Title: Innexins Ogre and Inx2 are required in glial cells for normal postembryonic development of the Drosophila central nervous system

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Summary

Innexins are one of two gene families that have evolved to permit neighbouring cells in multicellular systems to communicate directly. Innexins are found in prechordates and persist in small numbers in chordates as divergent sequences termed pannexins. Connexins are functionally analogous proteins exclusive to chordates. Members of these two families of proteins may form intercellular channels, assemblies of which constitute gap junctions. Each intercellular channel is a composite of two hemichannels, one from each of two apposed cells. Hemichannels dock in the extracellular space to form a complete channel with a central aqueous pore that regulates the cell-cell exchange of ions and small signalling molecules. Hemichannels may also act independently by releasing paracrine signalling molecules. optic ganglion reduced (ogre) is a member of the Drosophila innexin family, originally identified as a gene essential for postembryonic neurogenesis. Here we demonstrate, by heterologous expression in paired Xenopus oocytes, that Ogre alone does not form homotypic gap-junction channels, however, co-expression of Ogre with Innexin2 (Inx2) induces formation of functional channels, with properties distinct from Inx2 homotypic channels. In the Drosophila larval CNS, we find that Inx2 partially co-localises with Ogre in proliferative neuroepithelia and in glial cells. Downregulation of either ogre or inx2 selectively in glia, by targeted expression of RNA interference transgenes, leads to a significant reduction in the size of the larval CNS and behavioural defects in surviving adults. We conclude that these innexins are critically required in glial cells for normal postembryonic development of the CNS.

Introduction

Intercellular communication via gap-junction channels is ubiquitous in the animal kingdom and involved in fundamental processes such as cell proliferation and differentiation, metabolic homeostasis and electrical coupling that are essential for normal tissue development and function. Defective gap-junctional communication is implicated in several human diseases including peripheral neuropathies, deafness and cataract (Wei et al., 2004). At the molecular level, gap junctions are assembled from the products of two gene families. Innexins (Inx), originally identified in Drosophila melanogaster (Phelan et al., 1998) and Caenorhabditis elegans (Landesmann et al., 1999), are the only known gap-junction genes in
prechordate organisms (Phelan, 2005). Divergent innexins, termed pannexins (Panx), are present in small numbers in chordates, including humans (Panchin et al., 2000; Bruzzone et al., 2003; Yen and Saier, 2007), where the bulk of gap junctions are formed from members of the connexin (Cx) family (White and Paul, 1999). Innexins and connexins have the same membrane topology: four transmembrane domains interconnected by two extracellular, and a single intracellular, loop(s) and intracellular N- and C-termini. The proteins assemble into multimeric hemichannels; hemichannels in neighbouring cell membranes dock in the intercellular space to form a complete channel with a central aqueous pore. Hemichannels may be homomeric or heteromeric, composed of one, or more than one, protein species, respectively. These may assemble three possible intercellular channel types: homomeric homotypic channels in which the two hemichannels are identical in subunit composition (hereafter referred to as homotypic), homomeric heterotypic channels in which each hemichannel is composed of a different innexin species (hereafter referred to as heterotypic) and heteromeric channels in which one, or both, hemichannel(s) contain(s) a mix of two or more different proteins.

optic ganglion reduced (ogre) is one of eight innexin genes in Drosophila melanogaster. It was discovered by conventional mutagenesis screening and implicated in neural development (Lipshitz and Kankel, 1985). Mutant flies had small nervous systems; in particular, the optic lobes were significantly smaller than those of wild type and the neural architecture highly disorganized. This phenotype was attributed to defects in postembryonic neurogenesis (Lipshitz and Kankel, 1985; Watanabe and Kankel, 1992). This wave of neurogenesis begins in early larvae with the reactivation of quiescent embryonic neuroblasts. These divide to produce new adult-specific neurons which, together with re-modelled embryonic neurons, form the adult CNS. Glial cells develop simultaneously and play an important part in supporting and compartmentalizing neurons (Younossi-Hartenstein et al., 2003; Awasaki et al., 2008). Since the initial work on the ogre gene, which pre-dated its identification as a member of the innexin family, there have been no further analyses of the role of this or other innexins in early postembryonic neural development.

In this study, we examine the competency of Ogre to form gap-junction channels by heterologous expression in Xenopus oocytes. We show that this innexin does not form homotypic intercellular channels but may interact with Inx2 in the same cell to regulate intercellular coupling. We describe the expression pattern of these two innexins in the early larval CNS and explore their functional significance by investigating the consequences of cell-specific downregulation of innexin expression.
Results

Expression of ogre and inx2 in Xenopus oocytes

To investigate the ability of Ogre protein to form gap-junction channels, we expressed the corresponding RNA in paired Xenopus oocytes and recorded channel activity by dual voltage clamp electrophysiology. Cell pairs in which both cells express Ogre fail to form functional channels. Stepping one cell through a series of hyperpolarising and depolarising transjunctional voltage (Vj) steps (Fig. 1A) does not elicit junctional currents in its paired neighbour (Fig. 1B; Table 1). Junctional conductance (gj) in these cells equates that in water-injected control oocytes (Fig. 1F; Table 1). RNA amounts of up to 20 ng were tested with no evidence of coupling. Thus, Ogre does not form homotypic channels. Inx2 is a good candidate for potential functional interactions with Ogre as transcripts encoding these proteins frequently are co-expressed in Drosophila tissues (Stebbings et al., 2002; this study).

Inx2 previously was shown to form voltage-sensitive homotypic channels in oocytes (Stebbings et al., 2000) and this is confirmed in Fig. 1C. Figs. 1D and E are recordings from cell pairs co-expressing Ogre with Inx2 in different configurations. Oocyte pairs in which one cell expresses Ogre and the other Inx2 are not electrically coupled (Fig. 1D); the average gj of Ogre/Inx2 pairs is not significantly different from that of water-injected pairs (Table 1), demonstrating that the proteins do not form heterotypic channels. Co-expression of Ogre and Inx2 in both cells of a pair, on the other hand, results in the formation of functional channels (Fig. 1E) with an average gj slightly, although not statistically, higher than that of Inx2/Inx2 pairs (Table 1). The voltage properties of these channels differ significantly from Inx2 homotypic channels. The latter show steep voltage sensitivity; steady state junctional current (Ij) declines in a time and voltage-dependent manner for Vj ≥ 10 mV (Fig. 1C and Gj/Vj plot, Fig. 1G, squares). Channels induced in cell pairs in which both cells express Ogre and Inx2 show very weak or no voltage sensitivity (Fig. 1E and G, triangles). In the trace shown (Fig. 1E), Ijs are linear at Vjs of 10-40 mV and show a slight decline at higher Vjs. This is the maximum level of voltage sensitivity observed; in several cell pairs there was no evidence of a voltage response as Ijs were linear at all Vjs.

