Temporal and spatial expression of *Drosophila* DLGS97 during neural development

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A B S T R A C T

The products of the *Drosophila discs-large* (*dlg) gene* are members of the MAGUK family of proteins, a group of proteins involved in localization, transport and recycling of receptors and channels in cell junctions, including the synapse. In vertebrates, four genes with multiple splice variants homologous to *dlg* are described. *dlg* originates two main proteins, DLGA, similar to the vertebrate neuronal protein PSD95, and DLGS97, similar to the vertebrate neuronal and epithelial protein SAP97. DLGA is expressed in epithelia, neural tissue and muscle. DLGS97 is expressed in neural tissue and muscle but not in epithelia. The distinctive difference between them is the presence in DLGS97 of an L27 domain. The differential expression between these variants, makes the study of DLGS97 of key relevance to understand the *in vivo* role of synaptic MAGUKs in neurons. Here we present the temporal and spatial expression pattern of DLGS97 during embryonic and larval nervous system development, during eye development and in adult brain. Our results show that DLGS97 is expressed zygotically, in neurons in the embryo, larvae and adult, and is absent at all stages in glial cells. During eye development DLGS97 starts to be expressed in photoreceptor cells at early stages of differentiation and localizes basal to the basolteral junctions. In the brain, DLGS97 is expressed in the mushroom bodies and optic lobes at larval and adult stages; and in the antennal lobe in the adult stage. In addition we show that both, *dlgS97* and *dlgA* transcripts express during development multiple splice variants with differences in the use of exons in two sites.

1. Results and discussion

The MAGUK family is a conserved group of proteins, which play major roles in the assembly and maintenance of membrane domains such as septate junctions in epithelial cells and synapses in neurons (Pawson and Scott, 1997; Garner et al., 2000; Sheng and Sala, 2001; Tepass et al., 2001); in particular, the subfamily of Synaptic Associated Proteins (SAP), which has four members in vertebrates (SAP90/95, SAP102/NEDLG, SAP97/hDLG, SAP93/Chapsyn110) and is highly expressed in the vertebrate brain (Funke et al., 2005). *Drosophila* contains a single SAP homologue in its genome, namely the discs large (*dlg*) gene. *dlg* is required for the control of cell proliferation, the maintenance of neuroblasts polarity, the formation of septate junctions in epithelia and the correct development and function of the neuromuscular synapse. Mainly due to the epithelial cell defects in polarity and proliferation, *dlg* mutants die at the end of larval development precluding the analysis of DLG function in the adult nervous system. The multiple defects observed in *dlg* mutants were originally ascribed to the loss of a single gene product, DLGA. DLGA has three PDZ (PSD95-DLG-ZO1) domains, one SH3 (Src homologue 3) domain and one GUK (Guanylate kinase) domain (Woods and Bryant, 1991). However, additional proteins, products of the *dlg* gene are expressed in the central nervous system and muscles (Mendoza et al., 2003). These proteins are not expressed in epithelial cells and have an amino terminal region called S97N, which contains an L27 domain, not present in DLGA. L27 domains form homo or hetero-tetrameric complexes, providing a conserved platform for supramolecular assemblies (Feng et al., 2004). Some of functions of L27 domains in vertebrate culture neurons include vesicle transport (Setou et al., 2000), the regulation of neurotransmitter release and AMPA receptor trafficking (Nakagawa et al., 2004). Recently, L27 domains have been associated with activity induced synaptic modification in neurons (Schluter et al., 2006). We have recently shown, using mutants that selectively eliminate the expression of either DLGA or DLGS97, that the L27 containing DLG variant has specific functions in the larval neuromuscular synapse and in adult brain, which are not replaceable by DLGA. Although we have shown that the function of DLGS97 is necessary for the normal function of the larval neuromuscular synapse and of the adult brain, a detailed expression pattern of DLGS97 is still missing. Here we report a study of *Drosophila* DLGS97 expression in the central nervous system during development. In addition, we analyzed the expression during eye development given that in

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this tissue, the transition from an undifferentiated epithelium to a differentiated tissue that include neurons, can be easily observed. Finally, we studied *dlgA* and *dlgS97* transcripts by RT-PCR, in different tissue showing the alternative use of some of the exons present in both variants.

1.1. dlgS97 during embryonic development

We first studied the expression of *dlgS97* transcripts during embryonic development by *in situ* hybridization. From embryonic stage 10 a diffuse expression in the neurogenic region was detected (Fig. 1B). The same diffuse expression was observed at stage 11 (not shown). At stage 12, *dlgS97* expression became more defined and concentrated along the future ventral nerve cord region, including the dorsal germ band before its retraction (Fig. 1C, early stage 12, arrow). Beyond stage 13 *dlgS97* was clearly concentrated in the ventral nerve cord and later also in the brain (Fig. 1D and E). Although the expression of *dlg* is known to have a strong maternal component (Perrimon, 1988), a probe directed to the S97N coding region failed to detect *dlgS97* transcripts before embryonic stage 9 (Fig. 1A)

Next, we analyzed DLGS97 protein expression. For this, we used an antibody directed to its L27 domain (anti-DLGS97N antibody, (Mendoza et al., 2003; Mendoza-Topaz et al., 2008). DLGS97 protein started to be detected in the embryonic ventral cord at late embryonic stage 12, when the germ band is almost completely retracted (Fig. 1J, arrow). In stages 13 and 14, DLGS97 was observed in the commissures crossing the midline (Fig. 1K–L and P–Q). From stages 15 to 17 the label grew more intense as the ventral nerve cord retracts and became clear in the longitudinal fibers (Fig. 1M–O and R–T, arrow). At these stages, the expression was also detected in the brain (Fig. 1M, arrow), gonads (Fig. 1N, arrow), and the segmental and intersegmental nerve roots that leave the central nervous system to the periphery (Fig. 1S, arrow). In agreement with the *in situ* hybridization results, the protein was not detected before stage 11 (Fig. 1F–I).

As we did not detect *dlgS97* transcripts or DLGS97 protein during early stages of development, we sought to confirm this result by a more sensitive approach. For this, we conducted RT-PCR and Western blot experiments of staged embryos. As the zygotic transcription starts around embryonic stage five, we used RNA from embryos before stage five and compared it to RNA from late stage embryos (stages 15–17) where DLGS97 and DLGA are expressed. Using forward primers specific for either *dlgS97* or *dlgA* and reverse primers common to both variants in two different regions (SH3 and GUK coding regions), we were able to amplify from early embryos *dlgA* transcripts, but not *dlgS97* transcripts (Fig. 2A), while full length transcripts for both isoforms were detected in late embryos (stages 15–17; Fig. 2A). At late developmental stages, we also observed additional bands for both variants (*dlgA* and *dlgS97*). The exclusive zygotic expression of DLGS97 was confirmed by Western blot (Fig. 2B).

