Gap junction proteins are not interchangeable in development of neural function in the *Drosophila* visual system

Kathryn D. Curtin*, Zhan Zhang and Robert J. Wyman
Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT 06511, USA
*Author for correspondence (e-mail: kathryn.curtin@yale.edu)

Accepted 12 June 2002
Journal of Cell Science 115, 3379-3388 (2002) © The Company of Biologists Ltd

Summary

Gap junctions (GJs) are composed of proteins from two distinct families. In vertebrates, GJs are composed of connexins; a connexin hexamer on one cell lines up with a hexamer on an apposing cell to form the intercellular channel. In invertebrates, GJs are composed of an unrelated protein family, the innexins. Different connexins have distinct properties that make them largely non-interchangeable in the animal. Innexins are also a large family with high sequence homology, and some functional differences have been reported. The biological implication of innexin differences, such as their ability to substitute for one another in the animal, has not been explored.

Recently, we showed that GJ proteins are necessary for the development of normal neural transmission in the *Drosophila* visual system. Mutations in either of two *Drosophila* GJ genes (innexins), *shakB* and *ogre*, lead to a loss of transients in the electroretinogram (ERG), which is indicative of a failure of the lamina to respond to retinal cell depolarization. Ogre is required presynaptically and shakB(N) postsynaptically. Both act during development.

Here we ask if innexins are interchangeable in their role of promoting normal neural development in flies. Specifically, we tested several innexins for their ability to rescue *shakB*^{2} and *ogre* mutant ERGs and found that, by and large, innexins are not interchangeable. We mapped the protein regions required for this specificity by making molecular chimeras between shakB(N) and ogre and testing their ability to rescue both mutants. Each chimera rescued either *shakB* or *ogre* but never both. Sequences in the first half of each protein are necessary for functional specificity. Potentially crucial residues include a small number in the intracellular loop as well as a short stretch just N-terminal to the second transmembrane domain.

Temporary GJs, possibly between the retina and lamina, may play a role in final target selection and/or chemical synapse formation in the *Drosophila* visual system. In that case, specificity in GJ formation or function could contribute, directly or indirectly, to chemical synaptic specificity by regulating which neurons couple and what signals they exchange. Cells may couple only if their innexins can mate with each other. The partially overlapping expression patterns of several innexins make this 'mix and match' model of GJ formation a possibility.

Key words: Gap junctions, Innexins, Chemical synapses

Introduction

Gap junctions (GJs) allow cytoplasm-to-cytoplasm communication via ions or other small molecules (Kumar and Gilula, 1996; Nicholson and Bruzzone, 1997). In vertebrates, GJs are composed of multimers of connexins (Swenson et al., 1989), and the connexin family has at least 25 different members (Simon and Goodenough, 1998). In invertebrates, innexins, which have no sequence homology to connexins, are the structural molecules for GJs (Krishnan et al., 1993; Starich et al., 1996; Phelan et al., 1998). The innexins are a large family with approximately 25 genes in *Caenorhabditis elegans* and eight genes in *Drosophila*. Mammalian genes with a very low homology to *innexin*s have been reported, which suggests that this may be a universal family (Panchin et al., 2000).

The reason for the large number of connexin genes seems to be related to their variable permeability and electrophysiological properties, as well as their ability to regulate cellular coupling through selective connexin mating (White and Bruzzone, 1996) (also see Discussion). Connexins are sometimes interchangeable, and sometimes not, as shown in mice when one *connexin* gene is replaced with another via genetic knock-in (Plum et al., 2000). Specifically, there is tissue-specific variability in the ability of Cx43 or Cx32 to functionally replace Cx40. Differences in intercellular communication requirements are suggested to be the main reason for failure of one connexin to substitute for another, although the exact causes of such failure are generally unknown (Plum et al., 2000). A large number of studies on connexin chimeras have implicated discreet portions of connexin proteins as responsible for the observed functional variability, from mating compatibility to ion selectivity (see Discussion).

Systematic studies examining innexin interchangeability have not been done as innexins are a relatively new GJ protein family. However, different innexins behave differently in experimental situations, suggesting that they have distinct properties. First, some innexins form GJs in a paired *Xenopus* oocyte assay, whereas others do not (Phelan et al., 1998;

Landesman et al., 1999; Stebbings et al., 2000) (K.D.C., T. White, R.J.W. and D. Paul, unpublished). Those innexins that fail to form GJs in this assay, nevertheless apparently code for GJs as their mutation leads to a loss of GJs between specific cells in animals (Krishnan et al., 1993; Starich et al., 1996) (Y.-A. Sun and R.J.W., unpublished). Second, pan-neural expression of GJ transgenes causes a variety of phenotypes. Such expression of two different, but highly homologous, splice variant products of *shakingB* (*shakB*), *shakB*(L) and *shakB*(N) (Krishnan et al., 1995; Zhang et al., 1999) leads, respectively, to lethality or to no obvious phenotype (Stebbings et al., 2000; Curtin et al., 2002). Pan-neural expression of D-inx-2 is lethal, whereas expression of D-inx-7 has no obvious phenotype (K.D.C. and R.J.W., unpublished). Pan-neural expression of ogre (optic ganglia reduced) causes cell loss in the eye, whereas expression of D-inx-2 in the eye along with ogre reduces ogre-induced cell loss (K.D.C. and R.J.W., unpublished).

These differences in innexin function could be due to specificity in innexin-innexin interactions. Some innexins form heteromeric junctions: the hemi-junction is composed of more than one innexin (Stebbings et al., 2000). Innexins may also form heterotypic junctions: hemi-junctions on apposing cells are made from different innexins. The observed overlapping innexin expression patterns in flies would allow such mixing and matching (Curtin et al., 1999; Stebbings et al., 2000; Zhang et al., 1999). Innexins could also differ in crucial permeability or biophysical properties. By whichever mechanism, innexin specificity must be mediated by protein sequence differences.

We examined innexins interchangeablity in the *Drosophila* visual system. We have recently shown that *shakB* and *ogre* are required for the development of normal neural information flow between retinal photoreceptors and their lamina synaptic partners in the adult (Curtin et al., 2002). Mutations in either gene eliminate neural transmission from the retina to the lamina (Homyk et al., 1980; Lipshitz et al., 1985; Curtin et al., 2002). Ogre is required in retinal neurons, whereas shakB(N) is required, at a minimum, in lamina neurons (Curtin et al., 2002). Expression of both proteins is required during development (Curtin et al., 2002).

During development, temporary GJs have frequently been observed between cells that will later form chemical synapses with each other (Fischbach, 1972; Lopresti et al., 1974; Allen and Warner, 1991). However, tools have not been available to determine whether this relationship is causal or coincidental. Our work suggests that developmental GJs, possibly between pre- and post-synaptic cells, are needed for normally functioning connections to form in the *Drosophila* visual system (Curtin et al., 2002). Specificity in innexin function could determine which cells form GJs and regulate what signals are exchanged. In connectivity/synaptogenesis, such molecular specificity could influence which cells interact to form functional chemical synapses, thus contributing, directly or indirectly, to synaptic specificity.

Here we show that innexins, though highly homologous (Curtin et al., 1999), are not interchangeable in their role to promote normal neural development. In addition, we show that we can map protein regions required for such innexin specificity and we discuss what specific residues may mediate the observed specificity.

Materials and Methods

Transgenic *Drosophila* lines

A 4.5 kB *Xho*I-*Hin*dIII fragment from upstream of the *ogre* message was cloned into the Gal4 vector pGatB (Brand and Perrimon, 1993). The *Xho*I site at the 5′ end was the same site as that in the 5′ end of the clone used by Watanabe and Kankel for ogre rescue (Watanabe and Kankel, 1990). The *Hin*dIII site is within the 3′ end just inside the *ogre* message start site. $ShakB^{N1}$ and *ogre* cDNAs were amplified by PCR and cloned into pUAST (Brand and Perrimon, 1993). Constructs were introduced into flies by P-element-mediated transformation (Spradling et al., 1982). The *elav-Gal4* enhancer trap line, C155, was obtained from Corey Goodman. $ShakB^2$ was recombined with C155, and this recombinant was used in rescue experiments by crossing to *UAS-geneX* lines. Gene X refers to any one of several innexins, connexin43 or chimeric genes used for rescue.

