistances between 1 and 6 MΩ

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when filled with pipet solution. The pipette solution contained (in mM) 140 KCl, 1.0 $MgCl_2$, 0.1 $GdCl_2$, 10 HEPES (pH 7.4 with KOH). In some experiments KCl was replaced with equimolar concentrations of NaCl (pH adjusted with NaOH). The extracellular solution contained (in mM) 140 KCl, 1.0 $MgCl_2$, 1.0 EGTA, 10 HEPES (pH 7.4 with KOH). Continuous data recordings were stored on VCR tape. For subsequent analysis data were filtered at 1 kHz by an 8-pole Bessel filter and sampled at 5 kHz using a Digidata 1200 A/D converter (Axon Instruments) and stored on computer. All recordings were performed at room temperature (18–24°C).

2.2. Molecular biology

All channel subunits were subcloned into the oocyte expression vector pBF (provided by Dr. B. Fakler) which provides 5′ and 3′ untranslated regions from the *Xenopus* β-globin gene flanking a polylinker containing multiple restriction sites. In vitro mRNAs were generated and evaluated as previously described [14]. For coexpression experiments with cloned G-$\beta_1\gamma_2$ subunits (generous gift of Drs. Paul Slesinger and Lily Jan), 5 ng of each mRNA were injected. K_{ir} 3.3 was generously provided by Dr. Henry Lester. The *wv* mutation was introduced into the coding sequence of K_{ir} 3.2 by oligonucleotide directed mutagenesis using the Altered Sites method (Promega), and confirmed by DNA sequence analysis.

2.3. Antibody production, membrane preparation, and western blot analysis

A synthetic peptide representing the C-terminal 20 amino acids of K_{ir} 3.2 was coupled to KLH and used as immunogen in rabbits. Bleeds were assayed by western blot analyses of in vitro translated K_{ir} 3.2 (Promega TNT-coupled lysate system). Total oocyte membranes were prepared for western blot analysis as described previously [14] and probed using the antibody described above.

3. Results

3.1. Cell-attached recordings show that weaver K_{ir} 3.2 produces a non-selective cation channel

Expression of *weaver* K_{ir} 3.2 (*wv*) in *Xenopus* oocytes results in macroscopic currents which differ from wild-type K_{ir} 3.2 (wt) in being both larger and non-selective to cations [8] (Fig. 1A,B). We used western blot analysis to determine whether the greater amplitude of *wv* K_{ir} 3.2 currents is due to an increase in the amount of protein expressed. As shown in Fig. 1C, approximately equal amounts of the wt and *wv* protein were detected in total oocyte membrane preparations. This suggests that the increased *wv* K_{ir} 3.2 currents result from an increase in either the single-channel conductance or open probability. We therefore compared single-channel currents recorded from cell-attached patches on oocytes injected with wt K_{ir} 3.2 or *wv* K_{ir} 3.2 mRNAs. With 140 mM K^+ in the external (pipette) solution, wt channels displayed very brief, flickery openings which could not be clearly resolved at negative membrane potentials ($n = 5$) [3,9] (Fig. 2, top left). No channel openings were observed at positive potentials. When external K^+ was replaced by Na^+, no channel openings were observed ($n = 5$) (Fig. 2, bottom left).

Oocytes injected with *wv* K_{ir} 3.2 mRNA displayed prominent channel activity at negative membrane potentials, which was much greater than that found for wt-injected oocytes ($n = 5$) (Fig. 2, right). Although single-channel openings could not be completely resolved, it is evident from the records that there is no marked difference in current amplitude between wt and *wv* channels. Consistent with previously reported macroscopic studies [8], significant channel activity was observed in cell-attached patches on *wv*-injected oocytes when Na^+ was substituted for external K^+ ($n = 5$) (Fig. 2, lower right).