We then centered our study in late stage embryos (stage 15) using confocal microscopy to study in more detail the DLGS97 expression in the ventral nervous system. At this stage, DLGS97 was clearly seen in the intersegmental (ISN) and segmental nerve roots (SN), the anterior (ac) and posterior commissures (pc) and in the ventral cells of the ventral nervous system (Fig. 3A). In co-labeling experiments, we observed that all the ventral cord nerve cells labeled cortically with DLG_{S97N} antibody were co-labeled with anti-ELAV antibody, a established nuclear marker for all mature neurons (Fig. 3E). In contrast, labeling with anti-REPO antibody, a nuclear marker for glial cells, we did not detect co-labeling with anti-DLG_{S97N} (Fig. 3B, arrow).

DLG is expressed in neuroblasts where is necessary for the process of asymmetric division (Ohshiro et al., 2000; Peng et al., 2000). To determine the variant expressed in neuroblasts we used anti-Miranda antibody as a marker of mitotic neuroblasts. We did not detect DLGS97 expression in neuroblasts (Fig. 3C). Moreover, DLGS97 staining was only detected in a cell layer more dorsally located (inner) compared with the cells positive for anti-Miranda staining. The light signal in glia and neuroblasts observed using the anti-DLG_{S97N} was not different from background (data not shown).

In addition to the neurons of the central nervous system, DLGS97 was detected in the neurons of the peripheral nervous system and in the developing somatic muscles (Fig. 3D) (Mendoza et al., 2003).

These results reveal that the embryonic expression of DLGS97 is not maternally contributed, starting at embryonic stage 12 in

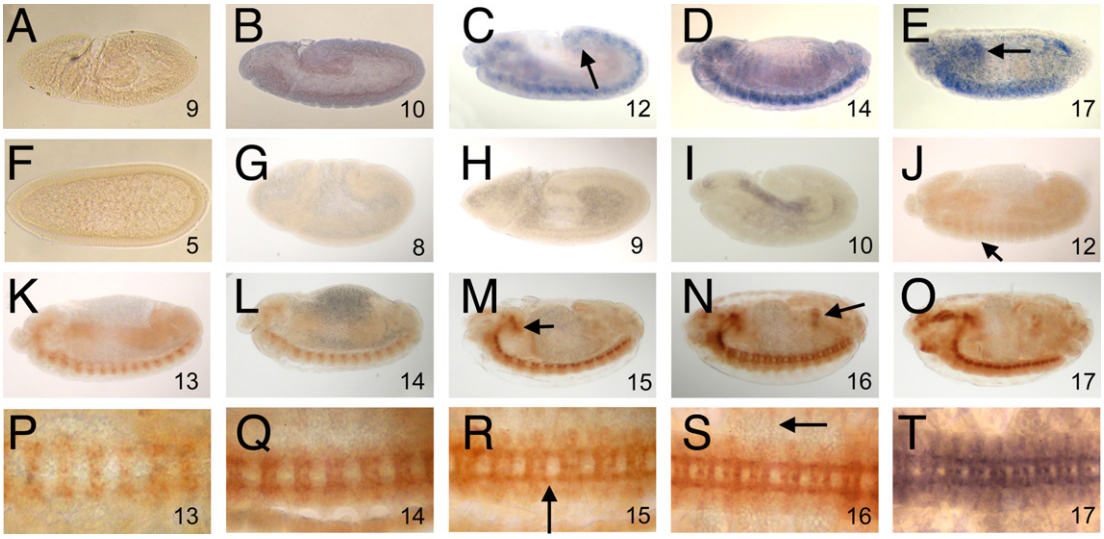

Fig. 1. DLGS97 during embryonic development. (A–E) *In situ* hybridization with a probe directed to the L27 region (see Section 2). (F–T) immunostaining with antiDLG_{S97N} antibody. The arrow in C points to the expression in the germ band before its retraction, and the arrow in E indicates the brain. (P–T) Higher magnification view of the ventral nerve cord. Arrowheads in J and M point to early DLGS97 protein expression in the ventral nerve cord and brain, respectively. Arrows in N, R and S indicate the gonads, the connectivity fibers in the anterior–posterior axis and the motor neurons that leave the CNS to the periphery, respectively. (A–O) are lateral and (P–T) are ventral views of whole embryos. The developmental stage is indicated in the bottom right corner in each panel. Anterior left (A–T) and dorsal up (A–O).

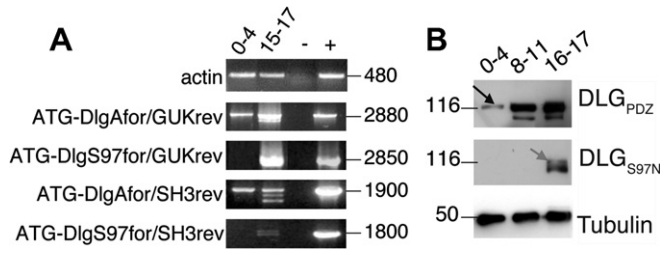

Fig. 2. DLGS97 is not maternally contributed. Temporal expression of DLGS97 and DLGA during embryogenesis (A) RT-PCRs and (B) Western blot from staged embryos. Embryos from stages 0–4 have only maternal contributed RNAs whereas in the oldest embryos (over stage 15) zygotically expressed RNAs are predominant (see Supplemental figure S1). The primers used are indicated on the left, and the band size of each product is indicated on the right. (−) Indicates the negative control without the reverse transcriptase enzyme, and (+) indicates the positive controls made with the respective clone; from top to bottom: actin (first panel), *dlgA* (second and fourth panel), and *dlgS97* (third and fifth panel). (B) All extracts were loaded and analyzed with the antibodies indicated on the right, the picture correspond to two different gels. The arrows point to the two bands detected from embryonic stage 8 with DLG_{PDZ} antibody. Molecular weight standards in kDa are indicated on the left.

mature neurons. This last statement is based on the observation that all DLGS97 positive cells are also ELAV positive cells and the fact that the expression of ELAV protein starts at stage 10 or before (Bier et al., 1988). In addition, our results show that during embryonic development DLGS97 is expressed, besides neurons, in somatic muscle and gonads and is not expressed in glial cells and neuroblasts.