To determine whether Ogre and Inx2 form non-junctional hemichannels, the RNAs were expressed in single oocytes which were recorded using two-electrode voltage clamp. The current/voltage (Im/Vm) relationships are shown in Fig. 1H. At RNA amounts (5ng),
equivalent to those expressed in the paired cells (Fig. 1A-G), membrane currents recorded in
*ogre*-injected cells (Fig. 1H, filled circles) were not significantly different from those in
water-injected controls (Fig. 1H, open circles). By contrast, cells injected with *inx2* (Fig. 1H,
squares) or both *ogre* and *inx2* (Fig. 1H, triangles) exhibited non-junctional currents, the
magnitude of which was greater in cells expressing both innexins. Maximum currents are
shown in Fig. 1H; *I_m* exhibited modest voltage sensitivity which was more marked in Inx2,
than in Ogre and Inx2-expressing cells. Although high amounts of *ogre* RNA failed to induce
intercellular coupling in paired cells, raising the amount injected (to 10 ng) in single cell
recordings did induce non-junctional currents, albeit unreliably (data not shown).

The observed differences in the properties of intercellular channels and hemichannels
in cells expressing Inx2 alone and those expressing Inx2 and Ogre are indicative of a
functional interaction between these proteins.

**Characterisation of innexin antibodies**

Affinity purified polyclonal antibodies raised to unique C-terminal peptides of Ogre and Inx2
(Fig. 2A) were characterised by Western blotting and immunofluorescence microscopy. In
Western blots of *Drosophila* larval nervous systems, anti-Ogre and anti-Inx2 recognise single
bands, which are eliminated by pre-incubation of the antibodies with an excess of the
corresponding unlabelled peptides (Fig. 2B). The proteins migrate to ~37 kDa, slightly below
their deduced molecular mass of 42 kDa; this is consistent with previous reports of slightly
anomalous migration of innexins in SDS-PAGE (Stebbings et al., 2000; Phelan et al., 1998;
2008) and may be accounted for by the hydrophobic nature of these proteins (Rath and
Deber, 2013). Since Ogre and Inx2 proteins are of identical size, the specificity of the
antibodies in Western blot was examined by expressing the corresponding RNAs in *Xenopus*
oocytes, which lack endogenous innexins, and probing these cells with the antibodies to
detect translated protein. Fig. 2C shows Western blots of oocyte membrane fractions labelled
with anti-Ogre (left panel) and anti-Inx2 (right panel). Anti-Ogre detects a ~37 kDa band in
cells expressing *ogre* RNA (Fig. 2C, left panel, lane 1) or an equal mix of *ogre* and *inx2*
RNAs (Fig. 2C, left panel, lane 3). This band is not detectable when *inx2*-expressing cells
(Fig. 2C, left panel, lane 2) or water-injected control cells (Fig. 2C, left panel, lane 4) are
probed with anti-Ogre, demonstrating that this antibody is specific for Ogre protein.
Similarly, anti-Inx2 detects a band at ~37 kDa in cells expressing *inx2* RNA (Fig. 2C, right
panel, lane 2) or a mixture of *inx2* and *ogre* RNAs (Fig. 2C, right panel, lane 3) but does not
label a corresponding band in cells expressing *ogre* (Fig. 2C, right panel, lane 1) or in water-
injected control cells (Fig. 2C, right panel, lane 4), confirming that this antibody is specific for Inx2 protein. Bands common to RNA-injected, and water-injected, cells are non-specific. Higher molecular mass bands may be innexin oligomers (see Fig. 2 legend).

Validation of the antibodies for immunofluorescence labelling of whole tissues was demonstrated by peptide competition (Fig. 2D) and RNAi-mediated knockdown of innexins (Fig. 6). Fig. 2D shows that the characteristic pattern of labelling seen with anti-Ogre (upper left panel) and anti-Inx2 (lower left panel) in early larval nervous systems (described in detail, Figs. 3-5) is abolished when the tissues are incubated with antibody in the presence of the corresponding peptide (Fig. 3D, right panels). Fig. 6 (described in detail below) demonstrates that knockdown of ogre or inx2 in glial cells by RNA interference (RNAi) dramatically reduces anti-Ogre (Fig. 6A, B) or anti-Inx2 (Fig. 6E, F) labelling, respectively, in these cells, further demonstrating that the antibodies recognise their target proteins.

Localisation of Ogre and Inx2 in the Drosophila CNS

Given the known role of ogre in postembryonic neurogenesis (Lipshitz and Kankel, 1985), we were interested to establish the potential of Inx2 to act with Ogre in this process. We, therefore, examined the relative distribution of the two proteins in larval nervous systems, 24-40 hours after larval hatching (ALH). At this stage of development, optic lobe neural precursors are actively dividing in two proliferative neuroepithelia. The outer optic proliferation centre (opc) gives rise to neurons of the adult lamina and distal medulla, which are the targets of retinal photoreceptors. The inner proliferation centre (ipc) produces neurons of the proximal medulla and the lobula complex, which interconnect the optic lobes and central brain (Hofbauer and Campos-Ortega, 1990). Watanabe and Kankel (1992) demonstrated that Ogre is expressed strongly in the opc and weakly in the ipc. Our data confirm these findings. The outer proliferation centre forms a characteristic dome-shaped structure with a central hole through which the optic stalk enters the brain. The ribbon-like inner proliferation centre lies medial to the opc (Fig. 3A-I). Ogre expression is clearly apparent in cells of the opc, with weaker expression in the ipc (Fig. 3A, D, G). Inx2 is also found in the proliferation centres with similar levels of expression in the opc and ipc (Fig. 3B, E, H). Both innexins localise predominantly to the plasma membrane (arrows, E, H) and, as the merged images (Fig. 3C, F, I) indicate, show significant overlap in expression. Ogre and Inx2 are also widely expressed and partially co-localise in other cells of the brain lobes (Fig. 3G-L) and the ventral ganglion (Fig. 3M-O). The pattern of expression is suggestive of glial cells, rather than neurons (confirmed in Figs. 4, 5).
To assess further the extent of overlap of Ogre and Inx2 signals in double labelled images, co-localisation analysis was performed. Calculated values of Pearson’s Correlation Coefficient (PCC) and the Intensity Correlation Quotient (ICQ) are consistent with one another and are given in Table S1. Highest correlation is observed for the cells along the ventral ganglion midline (arrowheads, Fig. 3N; PCC, 0.87), suggestive of near perfect co-localisation. PCC values for the OPC (0.52)>IPC (0.39) and presumptive glia of the brain lobes (0.53) are intermediate but nonetheless indicative of reliable partial co-localisation. Values for the proliferation centres may be underestimates as the whole region, which includes unstained cells, was quantified. There is no correlation between Ogre and Inx2 staining in the Ogre-positive bilateral puncta in the ventral ganglion (arrows, Fig. 3M; PCC 0.06) consistent with visual inspection of the merged images; coefficients are similar to those of unstained regions of the CNS (Table S1).