$ShakB^2$ rescue experiments

For $shakB^2$ rescue, we genetically recombined the *elav-Gal4* enhancer trap (C155) with the shaking $shakB^2$ mutation as both are on the X chromosome. One copy each of the driver and the UAS construct was adequate for rescue. We did the following type of cross for all rescue experiments of $shakB^2$:

elav-Gal4, shakB^2*/FM7* × *+/Y; UAS-geneX.*

Male progeny of the genotype, *elav-Gal4*, *shakB*^{2}/Y; *UAS-geneX*/+, were tested.

$Ogre^{cb8}$ rescue experiments

For $ogre^{cb8}$ rescue experiments, we found that animals with double copies of both the driver and UAS constructs showed noticeably better rescue (data not shown) than flies with single copies. To obtain double copies, the following cross was done with appropriately constructed lines:

ogre^{cb8}*/FM7; ogre-promoter-Gal4; ogre-promoter-Gal4* ×
+/Y; UAS-geneX; UAS-geneX.

We then tested male progeny of the following general genotype:

ogre^{cb8}*/Y; ogre-promoter-Gal4/UAS-geneX;*
ogre-promoter-Gal4/ UAS-geneX.

For two of the UAS constructs we obtained inserts on only one chromosome. So for those, we made fly lines of the following general genotype:

ogre^{cb8}*/FM7; ogre-promoter Gal4; UAS-geneX.*

Ogre^{cb8}/Y; ogre-promoter Gal4; UAS-geneX males were tested for rescue.

Making chimeric genes

We made chimeric genes by making two half-genes by PCR and ligating them together. Since we wanted to make a breakpoint in the coding part of each gene, we used *pfu* polymerase, which gives blunt-ended products (i.e. it does not tack on extra nucleotides). *pfu* also has 10× higher fidelity than *taq*. To make shakB(N)$^{1\text{-}97}$ogre$^{108\text{-end}}$ we used a 5′ primer that was homologous to the 5′ untranslated portion of shakB(N). This primer included sequences for an *Eco*RI restriction site at the very 5′ end. The other shakB(N)-specific primer was in a reverse direction and complementary to the codons for amino acids 97, 96 etc. (21 bp long). This primer contained a 5′ phosphate. These two primers were used to amplify shakB(N) sequences from the beginning of the gene through to amino acid 97 using a cloned *shakB(N)* gene as the template. Similarly, ogre was amplified from amino acid 108 to the end. The forward primer was homologous to ogre codons for amino acids 108, 109 etc. (20 bp long). This primer

contained a 5′ phosphate. The reverse primer was homologous to sequences in ogre just 3′ to the stop codon. This primer contained at it's 5′ end an *Eco*RI restriction site. These two PCR products were gel purified and cut with *Eco*RI. They were then ligated into bluescript cut with *Eco*RI in a three-way ligation, including a blunt-end ligation at the 'joint' between shakB(N) and ogre. IPTG/X-gal selection allowed us to detect clones that contained inserts. Three inserts are possible, and we used restriction analysis to choose clones with the correct insert. These were sequenced to insure that they were correct. ShakB(N)$^{1\text{-}172}$ogre$^{183\text{-end}}$ was made by introducing an *Eco*RI site at the junction between shakB(N) and ogre. Though neither gene has an *Eco*RI site at this position normally, ogre's sequence is one base different from an *Eco*RI site. Introducing an *Eco*RI site involved changing a base in the wobble position leading to no change in amino acid 185, which remains as F. The ogre primer at this spot was complementary to amino acids 184, 185 etc. (21 bp long), with the one base change at the wobble position for 185(F) to create the *Eco*RI site. The primer to the 3′ non-coding sequences of ogre was designed with a *Bgl*II site at its end. These two primers were used to amplify ogre from the codon from amino acid 183 to the end. To amplify shakB(N) from the beginning to the codon for amino acid 172, we designed a reverse primer that contained an *Eco*RI site with homology to codon 172, 171 etc. (21 bps long). The forward primer was homologous to the 5′ non-coding end of shakB(N), with a *Bgl*II site at the 5′ end. These two products were ligated via a three-way ligation into bluescript cut with *Bgl*II. Again, we used X-gal/IPTG screening to select for clones with inserts, followed by restriction analysis to find clones with the correct insert. These were subsequently sequenced. The inverse product ogre^{182}shakB(N)^{173} was made with the same basic strategy of introducing an *Eco*RI site into both genes at this position. In the forward primer for shakB(N), the *Eco*RI site replaces codons for amino acids 174 and 175, causing a sequence change from GAG CTG to GAA TTC. This changes residues E L in shakB(N) to the ogre sequence E F. The breakpoint for shakB(N)^{241}ogre^{252} was created by using an existing *Pst*I site in ogre at the codon for amino acid 249/250. This restriction site was introduced into the corresponding place in shakB(N) by including *Pst*I sequences in the primer used for PCR. A three-way ligation was done and clones selected as previously described. To make clones with more than one breakpoint, the same general strategy was used, only this time chimeric genes containing one breakpoint were used as templates for the second PCR reaction. All chimeric genes were subcloned into pUAST and introduced into flies by P-element-mediated transformation.

Electroretinograms

Flies were immobilized in a plastic pipet tip with the head protruding from the tapered end. Flies were oriented with one eye facing a fiber optic lamp positioned behind a shutter. Glass capillaries (1 mM diameter, with filament, Precision Instruments M1B100F-4) were pulled and filled with 130 mM NaCl, 50 mM KCl. A recording electrode was inserted just inside the cornea. A ground electrode was placed into the back of the head. A Grass stimulator was used to trigger an oscilloscope, and after a delay, to open a shutter to expose flies to the fiber optic light for 4 seconds. The response was recorded on an oscilloscope, the screen photographed on polaroid film and the pictures scanned into a computer.

Results

Electroretinogram phenotype of *shakB*^{2} and *ogre*^{cb8}

Electroretinograms (ERGs) record the presynaptic receptor potential and the postsynaptic response of the lamina. Fig. 1A shows the ERG from a wild-type (Canton-S) strain of *Drosophila*. ERG measurements were taken in a dimly lit room. Immobolized animals were subjetced to a bright light pulse from a fiber optic lamp for four seconds. As can be seen in Fig. 1, wild-type ERGs have at least three distinct phases. The cornea-negative (downward) response that lasts for the duration of the light pulse (the receptor potential) records the depolarization of the photoreceptor cells. In addition, there is a cornea-positive (upward) on-transient (arrow, Fig. 1A). A second downward transient at lights-on is usually masked by the receptor potential and is thus not further considered. When the lights go off there is a short cornea-negative (downward) transient (arrow, Fig. 1A). Subsequently, the receptor potential recovers to its pre-stimulus baseline.

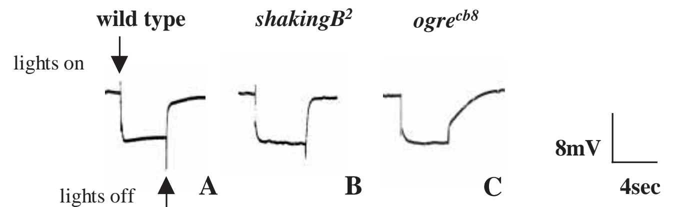

Fig. 1. Electroretinograms of *shakB*^{2} and *ogre*^{cb8} animals. ERGs of both mutants are defective in on- and off-transients, suggesting a reduction in or loss of functional chemical synaptic connections between the retina and lamina in these mutants. (A) ERG of wild-type (Canton-S) flies, vertical arrows mark the on- and off-transients, respectively. (B) ERG of *shakB*^{2} animals. (C) ERG of *ogre*^{cb8} animals.

