Supplemental Figures, Tables, and Figure Legends

Figure S1

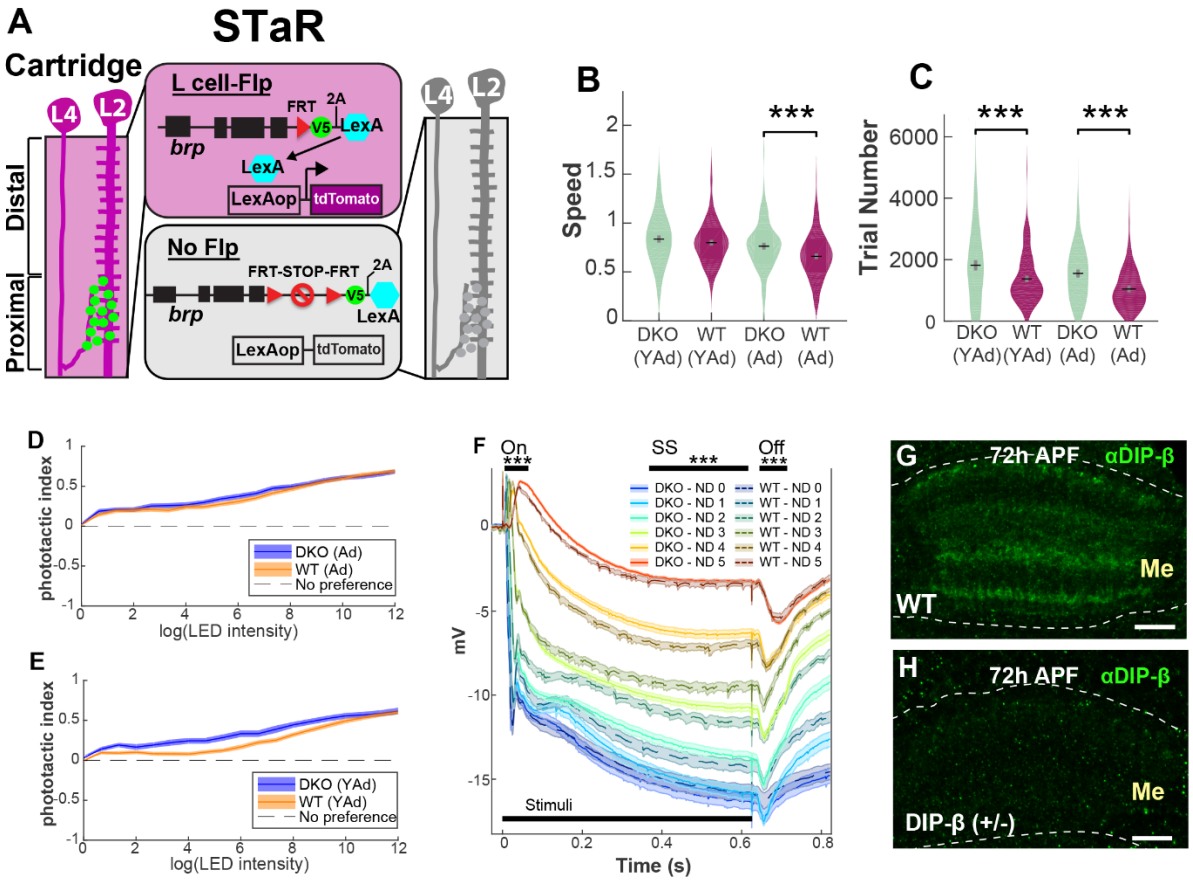

Figure S1. DIP proteins are required for proper visual function and synaptic connectivity (related to Figure 1)

(A) Labeling L2-L4 synapses using STaR. In the absence of Flp recombinase (gray panel) Brp is expressed from its native promoter within a bacterial artificial chromosome, but a transcriptional STOP sequence prevents incorporation of an epitope tag (smFPV5) (Viswanathan et al., 2015) and co-translation of LexA. (Magenta panel) When Flp recombinase is expressed in L cells (27G05-Flp) (Peng et al., 2018) the transcriptional STOP is excised, Brp becomes tagged and LexA is co-translated via the 2A peptide (Ryan and Drew, 1994). LexA then enters the nucleus to drive expression of myr-tdTOM which allows identification of the L cells expressing tagged Brp. As L2 and L4 are the only L cells that are pre-synaptic in the lamina, this allows selective visualization of synapses formed by these neurons.