To confirm that the innexin-expressing cells outside of the proliferation centres are indeed glia, and not neurons, the GAL4-UAS system was used to mark specific cells with GFP. UAS-\textit{mCD8::GFP}, a GFP fusion which localises to the plasma membrane, was expressed under the control of \textit{repo}-GAL4, to label glial cells, or \textit{elav}-GAL4, which drives expression in all postmitotic neurons. Larval nervous systems from these transgenic flies were double labelled with innexin and GFP antibodies (Figs. 4, 5). Repo expression, which is particularly well characterised in embryos, marks most glial cells, exceptions being the mesectoderm-derived ventral midline glia and a subset of nerve root glia (Halter et al., 1995; Stork et al., 2012). Fig. 4 shows innexin expression in relation to that of Repo in the CNS of early larvae. Both Ogre (Fig. 4A-F) and Inx2 (Fig. 4G-L) significantly co-localise with \textit{repo}-driven GFP, indicating that the proteins accumulate at the plasma membrane of glial cells. The distribution of innexin staining in the brain lobes (Fig. 4A-C and G-I) and ventral ganglion (Fig. 4D-F and J-L) suggests that cells in each of the broad categories of surface, cortex and neuropile glia (Hartenstein et al., 1998; Ito et al., 1995; Beckervordersandforth et al., 2008; Dumstrei et al., 2003) are labelled. Both proteins are present in prominent Repo-positive cells along the midline of the ventral ganglion (Fig. 4D-F and J-L, arrowheads in F, L); these are possibly channel (surface) glia that line the dorsoventral channel although their identity has not been confirmed using specific markers. Ogre alone is found in a bilaterally symmetrical row of Repo-negative puncta in the ventral ganglion (Fig. 4D-F, arrows in E; Fig. 3M, arrows); this possibly represents staining in the tracheal system, which develops in contact with glial cells. Clusters of Repo-negative, innexin-positive, cells visible in the brain
lobes (Fig. 4C) are likely to be scattered neuroblasts that are known to express Ogre (Watanabe and Kankel, 1992).

Neither Ogre (Fig. 5A-F) nor Inx2 (Fig. 5G-L) is expressed in Elav-positive neurons including cortical neurons on the surface of the brain lobes (Fig. 5A, arrowheads), mushroom body neurons deep in the lobes (Fig. 5G, arrowheads) and motor neurons of the ventral ganglion (Fig. 5D, J, arrowheads). Fig. 5 confirms some of the glial localisation of innexins seen in Figs. 3 and 4 and additionally shows expression of Ogre and Inx2 in glia associated with the axons of neurons exiting the ganglion (Fig. 5D-F and J-L, arrowheads in E-F and K-L).

Downregulation of *ogre* and *inx2* in the CNS

The strong expression of Ogre and Inx2 in glial cells of the larval CNS (Fig. 4) suggests that the proteins may be required in these cells for normal postembryonic development. To establish if this is the case, the GAL4-UAS system was coupled with RNAi to knockdown the expression of the proteins in a cell specific manner. Fig. 6 shows the consequences of repo-GAL4 directed expression of UAS-ogreRNAi or UAS-inx2RNAi specifically to glial cells. Ogre knockdown significantly attenuates protein levels in glial cells of the developing CNS; reduction of expression is less marked in cells near the midline of the ventral ganglion than elsewhere in the ganglion and brain lobes (Fig. 6A, B). The protein persists in non-targeted cells, including the optic proliferation centres (Fig. 6B, arrow) and the CNS-associated ring gland (rg, Fig. 6B). The loss of Ogre protein in transgenic flies is associated with a reduction in the size of the CNS of larvae (Fig. 6A, B) and surviving adults (Fig. 6C, D) and a variety of behavioural defects (see Movie 1, Supplementary Material). *ogre* knockdown adult flies exhibit impaired locomotor activity, characterised by inability to fly, difficulty walking and circling behaviour. Grooming behaviour is uncoordinated, indicative of sensorimotor defects. In extreme cases, the fly is unable to right itself and lies on its back continuously and rapidly moving its legs. These flies generally die within a few days of eclosion. The consequence of *inx2* knockdown (Fig. 6E, F) is remarkably similar to, albeit more severe than, that seen with *ogre* knockdown. Targeted expression of *inx2RNAi* to glial cells effectively reduces levels of Inx2 protein specifically in these cells and results in a dramatic reduction in the size of the developing nervous system (Fig. 6E, F). Typically, the early larval CNS of *inx2* knockdown flies is smaller than that of *ogre* knockdown flies at the same stage (compare Fig. 6B and F). Unlike *ogre* knockdown flies, transgenic flies with reduced glial Inx2 expression fail to eclose. Quantification of fluorescence intensity in immunolabelled nervous systems from
control and knockdown larvae reveals a 41% decrease in intensity in flies expressing the
\textit{ogreRNAi} transgene (Fig. 6G). This is associated with reductions of 27% and 33% in the size
of the larval brain (Fig. 6H, upper panel) and adult CNS (Fig. 6H, lower panel), respectively,
as indicated by measurements of surface area. In flies expressing \textit{inx2RNAi} transgenes, a 68%
reduction in immunofluorescence intensity (Fig. 6G) is coupled with a 51% reduction in
larval brain size (Fig. 6H). The more severe phenotype observed upon downregulation of
\textit{inx2} than of \textit{ogre} may reflect the level of knockdown or a greater requirement for Inx2 than
Ogre at early larval stages. The former is supported, in part, by the fact that \textit{ogre} null
mutants, lacking all functional protein, die as larvae (Lipshitz & Kankel, 1985).