1.2. DLGS97 expression during photoreceptors development

Photoreceptor differentiation initiates by mid third instar larval stage, starting at the posterior margin of the eye imaginal disc from where the morphogenetic furrow (MF) sweeps across in anterior direction leaving a trail of photoreceptor rows in its wake. Anterior to the MF, the epithelium is undifferentiated; posterior to it, the epithelium is organized into precursors of the adult ommatidia (Wolff and Ready, 1991). Each ommatidium in the adult is composed of eight photoreceptors (R) named R1 to R8. R8 is the first to express neural markers (ELAV for example) followed in successive rows after the furrow, by the pair R2–R5, then by the pair R3–R4 and then by the pair R1–R6. R7 is the last cell to express neuronal markers. In third instar larval stage, we detected DLGS97 exclusively posterior to the MF after the 4th row of differentiating R cells labeled with the neuronal marker ELAV (Fig. 4A–C). On the other hand, the DLG_{PDZ} antibody, which recognizes PDZ2 domain in all variants, stains all R cells, accessory cells and epithelial cells both anterior and posterior to the MF (Fig. 4E–G). At sub-cellular level DLGS97 was localized in the lateral membrane of the R cell, basal to the zonula adherens (ZA), which can be visualized by Armadillo (ARM) co-staining (Fig. 4H–K). At this stage we did not detect DLGS97 expression in axons of R cells (data not shown).

During pupal development (pd) the photoreceptors undergo a massive change in shape: their apical–basal axis rotates 90° into the central region of the forming ommatidia and the apical membranes differentiate into two regions: the rhabdomeres at the apical membrane and the stalk membrane connecting the rhabdomeres and ZA (Longley and Ready, 1995). During all pupae stage DLGS97 was restricted to R cells, where it was localized at the lateral membrane and the cytoplasm. Before apical rotation (30% pd), DLGS97 appeared concentrated at the center of the cluster basal to ARM (Fig. 5A–D). DLGS97 expression was also detected in the proximal region of R cell axons in a punctuated pattern (arrows in Fig. 5K) and co-localized partially with the axon marker Fasciclin II (FASII) (Fig. 5I–K). On the other hand, DLG_{PDZ} antibody labeled R cells in the same pattern as DLG_{S97N} antibody (Fig. 5E–G), but in addition labeled the differentiating cone and pigment cells located in the apical region (Fig. 5H) of the retina. After rotation (67% pd), DLGS97 remained basal to ARM and was localized at the cytoplasm surrounding the nucleus (Fig. 5L–O). An increased expression of DLGS97 in R cell axons, compared to 30% pd, was also observed (Fig. 5P, arrowhead).

Our results show that DLGS97 is specifically expressed in photoreceptor cells, which are co-labeled by ELAV, and it is associated, during larvae and early pupae stages to the lateral membrane, basal to the zonula adherens. On the other hand, DLGA is expressed additionally in cones and pigment cells (ELAV negative cells). We cannot discard that DLGA is expressed in R cells together with DLGS97 because DLG_{PDZ} antibody recognizes both DLGS97 and DLGA proteins.

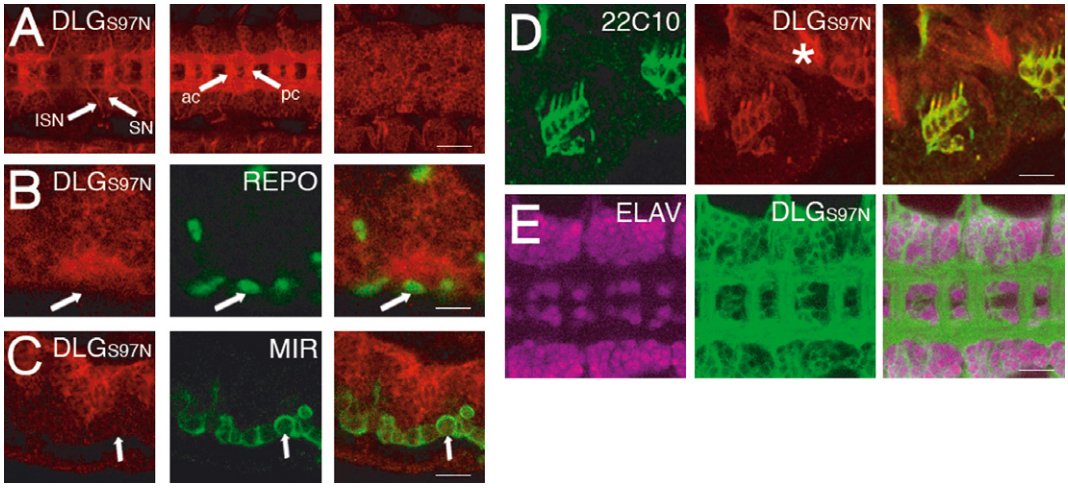

Fig. 3. DLGS97 is expressed in embryonic mature neurons from central and peripheral nervous system, but not in neuronal precursors or glial cells. Ventral (A and E) and lateral view (B, C) of ventral nerve cord and chordotonal organ (D) of whole embryos stained with DLG_{S97N} antibody (red in A–D and green in E) and (B) REPO, (C) Miranda, (D) 22C10 or Futsch antibodies (green in the three cases) and (E) ELAV (purple). (A) Three optical sections from dorsal to ventral of the same embryo. Note that DLGS97 was not expressed in embryonic glial cells (B) or in neuroblasts (C), but it was expressed in the nerve cord (A, E) and peripheral neurons (D). Anterior, left (A–E); dorsal, up (B, C, D). ISN = intersegmental nerve root, SN = Segmental nerve root, ac = anterior and pc = posterior commissures. Asterisk in D indicates DLGS97 expression in somatic muscles. Scale bars A = 25 μm; B–C = 8 μm; D = 6 μm and E = 12 μm.