The on- and off-transients are responses of the second order lamina cells when they are synaptically driven by the photoreceptors (Alawi and Pak, 1971; Heisenberg, 1971; Burg et al., 1993). In addition, mutants that disrupt neurotransmitter synthesis in the retina or eliminate synaptic vesicle release abolish transients but do not affect the receptor potential (Hotta and Benzer, 1969; Pak et al., 1969; Burg et al., 1993; Stowers and Schwarz, 1999). These synapses are purely chemical - there are no gap junctions between pre- and postsynaptic cells in the adult.

The ERG phenotypes of both *ogre*^{cb8} and *shakB*^{2} mutants indicate a failure of transmission between the retinal photoreceptor neurons and their post-synaptic partners, the lamina monopolar neurons (below) (Homyk et al., 1980; Lipshitz and Kankel, 1985; Curtin et al., 2002). For both mutants, the receptor potential was normal (Fig. 1B,C), indicating normal photoreceptor cell transduction and depolarization. However, both mutants show an absence or a reduction in transients, indicating that the lamina is not receiving its normal input from the retina. For *ogre*^{cb8} animals,

Table 1. ERG phenotypes of *shakB*^{2} and *ogre*^{cb8} animals under two lighting conditions

	Room lights on		
	On-transient (mV)	Off-transient (mV)	Receptor potential (mV)
Wildtype	1.9±0.3	7.1±0.8	9.5±1
shakB^2	0.9±0.2	1.8±0.4	9.1±0.7
ogre^{cb8}	0	0	8.8±0.8

Magnitude of on- and off-transients and receptor potentials for *shakB*^{2} and *ogre*^{cb8}. Each point is an average of results from eight or more animals with the standard deviation.

both transients were missing entirely (Fig. 1C, Table 1; see also Curtin et al., 2002). The phenotype for *shakB*^{2} was less severe (Fig. 1B; Table 1) (see also Homyk et al., 1980; Curtin et al., 2002). *ShakB*^{2} flies (Fig. 1B; Table 1) showed on-transients that were approximately 47% of wild-type, whereas the off-transients were approximately 25% of wild-type (Table 1).

Though *ogre*^{cb8} has optic ganglia that are reduced in size (Lipshitz and Kankel, 1985), the failure of transmission in this mutant is independent of the reduced ganglia. The ERG transients are absent even in mosaic animals in which just the retina is mutant for *ogre* and the optic ganglia is genotypically and morphologically normal (Curtin et al., 2002).

The ERG readings shown for *ogre*^{cb8} (Fig. 1C) also show delayed recovery of the receptor potential. Our *ogre*^{cb8} flies also carry mutations that reduce eye pigmentation. Flies with reduced screening pigment are more sensitive to blue light, which causes an after-depolarization that leads to a delayed recovery of the photoreceptor potential (Cosens and Briscoe, 1972). The delay in recovery we see can be eliminated by screening out blue light with an orange filter, which shows that this is due to the lack of screening pigment and not to the *ogre* mutation (data not shown).

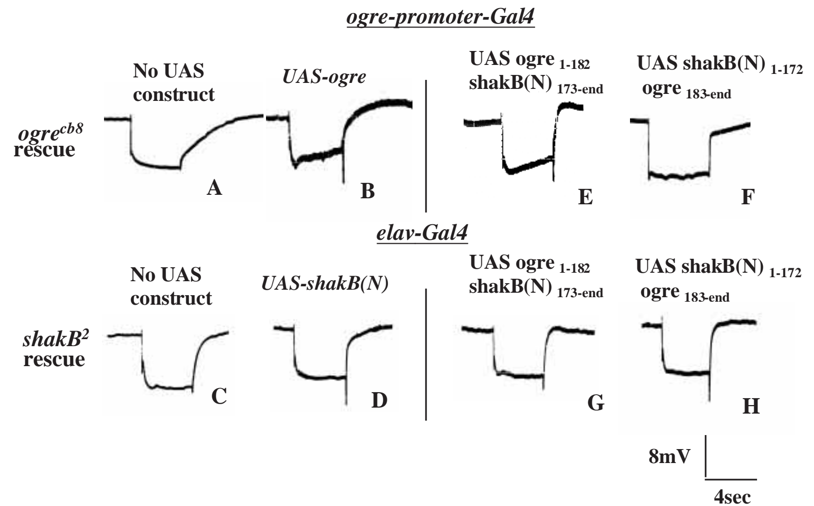

Fig. 2. Rescue of *ogre*^{cb8} and *shakB*^{2} ERGs. (A,C) Negative controls. (A) *ogre*^{cb8} with *ogre promoter-Gal4* driver only and (C) *shakB*^{2} with *elav-Gal4* driver only. (B) ERG of *ogre*^{cb8} animals rescued by expression of the ogre protein via an *ogre promoter-Gal4* driver. (D) ERG of *shakB*^{2} animals rescued by expression of shakB(N) in all neurons via the *elav-Gal4* driver. Compare these ERGs with wild-type in Fig. 1A. ShakB(N)-ogre chimeric proteins that contain the first half of ogre rescue *ogre*^{cb8} but not *shakB*^{2}, whereas those that contain the first half of shakB(N) rescue *shakB*^{2} but not *ogre*^{cb8}. (E) *ogre*^{cb8} is rescued by ogre$^{1\text{-}182}$shakB(N)$^{172\text{-end}}$. (F) *ogre*^{cb8} is not rescued by shakB(N)$^{1\text{-}172}$ogre$^{183\text{-end}}$. (G) *shakB*^{2} is not rescued by ogre$^{1\text{-}182}$shakB(N)$^{173\text{-end}}$. (H) *shakB*^{2} is rescued by shakB(N)$^{1\text{-}172}$ogre$^{183\text{-end}}$.

Rescue of *shakB*^{2} and *ogre*^{cb8} ERGs

To rescue both *shakB*^{2} and *ogre*^{cb8}, we made use of the versatility of the Gal4/UAS expression system developed previously (Brand and Perrimon, 1993). Briefly, GAL4 is a yeast transcription factor protein that binds to a sequence known as the upstream activating sequence (UAS). Binding turns on expression of any gene that is placed immediately downstream of the UAS. *Gal4* is cloned behind a *Drosophila* promoter that will turn on in the cells of interest, and the gene that we want to express in those cells is cloned behind UAS. These constructs are introduced separately into flies by P-element-mediated transformation. Both constructs are combined in the same fly by mating. Gal4 is expressed in the cells of interest, it binds to UAS and the target gene is turned on.

To rescue *shakB*^{2}, an *elav-Gal4* enhancer trap, which expresses in all neurons from neural birth through adulthood, was used to drive expression of wild-type shakB(N). The on-transient was rescued to 95% of wild-type levels; the off-transient was rescued to 76% of wild-type levels (compare Fig. 2D, Table 2 with wild-type animals in Fig. 1A, Table 1) (see also Curtin et al., 2002). As a negative control we used a parent with the driver but without the UAS-shakB(N), that is, *elav-Gal4*, *shakB*^{2} males (Fig. 2C); these exhibited no rescue and were identical to *shakB*^{2} (Fig. 1B).