In addition to knocking down \textit{ogre} and \textit{inx2}, we used \textit{repo-GAL4} to drive expression
of UAS-\textit{RNAi} constructs to all other innexins with the exception of \textit{inx7}, which is required in
subsets of glia in the embryonic CNS (Ostrowski et al., 2008) and \textit{inx4}, which is germ cell
specific (Tazuke et al., 2002). No phenotype was observed in flies expressing \textit{shakB}, \textit{inx3},
\textit{inx5} or \textit{inx6 RNAi} transgenes in glia (data not shown). In the case of Inx3, this was somewhat
surprising as its expression partially overlaps that of Ogre and Inx2 in the larval CNS (our
unpublished observations). These data suggest that these proteins are not critically required in
glia and, importantly, that the \textit{ogre} and \textit{inx2} knockdown phenotypes cannot be attributed to
off-target effects of the \textit{RNAi} constructs on related proteins. Specificity of the \textit{ogreRNAi}
transgene for its intended target is further evidenced by the similarity in phenotype of \textit{ogre}
knockdown flies (Fig. 6C-D) and \textit{ogre} loss-of-function mutants (Lipshitz and Kankel, 1985;
see Discussion).

Consistent with the finding that Ogre and Inx2 are not expressed in neurons,
transgenic flies expressing UAS-\textit{ogreRNAi} or UAS-\textit{inx2RNAi} under the control of \textit{elav-}
\textit{GAL4} complete development and the size and gross morphology of the adult CNS is normal.
Interestingly, although both innexins are expressed in the optic proliferation centres,
preliminary studies in which the GAL4 driver \textit{c855a} (Egger et al., 2007) was used to target
\textit{RNAi} constructs to these cells did not yield an obvious phenotype (data not shown). This
could be accounted for by inadequate levels of knockdown, the proteins having subtle roles
or by genetic redundancy, whereby the loss of one innexin is compensated for by the
presence of another. Further studies will be required to address the function of the proteins in
these cells.
Discussion

The genome of Drosophila has eight innexin encoding loci, transcripts from seven of which, shakB, ogre, inx2, inx3, inx5, inx6 and inx7, are found at some stage in the nervous system. Knowledge of precise spatial and temporal patterns of expression and functions of the individual genes is far from complete. ShakB (Phelan et al., 1996; 2008; Trimarchi and Murphey, 1997), Inx6 and Inx7 (Wu et al., 2011) mediate electrical signalling in defined adult neural circuits. These and the other innexins are widely and dynamically expressed during development (Stebbings et al., 2002) and previous studies have highlighted roles for Ogre (Lipshitz and Kankel, 1985; Curtin et al., 2002), ShakB (Curtin et al., 2002) and Inx7 (Ostrowski et al., 2008) in neural development. The key findings of this study are that Ogre and Inx2 are required in glia for normal postembryonic development of the CNS. Their expression patterns in these cells partially overlap and, in vitro, channel activity of one protein is influenced by the presence of the other. Thus, the proteins may act independently and/or in concert.

Ogre and Inx2 act co-operatively in vitro

Paired Xenopus oocytes have been used widely as a heterologous system to examine the ability of proteins to form gap-junction channels. In this system, Inx2 forms homotypic channels. Previous studies demonstrated that the properties of these channels are influenced by co-expression of Inx3, which itself is not competent to form intercellular channels (Stebbings et al., 2000). Here we demonstrate that Ogre, like Inx3, does not form intercellular channels independently, but when co-expressed also modifies intercellular conductance in Inx2-expressing cells. Specifically, co-expression of Ogre with Inx2 in both cells of a pair reduces the voltage sensitivity, without affecting the mean level, of intercellular conductance. When expressed in single oocytes, magnitude of hemichannel currents vary with Ogre+Inx2>Inx2>Ogre. There are a number of speculative mechanisms that may explain these findings. Trafficking of Ogre to the membrane may be aided by Inx2; once at the membrane, Ogre, like Inx2, may form homomeric hemichannels and homotypic intercellular channels. Recordings from co-expressing cells would then reflect the presence of two distinct populations of channels. Alternatively, Ogre and Inx2 may preferentially assemble heteromeric hemichannels that dock to form intercellular channels with properties distinct from Inx2 homotypic channels. We favour the latter because even at high levels of RNA ogre did not induce homotypic intercellular channels although increasing the amount of RNA
injected did induce non-junctional currents. There is also some precedent for such a
mechanism as Inx2 and Inx3 have been shown to form hetero-oligomers (Lehmann et al.,
2006).

Ogre and Inx2 are expressed in neural precursors and glial cells

Previous studies demonstrated that Ogre is expressed in the larval optic lobe proliferation
centres (Watanabe and Kankel, 1992). This is confirmed here and we find that Inx2 is also
expressed in the optic anlage. The two innexins show significant overlap particularly in the
opc and to a lesser extent in the ipc. Neither innexin is found in postmitotic neurons. By
contrast, both proteins are extensively expressed, in a largely overlapping pattern, in several
populations of glia cells in the larval brain lobes and ventral ganglion. The presence of two
Inxs in highly overlapping patterns in different cell types in the developing CNS prompts
several questions. Are both required for normal development? If so, in which cell types do
they act and what is the mechanism of action?

Ogre and Inx2 are required in glial cells for postembryonic neural development

We have demonstrated, by cell-specific knockdown, that Ogre and Inx2 are critically required
in glial cells for normal development of the postembryonic CNS. Downregulation of the
expression of either protein specifically in glia leads to a marked reduction in the size of the
larval CNS. In the case of Inx2, the flies die as larvae. Flies with reduced glial Ogre
expression, on the other hand, develop to eclosion and survive briefly despite a small and
morphologically abnormal CNS. Loss of ogre function in surviving adults leads to defective
locomotor and sensorimotor activity. The behavioural phenotypes (see Movie 1) presumably
are due to the failure of adult neural circuitry to develop and are reminiscent of those seen in
various neural degeneration or neural wiring mutants. Circling behaviour was first described
in pirouette mutants and associated with degeneration of the brain, particularly the optic lobes
(Eberl et al., 1997). Abnormal grooming is seen in flies with mutations in various molecules
involved in axon growth and synaptogenesis (Phillis et al., 1993). Flies with mutations in the
cyclin-dependent kinase activator, p35, have defects in axon patterning at early
developmental stages and as adults exhibit a supine phenotype (inability to right themselves)
(Connell-Crowley et al., 2007).