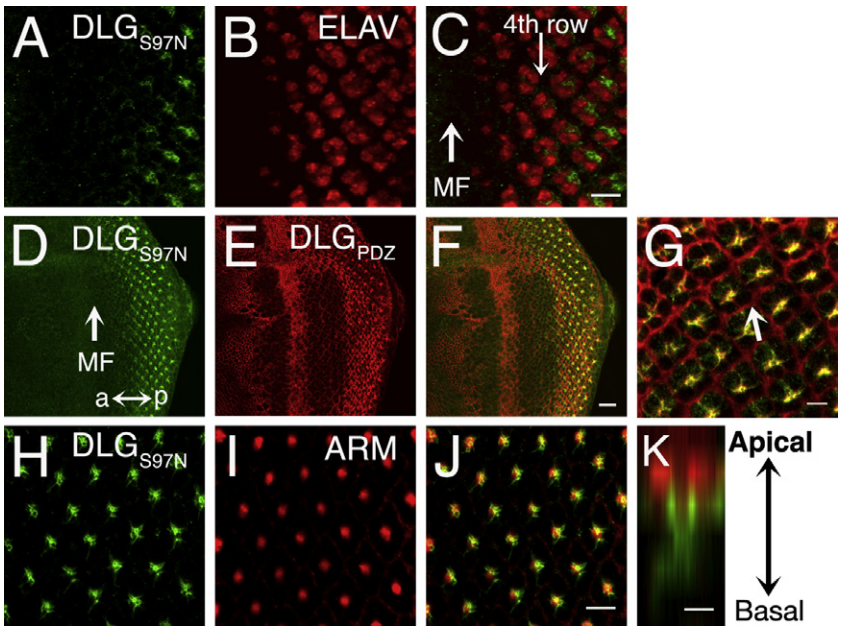

Fig. 4. Expression of DLGS97 in the eye imaginal discs. High magnification view of the morphogenetic furrow region of a third instar larval eye imaginal disc co-stained with DLG_{S97N} (A) and ELAV antibodies (B) and the merged image (C). DLGS97 was localized posterior to the MF from the 4th row (arrow in C) of R cells (stained with ELAV). Third instar larval eye imaginal discs co-stained with DLG_{S97N} (D) and DLG_{PDZ} antibodies (E) and the merged image (F); note that the DLG_{PDZ} antibody stains R and epithelial cells both anterior and posterior to the MF. (G) High magnification image of a region posterior to the MF co-stained with the same antibodies; note that DLG_{PDZ} antibody staining does not co-localize with DLG_{S97N} staining in the inter-ommatidial precursor cells (arrow in G). (H–K), co-staining with DLG_{S97N} (H) and ARM (I) antibodies and the merged image (J). (K) Z plane reconstruction of the double staining of these two antibodies. DLGS97 localizes basal to ARM (red) at the ZA. Scale bars F = 20 μm; C, G, J = 5 μm; K = 2 μm. a, anterior; p, posterior. Anterior is to the left. MF, morphogenetic furrow.

1.3. DLGS97 expression in the brain

The main structures of the adult *Drosophila* brain include the mushroom bodies (MBs) (Strausfeld et al., 1998), the optic ganglia (Jefferis et al., 2002) and the antennal lobe (Stocker, 1994). At larvae stage the brain is formed by several structures that are either replaced in the adult or are remodeled during metamorphosis (Truman, 1990). For example, the MBs at larvae stage appear as paired neuropiles with a structure mostly conserved in the adult. The gross morphology can be divided into discrete anatomical domains; a posterior cluster of cells, the Kenyon cells (Kc) that extends two types of processes, one type that forms a dendritic calyx (Ca) and receives olfactory information from the antennal lobes via the prominent inner antenno-cerebral tract (act), and a second type forming a fasciculated axonal tract (the peduncle), which extends ventrally toward the anterior surface of the brain, where it segregates into two main branches: the dorsal lobe (Dl) and the medial lobe (Ml) (Yang et al., 1995; Ito et al., 1997; Crittenden et al., 1998) (see scheme in Fig. 6B). DLGS97 was detected in the dorsal lobe (dl), medial lobe (ml), heel (h) and in the peduncle of the MBs in the central brain (CB) (Fig. 6F–K), co-localized with Fasciclin II (Fig. 6C). Other structures labeled with DLG_{S97N} were the antenno-cerebral tract, dendritic calyx and central complex (cx) (Fig. 6F–H). In the developing optic lobes (OL), a weak but detectable expression of DLGS97 was observed (Fig. 6C–E). Here, DLGS97 was localized in the larval optic neuropile (Lon), structure situated at the base of the medulla, co-localized with chaoptin (Fig. 6L–N). We did not detect the expression of DLGS97 in the optic stalk (os), lamina (Lam) and medulla (Me) at this stage (Fig. 6N). In the ventral nerve cord (VNC), DLGS97 is expressed in apparently all neurons and their projections (Fig. 6C–E). The only brain tissue not labeled by DLG_{S97N} antibody but positive for DLG_{PDZ} antibody was the epithelia covering the entire brain (Mendoza et al., 2003, and data not shown).

In the adult brain, as in embryos and larvae, all cells labeled by DLG_{S97N} antibody were co-labeled with the neuronal nuclear marker ELAV (Fig. 7A and B) and none of them was co-labeled with the glial nuclear marker REPO (data not shown). As glial cells are in intimate contact with neurons in some areas of the brain, making difficult to discard the nuclear REPO staining in DLGS97 positive cells, we studied the expression of DLGS97 in flies expressing CD8-GFP under the control of REPO-GAL4 driver and confirmed the lack of co-localization (Fig. 7C and D).

The more prominent structures of the adult brain labeled with DLG_{S97N} antibody, were the mushroom bodies and the antennal (AL) and optic lobes (OL) (Fig. 7G). In the adult the OL consists of four neuropile regions, lamina (Lam), medulla (Me), lobula (Lo) and lobula plate; DLG_{S97N} labeled all these structures including the projections that connect the lobula and the medulla neuropiles (Fig. 7K and data not shown). To test the synaptic localization of DLGS97 we double stained adult brains with DLG_{S97N} antibody and NC82 antibody, which recognizes the active zone protein Bruchpilot. We observed an almost complete overlapping of both staining highlighting the synaptic localization of DLGS97 (Fig. 7H–J). No differences between the signal observed with DLG_{PDZ} and DLG_{S97N} antibodies were detected (Fig. 7E–G), in agreement with previous results with *dlgS97* mutants that show that the main variant in the adult brain is DLGS97 (Mendoza-Topaz et al., 2008).

Then, we studied in more detail the distribution of DLGS97 in the neurons of the MB, given that the mushroom bodies (MB) are essential structures involved in complex behaviors as learning, memory, sleep and aggression (Joiner et al., 2006; Keene and Waddell, 2007). In the adult, the larval MB's medial lobe becomes the β, β′ and γ lobes, while the dorsal lobe becomes α and α′ lobes (Jefferis et al., 2002). We found DLGS97 localized in the α′/β′ and γ neurons, both known to be developed in larvae stage (Fig. 7L–O). In the α/β neurons, which are born during pupae stage, DLGS97 was mainly localized in the distal portion of the α lobe (asterisk) as can be seen by the almost no co-localization between DLG_{S97N} and FASII stain-