3.2. Expression of wv K_{ir} 3.2 results in cell death which can be prevented by removal of calcium

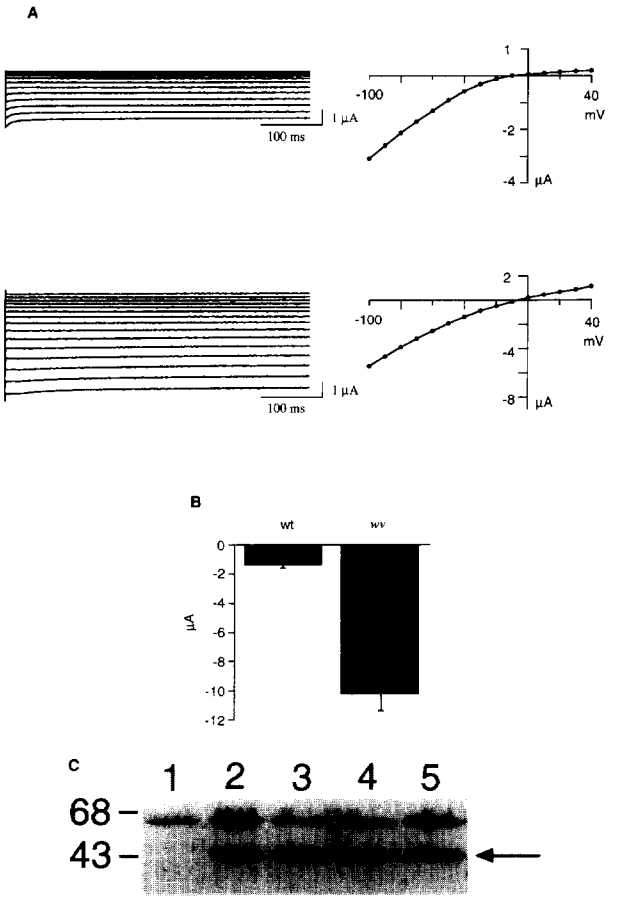


Fig. 1. (A) Current family evoked from an oocyte expressing the wt allele of K_{ir} 3.2 (top) or the *wv* allele (bottom). Steady state I–V relationships are shown on the right. (B) Histogram analysis of the steady-state current amplitudes at −100 mV from oocytes expressing equal amounts of wt or*wv* K_{ir} 3.2. (C) Western blot analysis of membrane proteins from non-injected oocytes (lane 1), oocytes expressing wt (lane 2), *wv* (lane 3), wt+K_{ir} 3.1 (1:5 ratio) (lane 4), or *wv*+K_{ir} 3.1 (1:5 ratio) (lane 5). Oocytes were injected with 2 ng of either wt or *wv* ± 10 ng K_{ir} 3.1 mRNA. The arrow identifies the predicted size of K_{ir} 3.2 subunits.

As reported previously [8], oocytes expressing *wv* begin to lose viability within 24 h of mRNA injection whilst those expressing wt remain viable for many days. Table 1 shows that the loss of viability after injection of *wv* mRNA is dose-dependent while injection of oocytes with the highest amount of wt mRNA does not compromise viability. Slesinger et al. [8] suggest that oocyte death results from chronic depolarisation produced by the large non-selective cation conductance. In agreement with this we found the mean resting membrane potential of oocytes expressing *wv* was -10 ± 0.3 mV ($n = 10$) in 2 mM external K^+, significantly more depolarised than found for oocytes expressing wt (-45 ± 0.7 mV; $n = 10$).

To explore further the cause of cell death, ionic substitutions were performed. As shown in Table 1 removal of Ca^{2+} from the extracellular medium prevents oocyte death in ND96. This suggests that the reduced viability induced by *wv* expression may result from an increased Ca^{2+} influx as a consequence of cell depolarization. To test this hypothesis, the