This is the first study to implicate inx2 (which is involved in epithelial
morphogenesis; Bauer et al., 2002, 2004) in neural development. ogre has a known role in
postembryonic neurogenesis from early studies of Lipshitz and Kankel (1985) that
characterised the phenotype of various mutations at the *ogle* locus. The major defect was a reduction in the size of the CNS, particularly the optic lobes, the extent of which correlated with the severity of the mutation. Hypomorphic mutants survived to adulthood but with highly abnormal neuronal architecture, at least in the visual system which was the focus of analysis. The CNS of putative null mutants was even smaller than that of hypomorphs and these flies died as late larvae or pupae. The site of *ogle* activity was mapped to the CNS but not to specific cell types therein (Lipshitz and Kankel, 1985). The strong expression of the protein in the wild type optic proliferation centres and scattered neuroblasts (Watanabe and Kankel, 1992; also this study), coupled with the profound reduction in the size of the nervous system in *ogle* mutants (Lipshitz and Kankel, 1985; Sing et al., 1989), was consistent with a requirement for the gene in the neural precursors. Interestingly, genetic mosaic analysis provided some evidence of cellular non-autonomy of the phenotype within the optic lobes (Lipshitz and Kankel, 1985). Our data support the latter in demonstrating that downregulation of *ogle* in glial cells largely reproduces the *ogle* mutant phenotype. Downregulation of *inx2* produces a remarkably similar phenotype. The accumulated data lead to the conclusion that these two innexins act in glial cells to regulate postembryonic neurogenesis and, quite possibly, subsequent stages of neuronal development.

**How do gap-junction proteins regulate postembryonic development of the CNS?**

Classically, gap-junction proteins act by forming intercellular channels that permit direct transfer of molecules between coupled cells. Increasingly, there is evidence that these proteins also have additional functions ranging from the formation of functional hemichannels that release signalling molecules such as ATP (Goodenough and Paul, 2003; Spray et al., 2006; Huang et al., 2007; Thompson et al., 2008; Samuels et al., 2010) to acting as cell adhesion molecules (Elias et al., 2007; Starich et al., 2003). In considering how glial-expressed Ogre and Inx2 might regulate neuronal development it is possible to hypothesize both junctional and non-junctional mechanisms, which are not mutually exclusive. Intercellular and/or hemichannel communication among neighbouring glial cells may be critical for development and maintenance of the extensive glial network which, in turn, supports neurons. Alternatively, the primary role of glial innexins may be in transfer of signals from these cells to developing neurons. The former is an attractive hypothesis as glial cells are known to regulate neuronal development through provision of signalling molecules including Anachronism and Perlecan (Ebens et al., 1993; Caldwell and Datta, 1998; Park et al., 2001; Voigt et al., 2002), E-cadherin (Dumstrei et al., 2003) and TGF-β family members.
Myoglianin, (Awasaki et al., 2011). However, both scenarios are possible and distinguishing these are key questions for future studies.

Materials and Methods

RNA synthesis, expression in Xenopus oocytes, and electrophysiology

DNA fragments containing the complete coding region of ogre or inx2 were subcloned into the plasmid vector pSPJC2L. Plasmids were linearised by restriction digestion with NotI (ogre-pSPJC2L) or XhoI (inx2-pSPJC2L) and transcribed in vitro from the SP6 promoter in the presence of m\(^7\)G(5')ppp(5')G (transcription kit and reagents from Roche Applied Science, UK) to produce capped RNAs.

Stage V-VI oocytes were isolated from Xenopus laevis, microinjected and cultured (Swenson et al., 1989; Phelan et al., 2008; Marziano et al., 2011) as single cells with the vitelline membrane intact (for measurement of non-junctional coupling) or cell pairs formed after removal of the vitelline membrane (for measurement of intercellular coupling). Recordings were made 24-48 hr after RNA injection. Each cell was impaled with two borosilicate glass electrodes (1-4 m\(\Omega\)) and recorded using single-cell (DePriest et al., 2011) or dual-cell (Spray et al., 1981; Phelan et al., 1998, 2008) two electrode voltage clamp. Details of the recording protocols, which applied sequential hyperpolarising and depolarising pulses, are provided in the legend to Fig. 1. Paired cell recording protocols which did not involve reversal of pulse polarity yielded the same results. Recordings were made using two GeneClamp500 amplifiers (paired cells) or an Axoclamp-2B amplifier (single cells) interfaced via a Digidata 1320A or 1322A digitizer (Axon Instruments/Molecular Devices, USA) to a PC. Data were acquired and analysed in pClamp software (versions 9.0 or 10.0; Axon Instruments) and graphed in Origin 7 (Silverdale Scientific Ltd, UK).

For Western blotting, oocytes were fractionated, 24-48 hr after RNA injection, using the ‘sucrose-cushion’ method (Colman, 1984).

Antibody generation and purification

Polyclonal antibodies were raised in rabbits (Neo-Multiple Peptide Systems Inc, San Diego, USA) to synthetic peptides corresponding to C-terminal amino acids 351-362 of Ogre (KQVEPSKHIDRAK) and 349-367 of Inx2 (LSREMSGDEHSAHKRPFD). Peptides were conjugated, via an added cysteine residue, to keyhole limpet haemocyanin for immunisation.
Antibodies were affinity purified on columns of peptide linked to SulfoLink gel (Pierce Products, ThermoFisher Scientific, UK).