Table 2. Rescue of ERG transients by innexin transgenes

	shakingB^{2} rescue (*elav-Gal4* driver)			*ogre*^{cb8} rescue (*ogre-promoter-Gal4* driver)		
UAS construct	On mV	Off mV	RP mV	On mV	Off mV	RP mV
No construct	0.9±0.2	1.8±0.4	9.1±0.7	0	0	8.8±0.8
ogre	0.85±0.5	1.7±0.3	8.8±1.5	1.5±0.5	5.0±1	9.5±2.0
shakB(N)	1.8±0.13	5.4+±0.5	9.0±1.6	0.3±0.2	0.15±0.5	9.0±1.8
D-inx-7	1.4±0.5	4.47±0.4	8.5±1.6	0.2±0.12	0	8.9±1.4
D-inx-2	ND	ND	ND	0.15±0.1	0.125±0.05	11.1±2.0
Cx43	0.7±0.2	1.9±0.3	8.5±1.8	0	0	9.4±1.6
shakB(N)$^{1\text{-}172}$ogre$^{183\text{-end}}$	1.7±0.2	5.1±0.4	10.0±2.0	0.10±0.15	0.2±0.13	8.6±1.1
ogre$^{1\text{-}182}$shakB(N)$^{173\text{-end}}$	0.8±0.2	1.9±0.3	9.8±1.7	0.75±0.18	3.3±0.25	12.6±0.98
shakB(N)$^{1\text{-}97}$ogre$^{108\text{-end}}$	1.24±0.5	2.9±1.7	11.1±1.8	0.12±0.1	0	10.2±1.2
ogre$^{1\text{-}107}$shakB(N)$^{98\text{-}172}$ogre$^{183\text{-end}}$	1.85±0.1	5.5±0.7	9.0±1.3	0	0	9.0±2.2
ogre$^{1\text{-}182}$ shakB(N)$^{173\text{-}241}$ogre$^{252\text{-end}}$	0.85±0.4	2.0±0.4	9.6±1.8	0.95±0.15	3.2±0.27	11.2±1.7
ogre$^{\text{TRUNC}}$	0.95±0.13	1.74±0.2	8.0±1.7	0	0	8.5±1.2
Wildtype	1.9±0.3	7.1±0.8	9.5±1.0			

Magnitude of on- and off-transients and receptor potentials in transgenic rescue experiments. Each point is an average of results from six or more animals with the standard deviation.

For rescue of the *ogre*^{cb8} ERG phenotype, we used an *ogre-promoter-Gal4* driver (Curtin et al., 2002), which should contain all the upstream elements needed for *ogre* rescue, to drive expression of wild-type ogre. This driver expresses in a subset of retinal neurons beginning in the early pupal stage (Curtin et al., 2002). We found that double copies of both the *ogre-promoter-Gal4* driver and *UAS-ogre* (or UAS-geneX) were needed to effect adequate rescue (see Materials and Methods). Animals that contained two copies of both the UAS-*ogre* and the *ogre-promoter-Gal4* showed rescue of on-transients that are approximately 80% of wild-type and off-transients that are approximately 70% of wild-type levels (Fig. 2B, Table 2). Control *ogre*^{cb8} mutants containing the driver alone show no rescue (Fig. 2A).

Are innexins interchangeable?

Once we demonstrated rescue of the *ogre* and *shakB*^{2} mutants with their respective wild-type genes, we went on to test the ability of other *innexin* genes, as well as *connexin43*, to rescue *ogre*^{cb8} and *shakB*^{2} using the same drivers.

The innexin genes we tested are shown in the pileup in Fig. 3. These proteins are all highly related (Curtin et al., 1999). They also show partially overlapping expression patterns. For example, D-inx-2, D-inx-3 and ogre all have largely overlapping expression patterns in the embryo (Curtin et al., 1999; Stebbings et al., 2000). ShakB(N) and D-inx-2 are both expressed in adult lamina neurons (Curtin et al., 1999; Zhang et al., 1999). ShakB(N) also has partially overlapping expression patterns with other innexins in the embryo (Zhang et al., 1999).

ShakB(N) and shakB(L) are made from alternately spliced mRNAs and are identical except for an approximately 20 amino acid difference in the first approximately 105 amino acids (Krishnan et al., 1995) and a slightly longer N-terminus in shakB(L) (see Discussion). Fig. 4 compares this divergent portion of shakB(N) and shakB(L). Mutations in shakB(N) lead to a loss of GJs in the Giant Fiber system (Phelan et al., 1996; Sun and Wyman, 1996), whereas mutations that eliminate shakB(L) lead to lethality (Krishnan et al., 1995). ShakB(L) also forms homotypic GJs in the paired *Xenopus* oocyte assay, whereas shakB(N) does not (Phelan et al., 1998).

Fig. 3. A pile-up of the *Drosophila* innexin family showing conserved residues in black. Putative transmembrane domains are underlined and marked as M1, M2 etc. Horizontal lines mark the breakpoints for the chimeras we tested and are labeled with the shakB(N) residue number on the left and the corresponding ogre residue number on the right. D-inx-7 rescues *shakB*^{2} whereas ogre does not. In addition, the chimeric studies show that residues 97-172 of shakB(N) are the residues needed for shakB(N) specificity. In this critical region those residues where D-inx-7 is identical or chemically similar to shakB(N), but chemically different from ogre, are marked with asterisks. These marked residues are likely to be the ones that are most crucial for shakB(N)-specific rescue.

Ogre^{cb8} mutants were rescued by wild-type ogre driven by the *ogre-promoter-Gal4*, as described above. However, there was no rescue when this construct was used to drive expression of shakB(N), shakB(L), D-inx-7 [prp7 (Curtin et al., 1999)] or D-inx-2 [prp33 (Curtin et al., 1999)] in an *ogre*^{cb8} background [Table 2; for shakB(L) data, see Curtin et al., 2002 (Curtin et al., 2002)]. Thus wild-type ogre protein rescues *ogre* mutants, but the four other innexins tested do not.

ShakB^2 was rescued when shakB(N) was driven in all

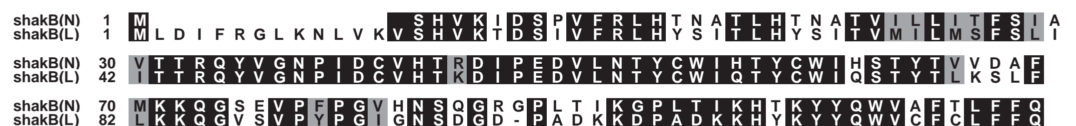

Fig. 4. A comparison of the amino-acid sequence of shakB(N) and shakB(L) coded by the divergent first coding exons. The sequences that differ between the two are all within the first approximately one third of the protein, from the start through the first extracellular loop. From this point on the two proteins are identical.

neurons via *elav-Gal4*, as described above. *ShakB^2* was not rescued at all by ogre using this same driver (Table 2). The failure of ogre to rescue is not due to an inadequate amount of ogre protein made via the *elav-Gal4* driver as we know we can rescue *ogre^{cb8}* with this same driver (Curtin et al., 2002). Interestingly *shakB^2* was partially rescued by D-inx-7 (prp7), which rescues the on- and off-transients to levels that are approximately 55% and 74%, respectively, of the rescue seen with shakB(N) (Table 2, see Fig. 5 legend for calculation scheme). We were unable to test whether D-inx-2 (prp33) or shakB(L) can rescue *shakB^2* because expression of either of these homotypic gap junction proteins in all neurons via the *elav-Gal4* driver is lethal (Stebbings et al., 2000) (K.D.C. and R.J.W., unpublished). We have been unable to find a driver that expresses in the developing visual system that does not lead to lethality when driving either of these two homotypic GJ proteins.

Cx43 does not rescue either mutant. However, immunohistochemistry shows that Cx43 is not stable, or not expressed well, in the visual system when expressed in all neurons (Z.Z. and R.J.W., unpublished).

The failure of each of the above tested innexins to replace shakB(N) or ogre in rescue experiments is not due to a failure to express a stable, functional protein. We have direct functional evidence that each protein is made in the visual system. ShakB(N) and D-inx-7 both rescue *shakB^2* and ogre rescues *ogre*, showing that these three proteins are all functionally expressed in this assay. We have also demonstrated, by immunohistochemistry, expression of shakB(N) in the nervous system of animals expressing shakB(N) via *elav-Gal4* (Curtin et al., 2002). In addition, ogre overexpression in the eye with several different drivers causes a rough eye phenotype, and co-expression of D-inx-2 reduces this roughness, indicating that D-inx2 is also functional when expressed in the eye (K.D.C. and R.J.W., unpublished). Lastly, we know that expression of both shakB(L) and D-inx-2 via *elav-Gal4* is lethal (Stebbings et al., 2000) (K.D.C. and R.J.W., unpublished). We have no way to directly compare the amount of each protein made in each rescue paradigm since most of the proteins do not include an antibody tag. Thus we cannot formally rule out the possibility that inadequate amounts of protein are made for rescue with some *Gal4*-UAS combinations.