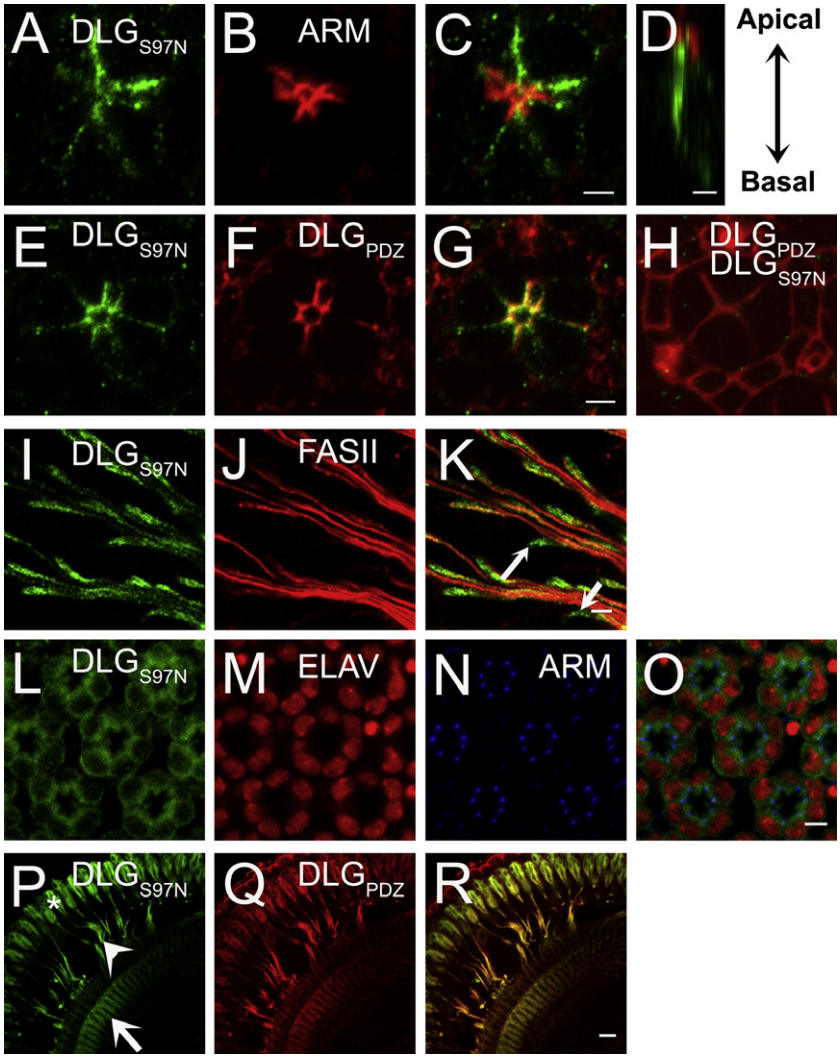

Fig. 5. DLGS97 expression in the eye during pupal development. (A to G) Single confocal images through a plane in the center of a 30% p.d. ommatidia co-stained with DLG_{S97N} (A) and ARM (B) or with DLG_{PDZ} (E and F) antibodies, and the merged images (C and G). DLGS97 is seen localized central but basal to ARM (red) as can be seen in the lateral view in D; and not co-localizing in support cells with DLGA. (H) Single confocal image through a superficial plane of a 30% p.d. ommatidia, where the cone cells are localized, DLG_{PDZ} antibody (red) but not DLG_{S97N} antibody (green) stained cone and pigment cells. (I–K) Longitudinal view of 30% p.d. eye disc showing the axons leaving the eye disc to the brain where DLGS97 is detected in the base of R cell axons in a punctated pattern (arrows in K) and was co-localized partially with FASII. (L to O) Single confocal images through a plane in the center of the 67% p.d. ommatidia, labeled with DLG_{S97N} (L), ELAV (M) and ARM (N) antibodies and the merged image. DLGS97 appeared localized at the cytoplasm surrounding the nucleus. (P–R) Longitudinal view of the retina at 67% p.d. labeled with DLG_{S97N} (P) and with DLG_{PDZ}. DLGS97 was localized in cell bodies (asterisk in P) and axons (arrowhead in P) of R cells and in the lamina (arrow in P). Scale bars R = 20 μm; K, O = 5 μm; C, D, G = 2 μm.

ing, which labels α/β neurons (Fig. 7N). Furthermore, in adults, DLGS97 was localized in the bodies of the Kenyon cells and in the calyx (data not shown).

The expression pattern in adult brain presented here shows that DLGS97 is a protein expressed in neurons with an intracellular distribution enriched in neuropiles but also present in the cell bodies. This distribution resembles the mammalian SAP97 protein more than PSD95 protein because SAP97 is found in dendrites and cell body, whereas PSD95 distribution is almost exclusively synaptic (Arnold and Clapham, 1999).

1.4. Drosophila dlgS97 and dlgA splice variants

DLG proteins are expressed in all developmental stages and in a variety of tissues, including neural tissue (Woods and Bryant, 1991; Woods et al., 1996). Although several *dlg* transcripts have been reported (Flybase; http://flybase.bio.indiana.edu) representing alternative splice variants, it is not clear if they are expressed in a tissue specific form or even translated. The analysis by Western blot assay with antibodies against PDZ2 domain (anti-DLGPDZ, Tejedor et al., 1997), GUK domain (anti-DLGGUK, Woods et al., 1996) or the N-terminal region of DLGS97 (anti-DLGS97N, Mendoza et al., 2003; Mendoza-Topaz et al., 2008) reveals only high molecular weight proteins (over 90 kDa) in embryo, larvae and adult tissues (Mendoza et al., 2003). However, the comparison of the relative sizes among the protein expressed in different tissues shows differences. These differences in the molecular weight could be due to post-translational modifications or to the expression of different splice variants. To analyze if at least some of variations are due to the alternative use of exons, we performed RT-PCR from ovaries, heads and bodies from adult flies as well as brains, eye discs and body walls from larvae using a primer in the ATG region of the *dlgS97* transcript together with a reverse primer in the coding region of the GUK domain. The PCR products obtained with this pair of primers are referred to as dlgS97-like. Additionally, we carried out RT-PCR using the same reverse primer in combination with a forward primer covering the ATG region of *dlgA* transcript for some of the above tissues. These PCR products