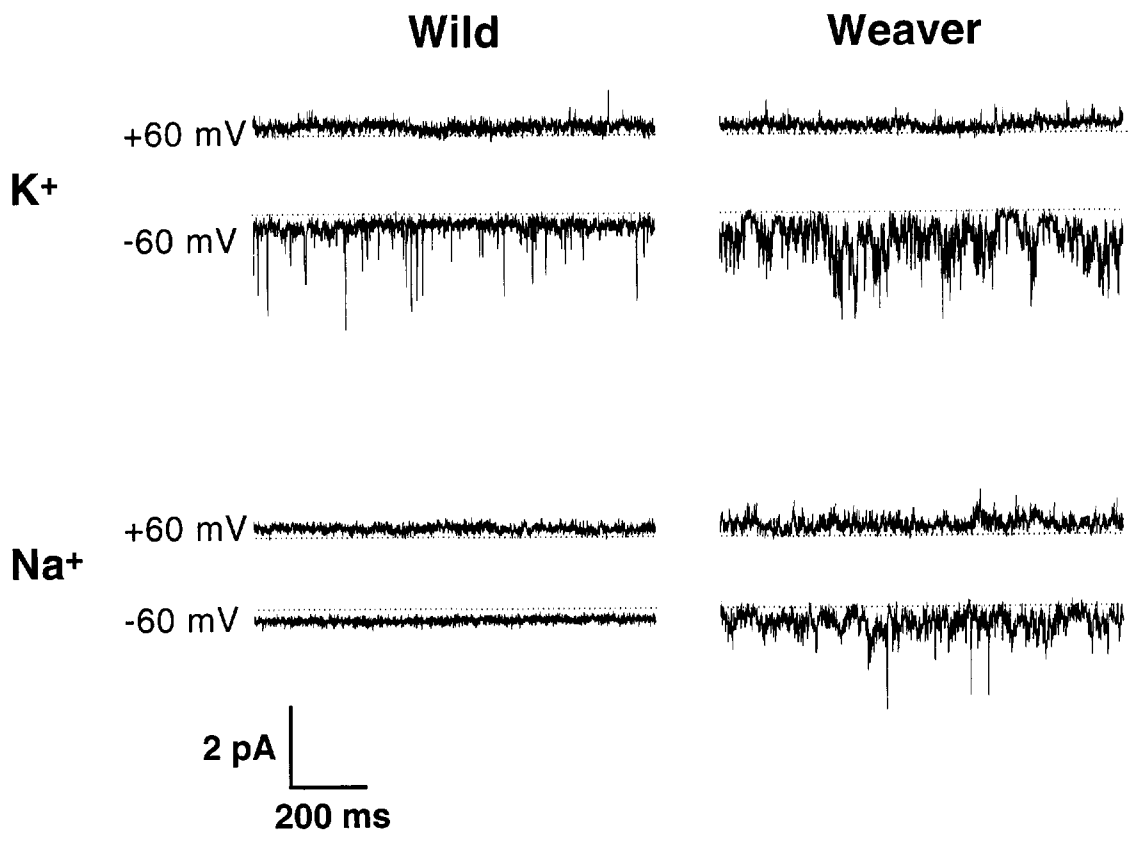


Fig. 2. Single-channel recordings. Currents recorded from cell-attached membrane patches on oocytes injected with wt (left) or *wv* (right) mRNA. Top panels show channel activity when the pipet solution contained 140 mM potassium. At −60 mV, wt channels showed rapid flickers with no clearly resolved openings (top left). *wv* channels showed more robust channel activity, at −60 mV the inward currents were again very flickery with no clear openings (top right). When the K^+ in the patch pipet solution was replaced by Na^+, no currents were detected in patches on oocytes expressing wt channels at membrane potentials between −60 and 60 mV (bottom left), while significant channel activity was observed in patches on oocytes expressing *wv* (bottom right).

viability of *wv*-injected oocytes was determined. Oocytes were maintained in solutions of either ND96 or a high K^+, low Cl^- depolarising solution in the presence or absence of 1.8 mM Ca^{2+}. The resting membrane potential was approx. 0 mV in the depolarising solution and −10 mV for the others. As shown in Table 1, *wv*-expressing oocytes remained viable for as long as controls when Ca^{2+} was eliminated from the ND96 extracellular solution. However, *wv*-expressing oocytes also remained viable in the depolarising solution, regardless of whether Ca^{2+} was present (not shown).