One might imagine that the absence of ERG transients in the above rescue experiments could be due to the exogenous proteins polymerizing with and thus disrupting the function of endogenous innexin proteins. However, when we express any of these proteins in non-mutant animals using either *elav-Gal4* or *ogre-promoter-Gal4* the ERGs are normal (data not shown). This shows that the failure of one innexin to rescue another is not a false negative caused by interference.

Mapping the protein regions required for specific functioning

Clearly, innexins are usually not interchangeable despite fairly high homology. For example, shakB(N) and ogre, which cannot substitute for each other, are 64% similar and 54% identical, with the homology being fairly evenly distributed across the protein. Next, we determined whether identifiable regions of the protein were crucial for functional specificity by making chimeras between shakB(N) and ogre and testing them for their ability to rescue ERGs for each mutant. This kind of information may be a first step in understanding what kinds of molecular functions are important for innexin specificity, that is, extracellular interactions, properties of the transmembrane pore, protein polymerization etc. No portion of innexin proteins have been found to be homologous to other protein families, thus innexins do not contain known structural motifs other than the hydrophobic transmembrane domains.

Barnes and Hekimi describe a putative tertiary structure for innexin proteins, predicting four transmembrane domains with the N and C termini both inside the cell (Barnes and Hekimi, 1997). Such folding would lead to two extracellular and one intracellular loop as well as a short N-terminal tail and a long C-terminal tail. Putative transmembrane domains are marked in the pileup in Fig. 3. We have some experimental evidence to suggest that the C-terminus is inside the cell as the model predicts. Specifically, when shakB(L) protein, with a C-terminal myc tag attached, is expressed in *Drosophila* salivary gland cells, the myc tag cannot be detected by antibody unless a detergent is added to permealize the cell membrane and thus allow access of antibody to the cell cytoplasm (data not shown).

We used the Barnes and Hekimi model as a guide to design chimeras between shakB(N) and ogre in which putative physical regions of these two proteins, such as extracellular loops etc, are exchanged. We used this model because it was based on the analysis of several family members from both *Drosophila* and *C. elegans*. Chimeras were tested to see if they could rescue *ogre^{cb8}* or *shakB^2* using the drivers and protocols already described. The three main 'break points' for our chimeras are marked on the pile-up in Fig. 3. These break points represent places where the coding sequence from one protein has been exchanged with a coding sequence from the other. There are gaps in the numbering only because shakB(N) is slightly shorter at the N-terminus than ogre so that, for example, amino acid 97 of shakB(N) is in a homologous position to residue 107 of ogre. The break points are as follows: (1) amino acid 107 of ogre (97 of shakB(N)), marking the end of the first putative extracellular loop; (2) amino acid 182 of ogre (172 of shakB(N)), located just after the presumed intracellular loop; and (3) amino acid 251 of ogre (241 of shakB(N)), which is about three quarters of the way through the presumptive second extracellular loop. Chimeras are named along the following lines: ogre$^{1\text{-}107}$shakB(N)$^{98\text{-}172}$ogre$^{183\text{-}end}$ encodes ogre from the N-terminus through amino acids 107, shakB(N) from amino acids 98-172 and ogre from amino acid 183 to the end. The chimeras are shown schematically in Fig. 5 where it is easy to see what portions of the proteins have been swapped.

First, it is important to note that all the chimeras were able to partially rescue either *ogre^{cb8}* or *shakB^2*, so all must be stable and make a functional protein. In addition, none of these proteins had any negative interfering effects; expressing each chimeric transgene in wild-type animals had no effect on the ERGs. Strikingly, although each chimera rescued either *shakB^2* or *ogre^{cb8}*, no chimera rescued both. This result suggests that there are distinct regions of each innexin protein that have diverged to provide specific functions. Such protein regions

may mediate defined molecular functions, such as innexin-innexin interaction or pore permeability.

ShakB(N) protein identity region

A chimera whose first half is ogre and second half is shakB(N), ogre$^{1-182}$shakB(N)$^{173\text{-end}}$ rescued *ogre^{cb8}*, but did not rescue *shakB^2* (Fig. 2E,G, Table 2, and schematically in Fig. 5F). The reverse chimera whose first half is shakB(N) and second half is ogre, shakB(N)$^{1-172}$ogre$^{183\text{-end}}$ did not rescue *ogre^{cb8}* but did rescue *shakB^2* (Fig. 2F,H, also Table 2, Fig. 5B). This chimera rescued *shakB^2* effectively, rescuing the on-transient 89% as effectively as shakB(N) protein and rescuing the off-transient 92% as effectively as shakB(N) (Fig. 5B). These results show that residues in the first half of shakB(N) are essential for shakB(N)-specific function.

We constructed and tested other chimeras to narrow down the portion of shakB(N) required for specific function. A chimera that contains only the second transmembrane domain, the intracellular loop, and the first half of the third transmembrane domain of shakB(N) (ogre$^{1-107}$shakB(N)$^{98-172}$ogre$^{183\text{-end}}$) rescued *shakB^2* to almost 100% of the level of full-length shakB(N) protein (Table 2, Fig. 5C). This would suggest that all the sequences that determine specificity of shakB(N) activity (the 'protein identity region') are in this intracellular loop or in the second transmembrane domain. Though a shakB(N)-specific first extracellular loop was not needed for specificity of development of retina-lamina transmission, it may be needed for specificity in other tissues where shakB(N) may make other protein-protein contacts (see Discussion). In addition, we saw some rescue (30-38%) with shakB(N)$^{1-97}$ogre$^{108\text{-end}}$ (Table 2, Fig. 5D). This construct rescued approximately one third of animals to a nearly complete level and failed to rescue approximately two thirds of animals at all. This suggests that shakB(N) sequences from the N-terminus through the presumptive first extracellular loop are capable of some degree of function. It also suggests that the requirement for sequences between 98-172 for shakB(N) specificity is not absolute.

Sequences between residues 98 and 172 contribute to shakB(N) specificity. In addition, D-inx-7 rescues *shakB^2* but ogre does not. Therefore, to identify possible key residues important for shakB(N) identity, we looked for residues between 98-172 where D-inx-7 and shakB(N) are chemically similar, but ogre is chemically different (Fig. 3, asterisks). There are six such residues. Four of these residues are found clustered just after the second transmembrane domain. In addition, five of the six represent a change in charge. These residues may turn out to be important for shakB(N) specificity.

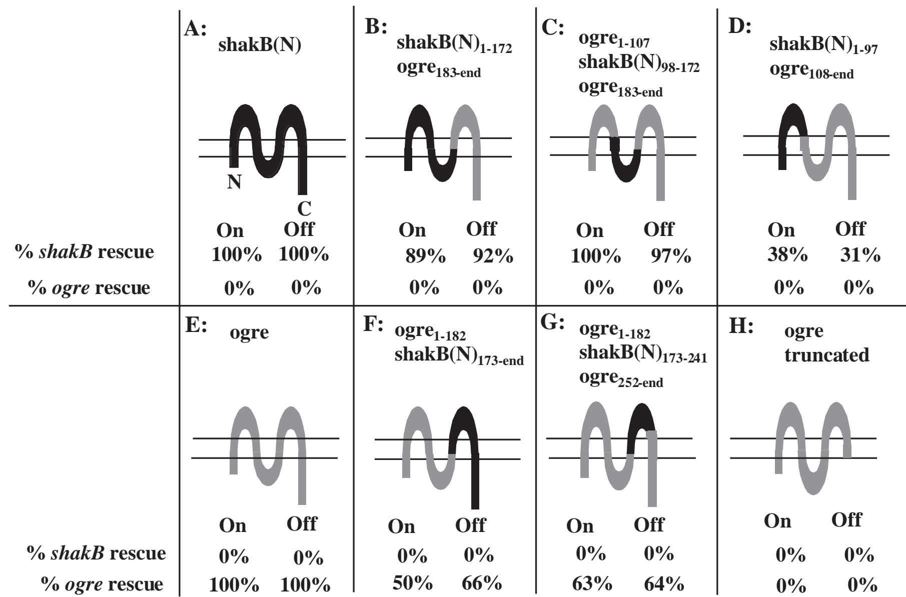

Fig. 5. A schematic of the shakB(N)-ogre chimeric proteins used to rescue *shakB^2* and *ogre^{cb8}* animals. shakB(N) sequences are represented in black and ogre sequences in grey. Beneath each schematic, the ability of each chimera to rescue the on- and off-transients of *shakB^2* and *ogre^{cb8}* mutants as compared with the wild-type protein is shown. The rescue ability is calculated as in the following example:

$$\frac{\text{On (or off) value of chimeraX rescuing } shakB^2 - \text{on (or off) of } shakB^2}{\text{On (or off) value of shakB(N) rescuing } shakB^2 - \text{on (or off) of } shakB^2} \times 100\% .$$

Small negative values and small positive values less than 5% were labelled as 0% rescue.