**Gel electrophoresis and Western blotting**

SDS-polyacrylamide gel electrophoresis and Western blotting were carried out using the Mini-Protean II system (Bio-Rad Laboratories Ltd, UK). Proteins were electrophoretically separated on 10-12.5% gels in standard Tris-glycine buffer and transferred to nitrocellulose paper (Hybond-ECL; Amersham, GE Healthcare Life Sciences, UK) in CAPSO buffer (Phelan et al., 1998). Western blots were incubated in blocking buffer (Phelan et al., 1998) prior to labelling with innexin antibodies (anti-Ogre, 1:50 or anti-Inx2, 1:1000 in blocking buffer) followed by anti-rabbit peroxidase-conjugated secondary antibody (Sigma Aldrich or Dako UK Ltd; 1:1000 in blocking buffer). In peptide competition experiments, anti-Ogre and anti-Inx2 antibodies were pre-incubated with synthetic Ogre (100 µM) and Inx2 (10 µM) peptides, respectively, for 30 minutes prior to labelling. Blots were washed 2 x 20 min in PBS containing 0.1% Tween-20 (PBT-W), with an intervening 20 min wash in 0.5 M NaCl in PBT-W, between primary and secondary antibodies and prior to development with ECL-Plus (Amersham).

**Immunofluorescence labelling**

Nervous systems, dissected in *Drosophila* saline, were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature and washed in PBS (Phelan et al., 1996). Prior to labelling, fixed whole-mount preparations were incubated for a minimum of 2 hr at room temperature in blocking buffer (Phelan et al., 2008). Antibodies, diluted in blocking buffer, were applied overnight at 4°C (primary antibodies) or for 2 hr at room temperature (secondary antibodies). Preparations were washed in PBS containing 0.5% Triton X-100 (6 x 10 min) between antibody incubations.

For single labelling, preparations were incubated in rabbit anti-Ogre (1:10) or anti-Inx2 (1:1000), followed by goat anti-rabbit Alexa Fluor secondary antibody (Molecular Probes, Life Technologies Ltd, UK; 1:500). In peptide competition experiments, primary antibodies were pre-incubated with excess peptide as described above for Western blot. For double labelling with anti-Ogre and anti-Inx2, preparations were sequentially labelled with anti-Ogre (1:10) and goat anti-rabbit Alexa Fluor 633 (1:500) followed by anti-Inx2 (1:40) conjugated to Zenon goat anti-rabbit Alexa Fluor 488 (Molecular Probes). The ratio of Zenon reagent to primary antibody and the final working dilution of the conjugate were determined.
empirically, using the manufacturer’s instructions as a guide. An approximately 25-fold increase in anti-Inx2 concentration was required to achieve the same signal with Zenon, as with conventional, labelling. Zenon-labelled preparations were post-fixed for 20 min in 4% paraformaldehyde. For double labelling with innexin and GFP antibodies, preparations were incubated simultaneously with rabbit anti-Inx (anti-Ogre, 1:10 or anti-Inx2, 1:1000) and mouse anti-GFP (Santa Cruz, Insight Biotechnology Ltd, UK; 1:100) followed by simultaneous application of secondary anti-rabbit Alexa Fluor 633 (1:500) and anti-mouse Alexa Fluor 488 (1:500).

Labelled preparations were dehydrated in glycerol and slide mounted in Vectashield (Vector Laboratories Ltd, UK).

Microscopy and Image Analysis

Fluorescently labelled preparations were imaged with a Leica TCS SP2 confocal microscope (Leica Microsystems (UK) Ltd). Unlabelled preparations were viewed and photographed under Nomarski optics (Leica DMR, IM50 software). Composite images were prepared in Adobe Photoshop 6.0 (Adobe, UK). Images were analysed in ImageJ (NIH, Bethesda, USA). Measurements of fluorescence intensity and surface area were made on maximum projections of confocal z-stacks. Intensity was calculated as the integrated density per unit area of the CNS preparation corrected for background, which was measured in the area around the preparation devoid of fluorescence. Surface area measurements were made to scale. Co-localisation analysis (Li et al., 2004) was performed using the ICA Plugin for WCIF ImageJ on confocal z-projections or single images. After background subtraction, Pearson’s Correlation Coefficient (PCC) and the Intensity Correlation Quotient (ICQ) were calculated for selected regions of interest as measures of overlap of red (Ogre) and green (Inx2) channels. As controls, the above parameters were quantified for unstained regions of the CNS and Co-localisation Tests were run to compare PCC values for the original red and green images (Robs) with values for one of the pair of images tested against multiple scrambled (randomized) versions of the other (Rrand). In all cases, %Robs>Rrand was 100%, providing confidence that positive measures of co-localisation did not arise by chance.

Drosophila Stocks

UAS-mCD8::GFP [#5137], c855a-GAL4 [#6990] and elav-GAL4 [#8765] were from the Bloomington Drosophila Stock Center, USA. UAS-inxRNAi lines were from the Vienna Drosophila Resource Center. Specificity scores for these are high: 0.98 for UAS-ogreRNAi
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[7136] and 1.0 for UAS-inx2RNAi [102194], where 1 predicts no off-target hits, and they contain few CAN triplet repeats, minimizing the likelihood of off-target effects (Dietzl et al., 2007). repo-GAL4 lines [repo-GAL4/TM6B,Tb and repo-GAL4/TM3,Sb] were gifts from J. Albert and I. Salecker, respectively.

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Author Contributions

CEH, Xenopus oocyte paired cell electrophysiology; WHL, RAB, hemichannel recordings; PP, WDJ, KB, phenotypic analysis of transgenic flies; PP, immunolabelling and microscopy, design and supervision of the project, and manuscript preparation.