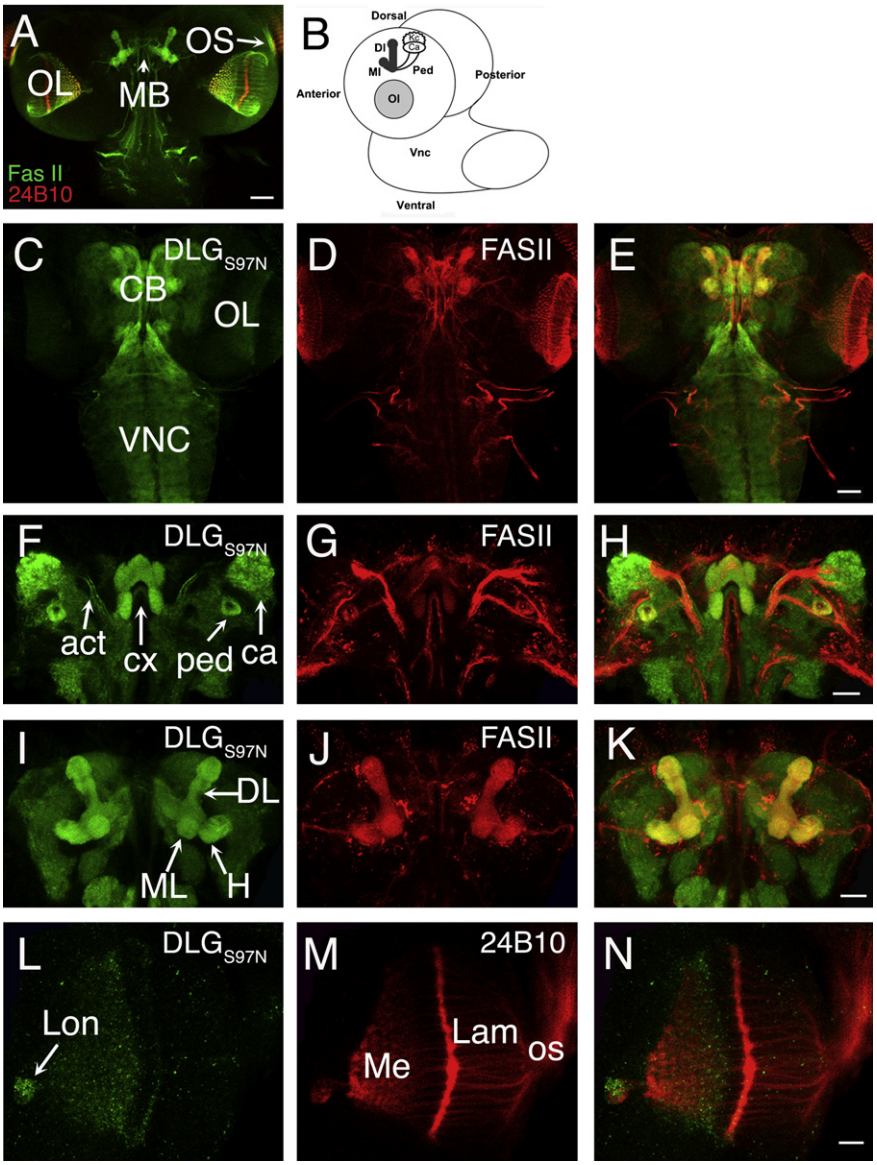

Fig. 6. DLGS97 expression in the larval brain. (A) Optical cross-section of third instar larval brain labeled with antibody against FASII (green) and Chaoptin (24B10) (red). (B) Schematic dorsolateral view of the larval brain. The mushroom bodies are composed by three distinct structures, the dendritic structure called calyx (ca), the axonal projection or peduncle (ped) and the axonal terminals lobes; dorsal lobe (dl) and medial lobe (ml). (C–K) DLG_{S97N} and FASII staining. (C–E) DLGS97 (green) was expressed in the central brain (CB), in the ventral nerve cord (VNC) and was weakly expressed in the developing optic lobes (OL). (F–K) Each row shows a different plane through the mushroom bodies, from posterior (F–H) to anterior (I–K). DLGS97 was detected in the calyx, peduncle, central complex (cx) and antenno-cerebral tract membrane (act) (F), it was also observed in dorsal lobe (DL), medial lobe (ML) and heel (H), (I). (L–N) Retinal axons in the optic stalk (os), lamina (Lam), medulla (Me) and larval optic neuropile (Lon) are stained by antibody against Chaoptin (24B10) (dorsal is up). DLG_{S97N} appeared to label only the Lon that is situated at the base of the medulla. Scale bars A, E = 50 μm; H, K, N = 20 μm.

are referred to as *dlgA-like*. All bands observed were isolated, cloned and sequenced. A scheme of the genomic organization of the *dlg* gene is presented in the Fig. 8A, a summary of the exon composition of the bands isolated from the different tissues is presented in Table 1 and a scheme of the variable regions and the protein domains is shown in Fig. 8B.

Only in adult tissue (heads and bodies) we observed two distinct products with both set of primers (Fig. 8B and C). The sequence of the high molecular products revealed that the *dlgS97-like* and *dlgA-like* type of transcripts presented differences compared with the reported transcripts isolated previously from embryo (see Table 1). The lower molecular weight products lacked the exons coding for PDZ3 and SH3 domains in *dlgS97-like* and *dlgA-like* transcripts (Fig. 8C). In larval tissues (body wall, brain and discs), only one band was obtained and the sequence of the clones isolated revealed only transcripts containing the exons coding for all DLG domains (PDZ, SH3 and GUK). The *dlg* sequenced transcripts expressed in embryonic, larval and adult tissues differentially incorporate exons, which potentially generate a diversity of products. The most noticeable usage of alternative exons comprised the inclusion of exons 9 and 12, and the combinations of exons 17, 18 and 19 (see Fig. 8B and Table 1). As this screen was not exhaustive because we did not sequence multiple clones of the same isolated band, we cannot ruled out that more combinations are present in each tissue. In addition, as the primers used in this analysis are specific for the ATG of *dlgA* and *dlgS97* variants we want to draw attention to the fact that we are not analyzing the presence of variants with exon 2 (which bears a different ATG), exon 7 and exon 20. These last two exons encode stop codons that generate truncated variants without GUK domain (MW: 21 kDa and 81 kDa, see Fig. 8A and Mendoza et al., 2003). All