The ogre protein identity region is in the first half of the protein

An ogre-shakB(N) chimeric protein that contains only the first half of ogre, ogre$^{1-182}$shakB(N)$^{173\text{-end}}$ rescued *ogre^{cb8}* ERG phenotype to an appreciable degree, retaining 50% to 66% of the function of the full-length ogre protein (Table 2, Fig. 2E, Fig. 5F). It did not rescue *shakB^2* (Fig. 2G, Table 2). This construct contains ogre sequences from the N-terminus through to the first few residues of the third transmembrane domain and thus includes the first extracellular loop, the first two transmembrane domains and the intracellular loop (Fig. 5F). Conversely, the chimera in which the first half of the protein encodes shakB(N), shakB(N)$^{1-172}$ogre$^{183\text{-end}}$, does not rescue *ogre^{cb8}* at all (Table 2, Fig. 2F, Fig. 5B). These results suggest that some residues in the first half of ogre are essential for the specificity of ogre function.

We made chimeras that subdivide the first half of ogre in an attempt to narrow down the sequences needed for *ogre* rescue. A chimera that contains shakB(N) sequences from the beginning of the molecule through the presumptive first extracellular loop, shakB(N)$^{1-97}$ogre$^{108\text{-end}}$ failed to rescue *ogre^{cb8}* (Table 2, Fig. 5D). Likewise, the chimera ogre$^{1-107}$shakB(N)$^{98-172}$ogre$^{183\text{-end}}$, which contains shakB(N) residues from the second transmembrane domain through to the first few residues of the third transmembrane domain (this includes the intracellular loop), failed to rescue (Table 2, Fig. 5C). This suggests that some residues on both sides of the second transmembrane domain breakpoint are essential for ogre-specific function.

Though some residues in the first half of ogre are essential for *ogre* rescue, chimeras that contained amino acids 1-182 rescued a third less than full-length ogre does, suggesting that some sequences in the last half of ogre may contribute to, but are not essential for, ogre specificity. In addition, ogre$^{1\text{-}182}$shakB(N)$^{173\text{-end}}$ (Fig. 5F) and ogre$^{1\text{-}182}$shakB(N)$^{173\text{-}241}$ogre$^{252\text{-end}}$ (Fig. 5G) rescued to the same level (Table 2), suggesting that amino acids 252-362 are not contributing to specificity at all. This means that the difference in activity between these chimeras and full-length ogre is due to replacement of ogre residues within amino acids 183-251 with shakB(N) residues. These sequences are in the putative second extracellular loop.

Lastly, since we know that the internal tail is not conferring unique identity on shakB(N) or ogre, we wondered if it was required at all. We cloned a truncated form of ogre that stops just after the last transmembrane domain. This protein was not able to rescue *ogre^{cb8}* ERGs (Table 2, Fig. 5H). Thus the internal tail is essential for innexin function.

Discussion

Although they show high sequence similarity, shakB(N) and ogre, as well as other innexins, are generally not functionally interchangeable. We have shown that mutations in *ogre* are rescued by ogre but not by shakB(N), shakB(L), D-inx-2 or D-inx-7. Mutations eliminating shakB(N) are rescued by shakB(N) and D-inx-7, but not by ogre. Hence, there must be some functional specialization of different innexins. In addition, we have mapped regions of innexins that are required for shakB(N) and ogre specificity.

Possible reasons for specific innexin action

Specificity in innexin function could be the result of differing biophysical properties. Evidence from *Xenopus* oocyte experiments shows that innexins differ in voltage gating and conductance (Phelan et al., 1998; Landesman et al., 1999; Stebbings et al., 2000). Junctions composed of different innexins may also allow passage of different sets of second messengers. Studies on connexins show that they show distinct preferences for ions of different charge (Beblo and Veesntra, 1997; Wang and Veenstra, 1997) as well as different permeabilities for non-charged molecules and second messengers (Bevans et al., 1998; Cao et al., 1998). Connexins also show notable differences in biophysical properties (White et al., 1994; Verselis et al., 1994).

Innexins may also engage in unique intermolecular interactions, with other innexins or with other classes of proteins. Two innexins form heteromeric GJs (Stebbings et al., 2000). In addition, although some form homotypic GJs, others do not (Phelan et al., 1998; Landesman et al., 1999; Stebbings et al., 2000) (K.D.C., T. White, R.J.W. and D. Paul, unpublished). The *Drosophila* innexins that form homotypic junctions in *Xenopus* oocytes, shakB(L) and D-inx-2, are lethal when expressed pan-neurally in *Drosophila* (Stebbings et al., 2000) (K.D.C. and R.J.W., unpublished), although those that do not form homotypic junctions do not cause lethality (D-inx-7, shakB(N) and ogre) (K.D.C. and R.J.W., unpublished). Overexpression of ogre in the eye, but not other innexins, causes a rough-eye phenotype, indicating cell loss or death (K.D.C. and R.J.W., unpublished). One likely explanation for these differences is that each innexin can polymerize or mate only with particular other innexins to form GJs. Most connexins are capable of making homotypic junctions in the *Xenopus* oocyte assay (White and Bruzzone, 1996), whereas data from both the *Xenopus* oocyte assay and from the ectopic expression studies in the animal just described suggest that more than half of innexins do not form homotypic junctions.

Since neither ogre nor shakB(N) seems to make homotypic GJs, it is possible that they form GJs by mating with other innexins. Both show highly overlapping expression patterns with other innexins in animals (Curtin et al., 1999; Zhang et al., 1999), making their involvement in heteromeric or heterotypic GJs a reasonable possibility.

Putative roles for intracellular sequences

The molecular functions that distinguish different innexins may be mediated by distinct protein regions. We tested this by making chimeras between shakB(N) and ogre and testing these for rescue of the *shakB^{2}* or *ogre^{cb8}* ERG phenotypes.

In order to function in this assay, both *ogre* and *shakB^{2}* need some gene-specific sequences from the second transmembrane domain or the putative intracellular loop. These sequences could play a role in polymerizing innexins to form a hemichannel. Both may normally form homomultimers so that replacing these sequences with residues from the other protein could prevent self-association. Alternatively, the sequences could specify polymerizing interactions with other innexins in the cell membrane to make a heteromeric channel. As a possible example of a heteromeric channel, we know that both shakB(N) and D-inx-2 are expressed in the lamina (Curtin et al., 1999; Zhang et al., 1999). We also know that shakB(N) expression in the lamina is required for normal retina-lamina synapses to form (Curtin et al., 2002;), and this may also be true for D-inx-2 (K.D.C. and R.J.W., unpublished). Thus shakB(N) could form heteromeric GJs with D-inx-2 in lamina neurons. A third possibility is that the sequences specify interactions with non-innexin proteins in the cell.