References


Figure Legends

Fig. 1. Differential properties of junctional and non-junctional conductance in *Xenopus* oocytes expressing Inx2 and Ogre, individually or in combination. *Xenopus* oocytes were microinjected with *ogre* (5 ng), *inx2* (5 ng) or a mix of *ogre* and *inx2* (5 ng of each) RNAs. (A-G) Cells were paired in homotypic or heterotypic configuration and recorded by dual cell voltage clamp. Both cells of a pair were clamped at a holding potential (Vh) of -40 mV. One cell (cell 1) was then depolarised or hyperpolarised in 10 mV steps (5 sec duration) to generate transjunctional voltage (Vj = V1-Vh, mV). Simultaneously, the current (I2, nA) required to maintain the other cell (cell 2) at the holding potential was recorded. Junctional conductance (gj, μS) is junctional current (Ij = –I2)/Vj. (A-F) Representative traces from paired cell recordings. (A) Voltage step protocol. (B) Pairs in which both cells express Ogre fail to form functional intercellular channels. (C) Expression of Inx2 in both cells of a pair induces the formation of channels that are strongly sensitive to voltage; steady state gj decreases in a time and voltage dependent manner for Vj steps ≥10 mV. (D) Cell pairs in which one cell expresses Ogre and the other Inx2 do not form functional channels. (E) Pairs in which both oocytes express Ogre and Inx2 form channels with no, or very weak, voltage sensitivity; gj is constant at Vjs up to 40 mV and declines slightly, in some pairs, at higher Vjs. (F) Water-injected controls are not electrically coupled. (G) Relationship between junctional conductance and transjunctional voltage for Inx2 homotypic channels (squares) and channels in Ogre and Inx2-expressing cell pairs (triangles). For each Vj step, steady-state gj was calculated from Ij measurements made close to the end of the step. gjs, normalized to their values at Vj = 10 mV, are plotted as mean ± s.e.m. for n = 4-5 pairs. Some error bars are contained within the symbols. (H) Two-electrode voltage clamp recordings from single oocytes. Whole cell membrane currents (Im) were recorded as the cells were stepped through a series of hyperpolarising and depolarising membrane potentials (Vm; 20 mV pulses applied for 5 sec with a 20 sec gap between successive steps). Im/Vm relationships are plots of initial current, recorded at the beginning of the step, versus voltage, after subtraction of holding values. Currents in water-injected controls (open circles) and *ogre*-injected cells (filled circles) are indistinguishable. Currents significantly greater than control are present in cells injected with *inx2* (squares) or *ogre* and *inx2* (triangles). Im values are mean ± s.e.m. for n = 5-7 cells; some error bars are contained within the symbols.
Fig. 2. Characterisation of anti-innexin antibodies. (A) Transmembrane (TM) topology of innexins. Antibodies were raised to short peptides corresponding to unique C-terminal sequences of Ogre and Inx2 proteins. (B-C) Western blots labelled with anti-innexin antibodies. Positions of molecular mass markers (kDa) are indicated on the left. (B) *Drosophila* larval nervous systems. The panels are neighbouring lanes (8 CNS equivalents per lane) cut from the same blot and probed with anti-Ogre (left panel) or anti-Inx2 (right panel) in the absence (-) or presence (+) of an excess of Ogre or Inx2 peptide, respectively. Both antibodies detect a single band at ~37 kDa in the absence of competing peptide; no bands are visible in the presence of peptide. (C) Membrane proteins from *Xenopus* oocytes (one cell equivalent per lane), injected with innexin RNAs or water, were probed with anti-Ogre (left panel) and anti-Inx2 (right panel). Left panel, anti-Ogre detects a ~37 kDa protein in cells injected with *ogre* RNA (10 ng, lane 1) or a mixture of *ogre* and *inx2* RNAs (5 ng each, lane 3), which is not present in oocytes injected with *inx2* RNA (lane 2) or water (lane 4). Right panel, anti-Inx2 detects a ~37 kDa protein in oocytes injected with *inx2* RNA (10 ng, lane 2) or a mixture of *ogre* and *inx2* (5 ng each, lane 3), but not in oocytes injected with *ogre* RNA (lane 1) or water (lane 4). The weaker bands just below the innexins common to all lanes, including water-injected cells, are non-specific. The higher molecular weight bands (left panel, lane 1; right panel, lane 2) may be oligomers of Ogre and Inx2, respectively. The lower intensity of these, and the 37 kDa, bands in lane 3 (left and right panels) is consistent with the fact that the co-injected cells received lower levels of the individual RNAs than the cells injected with a single RNA. (D) Immunofluorescence labelling of *Drosophila* larval nervous systems. Images are single images from a confocal z-series. Nervous systems were labelled with anti-Ogre (upper panels) or anti-Inx2 (lower panels) in the absence (left panels) or presence (right panels) of an excess of Ogre or Inx2 peptide, respectively. Peptide antigens effectively compete with the corresponding antibody for binding to cells of the brain lobes and ventral ganglion (see Figs. 3-5). Scale bar: 50 µm.

Fig. 3. Ogre and Inx2 partially co-localise in the *Drosophila* larval CNS. Nervous systems of *Drosophila* larvae (24-40 hours ALH) double labelled with anti-Ogre and anti-Inx2 antibodies. Shown are single images, or projections of a maximum of five images, from a confocal z-series taken at 1 µm steps. (A-O) Ogre distribution (left panels; A, D, G, J, M), Inx2 distribution (middle panels; B, E, H, K, N) and the overlay of Ogre (red) and Inx2 (green) with the merge represented in orange/yellow (right panels; C, F, I, L, O). (A-C) Low magnification view of the developing nervous system showing the brain lobes and ventral
ganglion; the ring gland (rg, B) is attached anteriorly. (D-O) Higher magnification views of the brain lobes (D-L) and ventral ganglion (M-O). (D-F) The left lobe, as depicted in A-C, focusing on the optic proliferation centres. Ogre and Inx2 partially co-localise in the proliferation centres. Ogre is strongly expressed in the outer centre (opc) and more weakly in the inner centre (ipc). Inx2 staining is similar in both centres. (G-L) The right lobe, as depicted in A-C, shown in two different focal planes (G-I and J-L). Ogre and Inx2 localise at the plasma membrane of cells of the proliferation centres (G-I, arrows, H) as seen in D-F (arrow, E). Plasma membrane localisation of the proteins is also evident in the large cells of the ring gland, situated between the brain lobes (G-I). Ogre and Inx2 are extensively expressed in presumptive glial cells throughout the lobes, some of which are indicated by arrowheads. (M-O) In the ventral ganglion, Ogre and Inx2 co-localise in cells along the midline (arrowheads, N) whereas Ogre alone is found in a row of puncta on either side of the midline (arrows, M). See Fig. 4 for confirmation of the glial identity of some of the innexin-expressing cells. Scale bars: A-C, 40 µm; D-O, 20 µm.

**Fig. 4. Ogre and Inx2 are expressed in glial cells in the larval CNS.** Glial cells were marked by expression of UAS-mCD8::GFP under the control of repo-GAL4. Nervous systems (24-40 hours ALH) were double labelled with innexin and GFP antibodies. Images are projections of a confocal z-series taken at 1 µm steps. In each case, repo-GFP labelling is shown in the left panel, innexin labelling in the middle panel and the overlay of GFP (green) and innexin (red) in the right panel with the merge in orange/yellow. (A-F) Preparations labelled with anti-Ogre and anti-GFP. (A-C) In the brain lobes, repo-GFP is widely expressed in glial cells (A), many of which also express Ogre (B, C). (D-F) In the ventral ganglion, Ogre expression overlaps that of repo-GFP in glial cells at the lateral margins of the ganglion and the neuropile and along the midline (arrowheads, F). Ogre-labelled puncta on either side of the ventral midline (arrows, E) are Repo-negative and may be part of the tracheal system. (G-L) Preparations labelled with anti-Inx2 and anti-GFP. Inx2 is expressed in a similar pattern to Ogre in Repo-positive cells of the brain lobes (G-I) and ventral ganglion (J-L). It is not expressed in the presumptive tracheal cells of the ventral neuropile which express Ogre (compare E and K). Scale bars: 25 µm.