3.3. Coexpression of $K_{ir}3.0$ and *wv* subunits reduces current amplitudes and preserves cell viability

Mice which are heterozygous at the K_{ir} 3.2 locus may be expected to produce equal amounts of wt and *wv* mRNAs, suggesting that heteromeric channels form between wt and *wv* subunits. We therefore examined the effects of coexpression of wt K_{ir} 3.2 with *wv* K_{ir} 3.2. Fig. 3A shows that wt K_{ir} 3.2 mRNA produced a mRNA ratio-dependent decrease in the amplitude of *wv* macroscopic currents. When maintained in ND96 oocytes coinjected in a 1:1 ratio remained viable for longer than oocytes injected with an equivalent amount of *wv* K_{ir} 3.2 mRNA alone (Table 1) and the remaining reduced currents could be carried by Na^+ as well as K^+ (not shown).

Coexpression of K_{ir} 3.2 and K_{ir} 3.1 produces large potentiated G-protein stimulated currents when compared to expression of either subunit alone [10], an effect of heteromeric channel assembly. However, no potentiated currents are observed upon coexpression of *wv* K_{ir} 3.2 with K_{ir} 3.1 and the currents are not significantly increased upon G-protein stimulation [8]. This suggests that heteromeric *wv*/K_{ir} 3.1 channels are non-functional.

To examine this further we investigated the effect of coexpression of other members of the K_{ir} 3.0 subfamily with *wv* K_{ir} 3.2. As previously reported [8], we found that coexpression of *wv* K_{ir} 3.2 with K_{ir} 3.1 (1:5 ratio) did not produce large potentiated currents as seen with coexpression of wt K_{ir} 3.2 with K_{ir} 3.1 in the same ratio (Fig. 3B) and that coexpression with G-$\beta_1\gamma_2$ failed to enhance this reduced channel activity (not shown). However, we also found that coexpression of *wv* K_{ir} 3.2 and K_{ir} 3.3, or 3.4 (1:5 ratio) resulted in markedly reduced current amplitudes (Fig. 3B) and increased oocyte viability compared to expression of *wv* alone.

4. Discussion

The results presented here confirm that the *weaver* mutation in K_{ir} 3.2 reduces the K^+ selectivity of homomeric *wv* channels, allowing significant permeability to other monovalent cations such as Na^+. The results also suggest that the increased macroscopic currents observed for oocytes expressing *wv* K_{ir} 3.2 are due to an increased open probability of these channels rather than a substantial increase in the single-channel conductance or number of channels.