Intracellular or transmembrane sequences could also affect biophysical properties or channel permeability to second messengers. For example, chimeric studies suggest that the first transmembrane domain of Cx46 confers its unique channel kinetics and single channel conductance properties (Hu and Dahl, 1999). Intracellular sequences of Cx26 and Cx30 also influence biophysical properties (Manthey et al., 2001).

Some of the crucial residues within shakB(N) that are likely to be required for shakB(N) identity are marked with asterisks in Fig. 3. Five of the six represent a change in charge. Four of these residues are found clustered just after the second transmembrane domain, with three of the four having a difference in charge. These differences in charge close to the transmembrane domain may be more consistent with a role for these sequences in biophysical or permeability differences between the proteins.

Interestingly, the total sequence difference between shakB(N) and ogre in the first 97 amino acids is much larger, even though these sequences are not crucial for shakB(N) 'identity'. Therefore overall homology is not a good indicator

of what portions of these proteins are functionally important for specificity. As a further example of this, the innexin proteins, as a family, differ most in the C-terminal tail, which plays no role in specificity in this assay.

Putative roles for the extracellular loop

For both shakB (N) and ogre, sequences in the first half of the protein, but not the second half, are essential for rescue. Possibly crucial residue differences between the two proteins in the intracellular loop have been identified (see above). In addition, some sequences N-terminal to the second transmembrane domain are also essential for ogre identity. We proposed that ogre-containing junctions in the retina mate with junctions in the lamina (Curtin et al., 2002). In that case, one might expect extracellular sequences in ogre to be crucial for specificity in mating with apposing hemi-junctions.

Other evidence suggests that mating specificity or innexin multimerization may be mediated by the first extracellular loop. ShakB(L) forms GJs when expressed alone in the paired *Xenopus* oocyte assay and is lethal when expressed pan-neurally (Stebbings et al., 2000; Phelan et al., 1998) (K.D.C. and R.J.W., unpublished). ShakB(N) does not form junctions when expressed alone in *Xenopus* oocytes and is not lethal when expressed pan-neurally (Phelan et al., 1998). Thus it seems that shakB(L) can form homomeric/homotypic GJs whereas shakB(N) cannot. Because shakB(N) and shakB(L) only differ from the beginning of the protein through the first putative extracellular loop (see Fig. 4) (Krishnan et al., 1995), some residues within this portion of protein probably mediate specificity of mating or polymerization.

Although shakB(N) and shakB(L) differ in the length of the N-terminal intracellular tail (see Fig. 4), this difference in length is not likely to contribute significantly to shakB(N) function. ShakB(N+16) is 16 amino acids longer at the very N-terminus than shakB(N), but otherwise identical and it is interchangeable with shakB(N) in the ERG rescue assay (data not shown). However, specific residues within the N-terminal tail could play a role in homo-multimerization of shakB(L) compared with shakB(N).

In the first extracellular loop, there are 20 residues that are dissimilar between shakB(N) and shakB(L). Eight of these are clustered together immediately prior to the second transmembrane domain and four of these eight lead to a change in charge (Fig. 4). A comparison of shakB(N) and ogre in this same stretch shows that 12 out of 15 residues preceding the transmembrane domain are different in the two proteins, and three of these represent a difference in charge (Fig. 3). It should be noted that shakB(N) and D-inx 7 also show divergence in this short stretch.

Neither ogre or shakB(N) proteins make homotypic GJs in the *Xenopus* oocyte assay (Phelan et al., 1998) (K.D.C., T. White, R.J.W. and D. Paul, unpublished). They also do not lead to lethality when expressed pan-neurally as do the known homotypic innexins, shakB(L) and D-inx-2 (Stebbings et al., 2000; Curtin and Wyman, 2002). Lastly, they do not make heterotypic or heteromeric GJs with each other in oocytes (K.D.C., T. White, R.J.W. and D. Paul, unpublished). Hence ogre and shakB(N) probably polymerize with other innexins to form gap junctions. Since they are not interchangeable, the required gene-specific sequences may be involved in heteromeric or heterotypic interactions. The first extracellular loop may be crucial for homo/heteropolymer formation within the cell membrane, or it may help determine which innexins recognize and mate with each other on apposing cells. In chimera studies on connexins, it is the second extracellular loop that has been suggested to play an important role in mating specificity to regulate what connexins can form heterotypic GJs (White et al., 1994).

The C-terminal tail

Although the C-terminal tail of the innexin proteins is not required for functional specificity in this assay, it is required for function. An *ogre* truncation lacking this tail fails to rescue mutants. When we overexpress shakB(N) in the nervous system with the *elav-Gal4* driver it localizes to the lamina neuropil roughly where synapses form (Curtin et al., 2002). Synaptic localization often requires an interaction of the C-terminal tail of a channel protein with an anchoring protein, such as a PDZ protein or other scaffold protein (e.g. Kim et al., 1995). Thus truncated ogre may not be correctly localized. Since we lack good antibodies we were unable to address this hypothesis directly. The C-terminal tail could also affect channel biophysics. Truncations of the cytoplasmic tail of Cx43 alter junctional conductance (Fishman et al., 1991), and the C-terminal tail of the shaker channel is important for voltage-gated inactivation (Miller, 1991).

The biological implications of a lack of innexin interchangeability

The lack of interchangeability of these proteins is an important finding. Developmental GJs, possibly occurring between pre- and post-synaptic cells, are required for development of normal neural transmission in the *Drosophila* visual system (Curtin et al., 2002). Specifically, both shakB(N) and ogre are required during development, with ogre required presynaptically and shakB(N) required, at a minimum, postsynaptically (Curtin et al., 2002). Both mutants show a functional failure of retina to lamina neural transmission as determined by the ERG as well as some retinal axon projection errors (Curtin et al., 2002). One hypothesis to explain this is that developmental GJs between pre- and postsynaptic cells may be necessary for final target selection and/or the development of functional chemical synapses (Curtin et al., 2002). During development, while searching for their final synaptic targets, retinal cell axons contact several neural types in the optic lamina. They interact with each other, with their synaptic partners and with other neurons and glia in the optic lamina. The selective formation of GJs between neurons via selective interaction or activity of innexin proteins could thus have important effects on target selection and synaptic specificity.

We are greatly indebted to Yi-An Sun for technical expertise and wise advice. We also thank Haig Keshishian for assistance and support. This work was funded by NIH grant # GM57889 and by the Charles E. Culpeper Foundation.

References

Alawi, A. A. and Pak, W. L. (1971). On-transient of insect electroretinogram: its cellular origin. *Science* **172**, 1055-1057.

Allen, F. and Warner, A. (1991). Gap junctional communication during neuromuscular junction formation. *Neuron* **6**, 101-111.

Barnes, T. M. and Hekimi, S. (1997). The *Caenorhabditis elegans* avermectin resistance and anesthetic response gene unc-9 encodes a member of a protein family implicated in electrical coupling of excitable cells. *J. Neurochemistry* **69**, 2251-2260.

Beblo, D. A. and Veenstra, R. D. (1997). Monovalent cation permeation through the connexin 40 gap junction channel: Cs, Rb, Na, Li, TEA, TMA, TBA, and effects of anions Br, Cl, F, acetate, aspartate, glutamate, and NO_3. *J. Gen. Physiol.* **109**, 509-522.

Bevans, G. G., Kordel, M., Rhee, S. K. and Harris, A. L. (1998). Isoform composition of Connexin channels determines selectivity among second messengers and uncharged molecules. *J. Biol. Chem.* **273**, 2808-2816.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Burg, M. G., Sarthy, P. V., Koliantz, G. and Pak, W. L. (1993). Genetic and molecular identification of a *Drosophila* histidine decarboxylase gene required in photoreceptor transmitter synthesis. *EMBO J*. **12**, 911-919.

Cao, F., Eckert, R., Elfgang, C., Nitsche, J. M., Snyder, S. A., Hulser, D. F., Willecke, K. and Nicholson, B. J. (1998). A quantitative analysis of connexin-specific permeability differences of gap junctions expressed in HeLa transfectants and *Xenopus* oocytes. *J. Cell Sci*. **111**, 31-34.