**Fig. 5. Ogre and Inx2 are not expressed in differentiated larval neurons.** Postmitotic neurons were marked by expression of UAS-mCD8::GFP under the control of elav-GAL4. Nervous systems (24-40 hours ALH) were double labelled with innexin and GFP antibodies.
Images are projections of a confocal z-series taken at 1 µm steps. In each case, elav-GFP labelling is shown in the left panel, innexin labelling in the middle panel and the overlay of GFP (green) and innexin (red) in the right panel. (A-F) Preparations labelled with anti-Ogre and anti-GFP. (A-C) In the optic lobe, Elav-positive neurons in the anterior region of the lobe and at the site of insertion of the optic stalk (arrowheads, A) do not express Ogre (B, C). The latter group are surrounded by Ogre-labelled cells of the optic proliferation centre (arrows, B). Ogre expression in glial cells is as shown in Figs. 3, 4. (D-F) In the ventral ganglion, elav-GFP, but not Ogre, is expressed in neural cell bodies and processes (arrowheads, D). Ogre-labelled glial cells associate with the neuronal axons (arrowheads, E, F). (G-L) Preparations labelled with anti-Inx2 and anti-GFP. (G-I) In the optic lobes, Elav-positive neurons of the mushroom bodies (arrowheads, G) do not express Inx2 (H, I). Inx2 is expressed in the optic proliferation centre and glial cells of the lobes as shown in Figs. 3, 4. (J-L) In the ventral ganglion, Inx2, like Ogre, is not expressed in Elav-positive neurons (arrowheads, J) but is found in glial cells surrounding the neuronal processes (arrowheads, K, L). Scale bars: 25 µm.

**Fig. 6. RNAi-mediated downregulation of innexin expression in glial cells significantly reduces protein levels and impairs CNS development.** UAS-innexinRNAi constructs were expressed in glial cells under the control of repo-GAL4. (A-D) ogre knockdown. (A-B) Nervous systems (36 hr ALH) labelled by immunofluorescence with anti-Ogre antibodies. (A) In controls, Ogre is strongly expressed in glial cells of the brain lobes (br) and ventral ganglion (vg) and in the optic proliferation centres. (B) Glial cell expression is significantly reduced in ogre knockdowns; some protein is still detectable in cells at the ventral midline. Expression persists in the optic proliferation centres (arrow) and in the ring gland (rg). Loss of Ogre protein results in a reduction in the size of the developing brain lobes and ventral ganglion (compare A and B). (C-D) Adult nervous systems. (C) The characteristic size and morphology of the normal adult CNS consisting of the central brain (cbr), optic lobes (ol) and ventral nervous system (vns). (D) The nervous system of ogre knockdowns is significantly smaller than that of controls. (E-F) inx2 knockdown. Nervous systems (36 hr ALH) labelled by immunofluorescence with anti-Inx2 antibodies. (E) Inx2 is strongly expressed in glial cells and in the optic proliferation centres in controls. (F) Expression persists in the optic proliferation centres (arrow) in inx2 knockdowns but is significantly reduced in glial cells. The loss of Inx2 expression correlates with a dramatic reduction in the size of the developing larval CNS (compare E and F). inx2 knockdown flies do not survive to adulthood. Images of
immunolabelled preparations (A-B and E-F) are projections of confocal z-series taken through the nervous systems. The same image acquisition settings were used for control and knockdown preparations, hence the saturation in control images. The ventral ganglion is outlined in (F). Scale bars: A-B and E-F, 50 µm shown in (A); C-D, 75 µm shown in (C). (G) Fluorescence intensity in immunolabelled nervous systems, quantified by measuring integrated density per unit area of tissue. (H) Relative differences in nervous system size, estimated by measuring the surface area of 2D images, such as those presented in A-F. The brain lobes of larval preparations were measured (upper panel); for adult preparations, the full CNS was measured (lower panel). Error bars, s.e.m., n = 3-7. *p = 0.0015, **p ≤ 0.0005, unpaired Student’s t-test. Fly genotypes: UAS-\textit{ogreRNAi}/TM6B,Tb or UAS-\textit{ogreRNAi}/TM3,Sb (\textit{ogre} control); UAS-\textit{ogreRNAi}/repo-GAL4 (\textit{ogre} knockdown); UAS-\textit{inx2RNAi}/TM6B,Tb (\textit{inx2} control); UAS-\textit{inx2RNAi}/repo-GAL4 (\textit{inx2} knockdown).
Table 1. Junctional coupling in Xenopus oocyte pairs injected with innexin RNAs

<table>
<thead>
<tr>
<th>Cell 1/Cell 2</th>
<th>Pairs coupled/Total (%)</th>
<th>Mean gj (µS ± s.e.m.)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ogre/Ogre</td>
<td>0/23 (0%)</td>
<td>0.17 ± 0.03</td>
<td>23</td>
</tr>
<tr>
<td>Inx2/Inx2</td>
<td>17/26 (65%)</td>
<td>3.41 ± 1.16</td>
<td>26</td>
</tr>
<tr>
<td>Ogre/Inx2</td>
<td>0/17 (0%)</td>
<td>0.16 ± 0.02</td>
<td>17</td>
</tr>
<tr>
<td>Ogre+Inx2/Ogre+Inx2</td>
<td>25/39 (64%)</td>
<td>4.49 ± 1.27</td>
<td>39</td>
</tr>
<tr>
<td>H₂O/H₂O</td>
<td>0/45 (0%)</td>
<td>0.11 ± 0.02</td>
<td>45</td>
</tr>
</tbody>
</table>

Junctional conductance, gj (mean for n cell pairs), was calculated as described in the legend to Fig. 1 at a 10 mV transjunctional voltage step. Pairs were considered coupled if gj >0.5 µS.