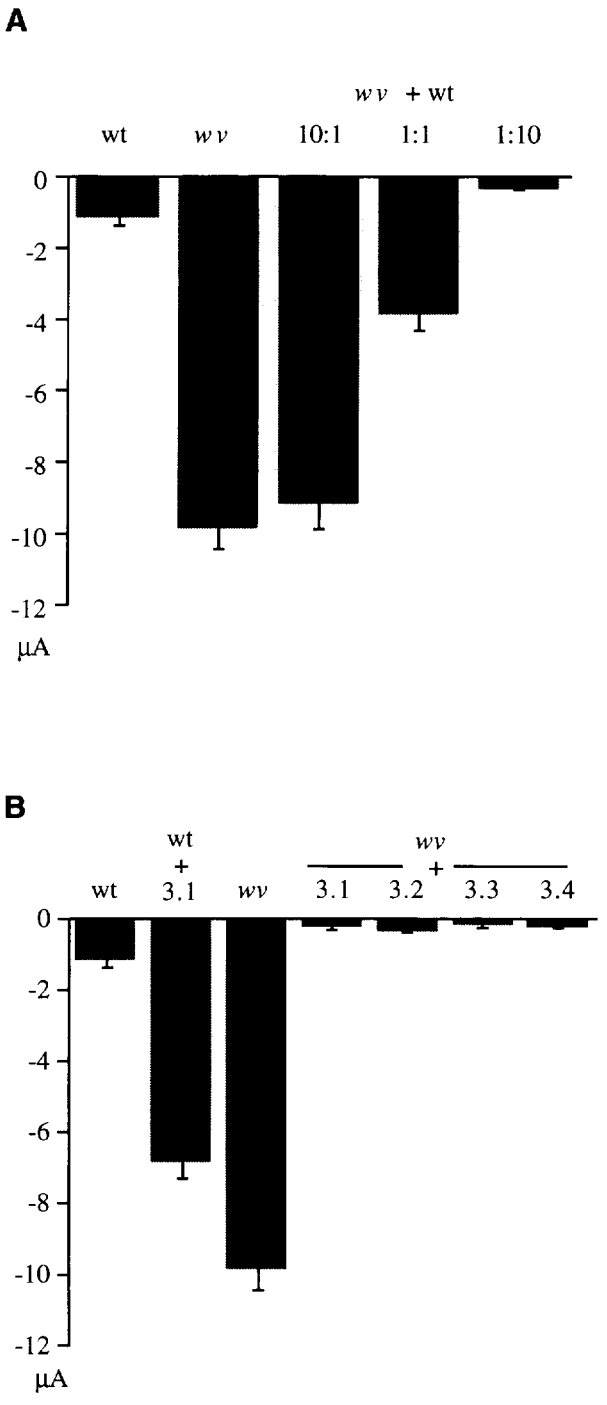


Fig. 3. Coexpression of *wv* with wt or other members of the K_{ir} 3.0 subfamily reduces current amplitudes. (A) Coexpresion of *wv* and wt subunits results in a mRNA ratio-dependent reduction of the current amplitudes compared to expression of *wv* alone (measured at −100 mV, $n=18$ for each group). Oocytes were injected with 2 ng of either wt or *wv* mRNA. In the coinjections the amount of *wv* mRNA was held constant at 2 ng and the wt varied in the ratios indicated. (B) Coexpression of *wv* with other members of the K_{ir} 3.0 subfamily also reduces the current amplitudes. In contrast, coexpression of wt K_{ir} 3.2 and K_{ir} 3.1 results in potentiated current amplitudes compared to expression of either subunit alone (measured at −100 mV, $n=18$ for each group). Oocytes were injected with 2 ng of either wt or *wv* mRNA. In the coinjections a 5-fold excess of the K_{ir} 3.1, 3.2, 3.3 or 3.4 mRNA was used (10 ng).

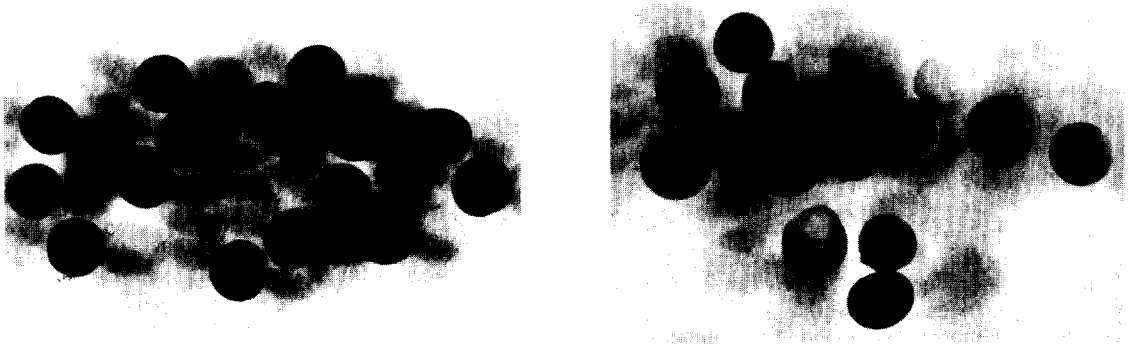
Interestingly, expression of *wv* channels in *Xenopus* oocytes results in cell death when the cells are maintained in a physiological solution such as ND96. Viability may be prolonged by elimination of calcium, or replacement of the external solution with a high K^+, low Cl^- depolarising solution. These results suggest that tonic depolarisation and Ca^{2+} influx is not per se responsible for cell death but that Na^+ influx is a necessary prerequisite for the subsequent, lethal effects of Ca^{2+}. These results are also consistent with the idea that the increased sodium permeability drives the cell towards the Gibbs-Donnan equilibrium resulting in cell swelling and death. This process is probably accentuated in oocytes by a depolaristion-induced calcium entry which activates chloride channels, allowing for a more rapid chloride influx. It is likely that in neuronal cells calcium influx will have additional neurotoxic effects.