Cosens, D. J. and Briscoe, D. (1972). A switch phenomenon in the compound eye of the white eyed mutant of *Drosophila melanogaster*. *J. Insect Physiol.* **18**, 627-632.

Curtin, K. D., Zhang, Z. and Wyman, R. J. (1999). *Drosophila* has several genes for gap junction proteins. *Gene* **232**, 191-201.

Curtin, K. D., Zhang, Z. and Wyman, R. J. (2002). Gap junction genes expressed during development are required for adult neural function in the *Drosophila* optic lamina. *J. Neurosci.* (in press).

Fischbach, G. D. (1972). Synapse formation between dissociated nerve and muscle cells in low density cell cultures. *Dev. Biol.* **28**, 407-429.

Fishman, G. I., Moreno, A. P., Spray, D. C. and Leinwand, L. A. (1991). Functional analysis of human cardiac gap junction channel mutants. *Proc. Natl. Acad. Sci.* **88**, 3525-3529.

Heisenberg, M. (1971). Separation of receptor and lamina potentials in the electroretinogram of normal and mutant *Drosophila*. *J. Exp. Biol.* **55**, 85-100.

Homyk, T., Jr, Szidonya, J. and Suzuki, D. T. (1980). Behavioral mutants of *Drosophila melanogaster*. III. Isolation and mapping of mutations by direct visual observations of behavioral phenotypes. *Mol. Gen. Genet.* **177**, 553-565.

Hotta, Y. and Benzer, S. (1969). Abnormal electroretinograms in visual mutants of *Drosophila*. *Nature* **222**, 354-356.

Hu, X. and Dahl, G. (1999) Exchange of conductance and gating properties between gap junction hemi-channels. *FEBS lett.* **451**, 113-117.

Kim, E., Niethammer, M., Rothschild, A., Jan, Y. N. and Sheng, M. (1995). Clusterting of Shaker-type K+ channels by interaction with a family of membrane-associated guanylate kinases. *Nature* **378**, 85-88.

Krishnan, S. N., Frei, E., Swain, G. P. and Wyman, R. J. (1993). *Passover*: a gene required for synaptic connectivity in the giant fiber system of *Drosophila*. *Cell* **73**, 967-977.

Krishnan, S. N., Frei, E., Schalet, A. P. and Wyman, R. J. (1995). Molecular basis of intracistronic complementation in the *Passover* locus of *Drosophila*. *Proc. Natl. Acad. Sci. USA* **92**, 2021-2025.

Kumar, N. M. and Gilula, N. B. (1996). The gap junction communication channel. *Cell* **84**, 381-388.

Landesman, Y., White, T. W., Starich, T. A., Shaw, J. E., Goodenough, D. A. and Paul, D. L. (1999). *Innexin-3* forms connexin-like intercellular channels. *J. Cell Sci.* **112**, 2391-2396.

Lopresti, V., Macagno, E. R. and Levinthal, C. (1974). Structure and development of neuronal connections in isogenic organisms: transient gap junctions between growing optic axons and lamina neuroblasts. *Proc. Natl. Acad. Sci. USA* **71**, 1098-1102.

Lipshitz, H. D. and Kankel, D. R. (1985). Specificity of gene action during central nervous system development in *Drosophila* melanogaster: analysis of the *lethal (1) optic ganglion reduced locus*. *Dev. Biol.* **108**, 56-77.

Manthey, D., Banach, K., Desplantez, C. G., Kozak, C. A., Traub, O., Weingart, R. and Willecke, K. (2001). Intracellular domains of mouse connexin 26 and –30 affect diffusional and electrical properties of gap junction channels. *J. Membrane Biol.* **181**, 137-148.

Miller, C. (1991). 1990: annus mirabilis of potassium channels. *Science* **252**, 1092-1096.

Nicholson, S. M. and Bruzzone, R. (1997) Gap junctions: Getting the message through. *Curr. Biol.* **7**, R340-R344.

Pak, W. L., Grossfield, J. and White, N. V. (1969). Nonphototactic mutants in a study of vision of *Drosophila*. *Nature* **222**, 351-354.

Panchin, Y., Kelmanson, I., Matz, M., Lukyanov, K., Usman, N. and Lukyanov, S. (2000). A ubiquitous family of putative gap junction molecules. *Curr. Biol.* **10**, R473-R474.

Phelan, P., Nakagawa, M., Wilkin, M. B., Moffat, K. G., O'Kane, C. J., Davies, J. A. and Bacon, J. P. (1996). Mutations in *shaking-B* prevent electrical synapse formation in the *Drosophila* giant fiber system. *J. Neurosci.* **16**, 1101-1113.

Phelan, P., Stebbings, L. A., Baines, R. A., Bacon, J. P., Davies, J. A. and Ford, C. (1998). *Drosophila* Shaking-B protein forms gap junctions in paired *Xenopus* oocytes. *Nature* **391**, 181-184.

Plum, A., Hallas, G., Magin, T., Dombrowski, A., Hagendorff, A., Schumacher, B., Wolpert, C., Kim, J.-S., Lamers, W. H., Evert, M. et al. (2000). Unique and shared functions of different connexins in mice. *Curr. Biol.* **10**, 1083-1091.

Simon, A. M. and Goodenough, D. A. (1998). Diverse functions of vertebrate gap junctions. *Trends Cell Biol.* **12**, 477-483.

Spradling, A. C. and Rubin, G. M. (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. *Science* **218**, 341-347.

Starich, T. A., Lee, R. Y., Panzarella, C., Avery, L. and Shaw, J. (1996). Eat-5 and unc-7 represent a multigene family in *Caenorhabditis elegans* involved in cell-cell coupling. *J. Cell Biol.* **134**, 537-548.

Stebbings, L. A., Todman, M. G., Phelan, P., Bacon, J. P. and Davies, J. A. (2000). Two *Drosophila innexins* are expressed in overlapping domains and cooperate to form gap-junction channels. *Mol. Biol. Cell* **11**, 2459-2770.

Stowers, R. S. and Schwarz, T. L. (1999). A genetic method for generating *Drosophila* eyes composed exclusively of mitotic clones of a single genotype. *Genetics* **152**, 1631-1639.

Sun, Y. A. and Wyman, R. J. (1996). Passover eliminates gap junctional communication between neurons of the giant fiber system in *Drosophila*. *J. Neurobiol*. **30**, 340-348.

Swenson, K. I., Jordan, J. R., Beyer, E. C. and Paul, D. L. (1989). Formation of gap junctions by expression of connexins in *Xenopus* oocyte pairs. *Cell* **57**, 145-155.

Verselis, V. K., Ginter, C. S. and Bargiello, T. A. (1994). Opposite voltage gating properties of two closely related connexins. *Nature* **368**, 348-351.

Wang, H.-Z. and Veenstra, R. D. (1997). Monovalent ion selectivity sequences of the rat connexin 43 gap junction channel. *J. Gen. Physiol.* **109**, 491-507.

Watanabe, T. and Kankel, D. R. (1990). Molecular cloning and analysis of *l(1)ogre*, a locus of *Drosophila melanogaster* with prominent effects on the postembryonic development of the central nervous system. *Genetics* **126**, 1033-1044.

White, T. W., Bruzzone, R., Wolfram, S., Paul, D. L. and Goodenough, D. A. (1994) Selective interactions among multiple connexin proteins expressed in vertebrate lens: the second extracellular domain is a determinant of compatibility between connexins. *J. Cell Biol.* **125**, 879-892.

White, T. W. and Bruzzone, R. (1996). Multiple connexin proteins in single intercellular channels: connexin compatability and functional consequences. *J. Bioenerg. Biomemb.* **28**, 339-350.

Zhang, Z., Curtin, K. D., Sun, Y. A. and Wyman, R. J. (1999). Nested transcripts of gap junction gene have distinct expression patterns. *J. Neurobiol.* **40**, 288-301.