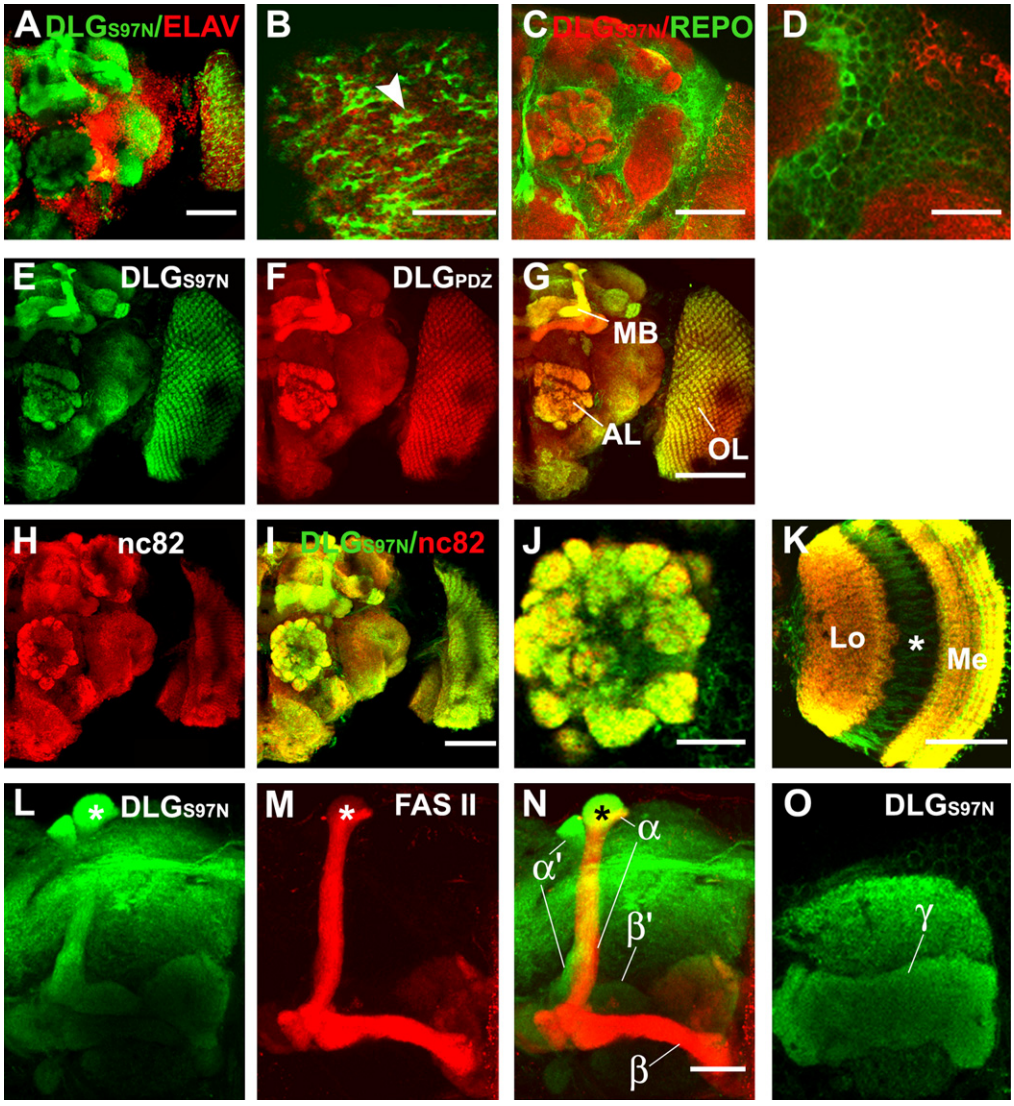

Fig. 7. DLGS97 expression in the adult brain (A) Whole brain double labeled with anti-DLG_{S97N} (green) and anti-ELAV (red). (B) Magnified view of the optic lobe in A (single optical cross section); arrowhead indicates a neuronal nucleus stained by anti-ELAV surrounded by DLG_{S97N} cytosolic labeling, (C) Single confocal image of a brain expressing CD8-GFP under the control of REPO-Gal4 double labeled with anti-DLG_{S97N} (red) and anti-CD8 (green). (D) Zoom of a region in the central brain of C (single confocal image) note that DLGS97 is absent of glial cell bodies and its projections. (E–G) Double staining with anti-DLG_{S97N} (E) and anti-DLG_{PDZ} (F) and the merge of both staining (G), MB: mushroom bodies, AL: antennal lobes and OL: optic lobes, (H) Brain labeled with anti-Bruchpilot (nc82 antibody) (I–K) Co-labeling with anti-DLG_{S97N} and nc82 antibody (I) same view as H, (J) single optical cross-section of antennal lobe and (K) optical lobe showing the co-localization of these two proteins, Lo: lobula, Me: medulla, asterisk indicate axon projections. (L–O) Zoom of mushroom bodies stained with anti-DLG_{S97N} (green) and FASII antibody (red) (the asterisk shows the distal region of the α/α' lobe), (O) a more anterior optical section showing the γ lobe. Scale bars A, C, G, I, K = 50 μm; B, D, J, N = 20 μm.

this transcripts have been reported as ESTs. Nevertheless, the expression of truncated variants (molecular weight lower than 90 kDa) seems to be very low or absent given that they are not detected by Western blot (Mendoza et al., 2003; Mendoza-Topaz et al., 2008). In addition, we did not find any transcript with exon five, supporting the data reporting it as an alternative exon of transcripts with exon 2 (Flybase; http://flybase.bio.indiana.edu).

The variation in protein molecular size described in previous publications is consistent with the translation of different splicing products, although tissue-specific posttranslational modifications in addition to the molecular size variations as the result of the use of alternative exons described here, cannot be ruled out. The splice variants found in this study show the high variability of the HOOK region (located between SH3 and GUK domains, Fig. 8). For mammalian SAPs two sites of splicing have been reported that are in the same position where we find the use of alternative exons in *Drosophila*, one before PDZ1, in an equivalent position to exon nine and one in the HOOK region. For SAP97 protein, different insertions in the HOOK region have been proposed to associate to functional changes; a SAP97 splice variant (with the insert called I2) is expressed in the neuronal soma and dendrites, whereas an other one (with insert I3) is expressed mainly in dendritic spines. Only the variant with the insert I3, which binds the protein 4.1, associates to an increased synaptic transmission and AMPA receptor expression upon SAP97 over-expression (Rumbaugh et al., 2003).

2. Experimental procedures

2.1. Flies

Wild type strains *cs* or *yw* strains (for photoreceptors) and REPO-GAL4 (7415) were obtained from Bloomingtson Drosophila Stock Center at Indiana University. UAS-CD8-GFP line is a gift from Dr. T. Lee (U. Mass. Medical School)