Coexpression of equal amounts of *wv* and wt K_{ir} 3.2 in *Xenopus* oocytes reduced whole cell currents and prolonged oocyte viability even when the cells were maintained in ND96. This has important implications for *wv*/wt heterozygotes. Assuming equal amounts of wt and *wv* proteins and random assembly of subunits into tetrameric channels [11], these conditions will result in 15/16 of channels containing at least one wt subunit. Many members of the K_{ir} 3.0 subfamily of G-protein-sensitive inward rectifiers form heteromeric channels [12,13] and this may reflect the preferred situation [10]. Indeed, coexpression of all other members of the K_{ir} 3.0 subfamily with *wv* resulted in reduced, G-$\beta_1\gamma_2$-insensitive whole-cell currents and prolonged oocyte viability. In contrast, coexpression of wt K_{ir} 3.2 and K_{ir} 3.1 in a 1:5 ratio results in significantly potentiated G-$\beta_1\gamma_2$-sensitive currents.

Unlike the reduced currents seen upon coexpression of members of the K_{ir} 3.0 subfamily with K_{ir} 4.1, which is mediated by heteromeric subunit degradation [14], the mechanism of inhibition of *wv* current by coexpression with K_{ir} 3.0 subunits is likely due to the formation of non-conductive heteromeric channels. In support of this hypothesis: (a) no reduction in *wv* protein levels were seen upon coexpression with an excess of K_{ir} 3.1 (Fig. 1B), indicating the heteromeric channels are not degraded (cf. [3]); (b) the *wv*-Kir 3.1 heteromeric channels were G-$\beta_1\gamma_2$-insensitive; and (c) the remaining reduced channel activity could be carried by Na^+ as well as K^+.

These results suggest a model for the *weaver* phenotype. In this model, when K_{ir} 3.2 is expressed in the premigratory cerebellar granule cells for the *wv*/*wv* animals this leads to cell depolarization, calcium influx and death. However, in heterozygous animals (wt/*wv*), cell death is significantly delayed and at least some granule cells are able to migrate due to the mitigating effects of coassembled wt and *wv* subunits. Other members of the K_{ir} 3.0 subfamily, such as K_{ir} 3.1 and K_{ir} 3.3 are also expressed in the adult cerebellum [6] and developing cerebellum [8]. If their expression is initiated prior to or during migration, they may assist cell survival through heteromeric subunit assembly with *wv* subunits. In other brain regions in which *wv* is expressed, cell survival is governed by the developmental timing and relative abundance of other K_{ir} 3.0 subfamily members. For instance, other K_{ir} 3.0 subfamily members which are already expressed in the substantia nigra prior to expression of *wv*, may be efficiently coassembled with *wv* subunits, reducing the toxic effects of *wv* channels.

Table 1
Percentage of viable oocytes remaining 48 h after injection with wt or *wv* K_{ir} 3.2 mRNA



TYPE OF RNA INJECTED		No. ALIVE AT 48 HRS	%
wt	25ng	46/50	92
wv	25ng	0/50	0
wv **(Ca^{2+}-free)**	25ng	41/50	82
wv	5ng	24/50	48
wv **+ wt**	5ng each	45/50	90

50 oocytes for each group were injected with different mRNAs as indicated and viability was determined by visual inspection. Oocytes which had ruptured, or were both markedly swollen and had lost color demarkation between the poles were scored as inviable. Representative oocytes injected with wt (left) or *wv* (right) mRNA are shown above.

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