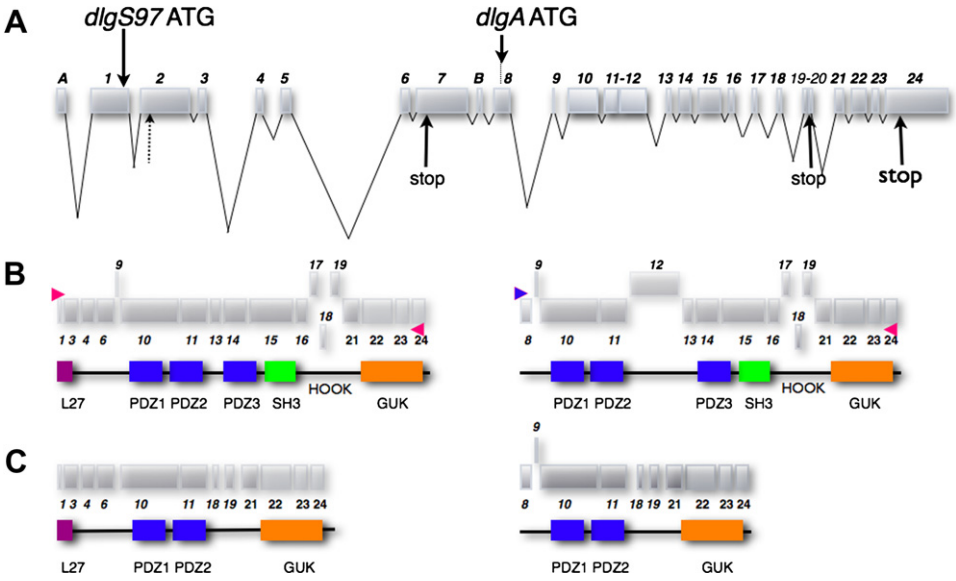

Fig. 8. Exon composition of *dlgS97* and *dlgA* and the main splice variants observed in embryo, larvae and adult tissues. (A) Genomic organization of the *dlg* gene, the translation initiation (ATG) for *dlgA* and *dlgS97* transcripts are indicated together with the stop codon in exon 24 that indicates the end of the translation for both isoforms. Two additional stop codons (smaller font) that result in truncated proteins are indicated. An arrow in punctuated lines indicates a third transcription start (exon 2) that was not studied in this work (BC) Scheme of the exon composition of *dlgS97-like* (left) and *dlgA-like* (right) transcripts; exons above and below the main line are alternative exons, see Table 1 for details. Below each transcript is the predicted protein domain. Small colored arrows in both transcripts indicate the region where the primers used for RT-PCR are located (C) Short splice variants lacking exons coding for PDZ3 and SH3 domains found in adults bodies and heads.

Table 1
Exon composition of *dlgS97* and *dlgA* transcripts in different tissues of *Drosophila* larva and adult

		Protein Domains																		
		L27						PDZ1-2			PDZ3		SH3		HOOK			GUK			
		EXONS																		
TISSUE	bp	1	3	4	6	8	9	10	11	12	13	14	15	16	17	18	19	21	22	23	24
		dlgS97																			
ADULT HEAD	2836	X	X	X	X			X	X		X	X	X	X		X	X	X	X	X	X
	2016	X	X	X	X			X	X							X	X	X	X	X	X
BODY	2830	X	X	X	X			X	X		X	X	X	X	X	X		X	X	X	X
	2016	X	X	X	X			X	X							X	X	X	X	X	X
BODY WALL	2810	X	X	X	X		X	X	X		X	X	X	X	X			X	X	X	X
LARVAL BRAIN	2755	X	X	X	X			X	X		X	X	X	X		X		X	X	X	X
EYE DISC	2911	X	X	X	X			X	X		X	X	X	X	X	X	X	X	X	X	X
OVARIES	2855	X	X	X	X		X	X	X		X	X	X	X	X	X		X	X	X	X
EMBRYO *	2861	X	X	X	X		X	X	X		X	X	X	X		X	X	X	X	X	X
		dlgA																			
ADULT HEAD	2427					X		X	X		X	X	X	X	X			X	X	X	X
	1537					X	X	X	X							X	X	X	X	X	X
	1512					X		X	X							X	X	X	X	X	X
BODY WALL	2940					X		X	X	X	X	X	X	X	X	X		X	X	X	X
	2497					X	X	X	X		X	X	X	X	X	X		X	X	X	X
EYE DISC	2920					X	X	X	X	X	X	X	X	X	X			X	X	X	X
OVARIES	2965					X	X	X	X	X	X	X	X	X	X	X		X	X	X	X
EMBRYO**	2895					X		X	X	X	X	X	X	X	X			X	X	X	X

Unshadowed are the exons common to all transcripts, in light grey the exons specific for *dlgS97*, in intermediate grey the exons specific for *dlgA* transcripts. In dark grey are the exons that are alternative for both groups of transcripts.
*Mendoza et al. (2003).
**Flybase, LD10659.

2.2. Probe preparation and whole mount in situ hybridization

In situ hybridization was performed by standard procedures with an antisense RNA probe labeled with digoxygenin (Boehringer, Mannheim, Germany) directed against the coding region of *dlg* L27 domain (exons 1, 3, 4 and 6). A control sense probe did not yield a signal above background level.

2.3. Immunohistochemistry

Embryos were stained as described (Mendoza et al., 2003). Adult and third instar larval brains and eye discs were dissected and stained with standard procedures (Walther and Pichaud, 2006; Wu and Luo, 2006). Primary antibodies were rat anti-Miranda (1:200, C.Q. Doe), rabbit polyclonal anti-DLG_{S97N} (1:500–1:2000,

Mendoza et al., 2003), rat anti-CD8 (1:100, Lee and Luo, 1999), mouse anti-GFP (1:100, Molecular Probes, Eugene, Oregon). The following antibodies were acquired from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA): polyclonal rat anti-ELAV (7E8A10, 1:10) and mouse monoclonal antibodies anti-FASII (1D4, 1:2), anti-DLG_{PDZ} (4F3, 1:500), anti-Futsh (22C10, 1:20), anti-REPO (8D12, 1:200), anti-ARM (N27A1,1:200), anti-Chaoptin (24B10, 1:100) and anti-ELAV (9F8A9, 1:10). HRP and fluorescent coupled secondary antibodies were from Jackson ImmunoResearch or Molecular Probes Eugene, OR. Images were captured using a digital camera (Nikon Coolpix E500) coupled to a Nikon E400 microscope, or a confocal microscope (LSM 510 Pascal; Carl Zeiss MicroImaging, Inc.).

2.4. RNA isolation and RT-PCR

cDNAs from staged embryos, larval brains, eye discs and adult heads were obtained by reverse transcription (Superscipt II, Invitrogen, California, CA) of polyA RNA obtained by Trizol extraction (Invitrogen, California, CA). Primers used in RT-PCRs were ATG-S97N-for (5′-aacaacgctcacagaatgcc), ATG-DlgA-for (ggtatcgccctgga caacgcccg), SH3rev (5′-ttcatcgtcggaggcattggt), GUKrev (5′-ggtatcgccctggacaac gcccg), 5′actin (5′-ggccggactcgtcgtactcctgc), 3′actin (5′-gagcaggagatggccaccgctgc).

2.5. Immunoblot detection

Embryos were homogenized in homogenization buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl), centrifuged for 10 min at 800*g* and the pellet resuspended in RIPA buffer (0.15 M NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA and 0.2 M Tris–Cl, pH 7.5) supplemented with a protease inhibitor cocktail (Boehringer, Mannheim, Germany). Proteins were separated in 10% acrylamide SDS–PAGE, transferred to nitrocellulose membranes and incubated with rabbit anti-Dlg_{S97N}, mouse anti-Dlg_{PDZ} (4F3) or mouse anti-b-tubulin (12G10; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) antibodies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gep.2008.04.001.

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