(B-C) Average speed (B) and number of trials per fly (C) for adult and young adult flies in the phototaxis assay.
(D-E) Phototactic choice as a function of intensity for DKO and control flies in adult (D) and young adult (E) flies. Dark lines show mean response and shaded region indicates 95% confidence intervals.
(F) ERG responses of young adult 1-2 day old DKO (n=9) and control (n=7) flies at different light intensities. Each fly was measured 10 times at each intensity.
(G and H) DIP-β immunolabeling (green) in the medullas (Me) of wild type flies (G) and DIP-β heterozygotes (β+/-) (H) at 72hAPF. The dotted lines delineate the medulla neuropil. n=5 brains per genotype. Scale bar = 10μm.
(Statistical significance- *<.05, **<.005, ***<.0005)

Figure S2

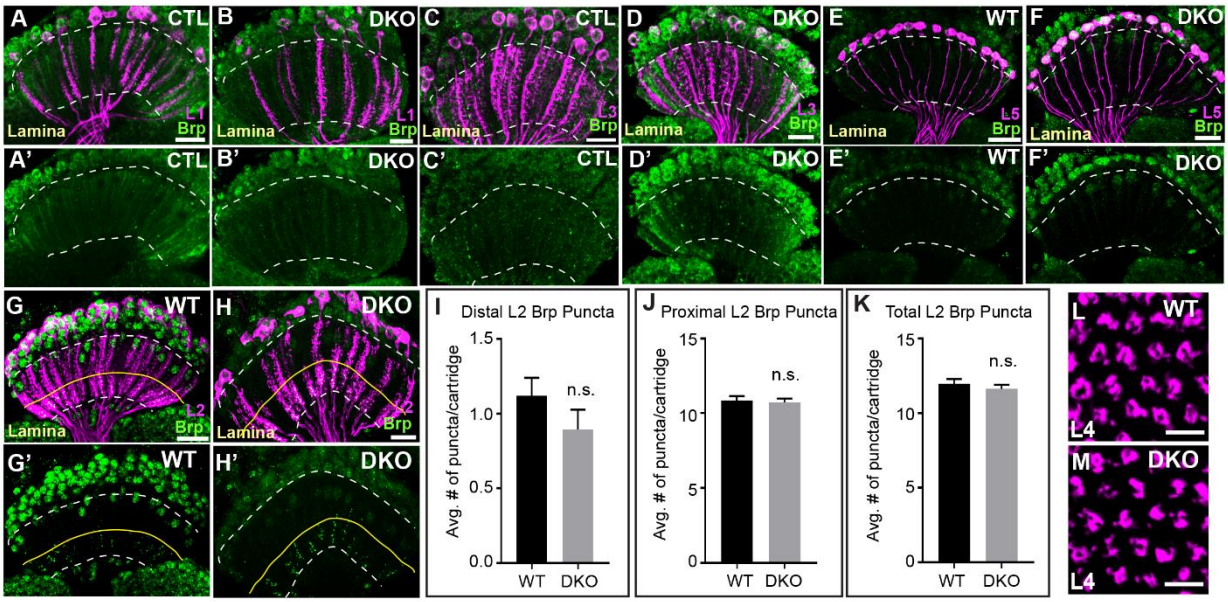

Figure S2. Cell type-specific STaR experiments (related to Figure 2)
(A-F') Confocal images (longitudinal plane of the lamina) showing the distribution of Brp (green-smGFPV5) expressed in L1 (A-B), L3 (C-D) or L5 (E-F) neurons (magenta- myr-tdTOM) in wild type (E-E'), Control (β-/+, γ -/+ (A-A', C-C') or DKO (B-B', D-D', F-F') flies. The dotted lines delineate the lamina neuropil. Scale bar = 10μm.
(A-B') Brp-smGFPV5 in L1 neurons. CTL (n= 6 brains), DKO (n= 6 brains).

(C-D') Brp-smGFPV5 in L3 neurons. CTL (n= 6 brains), DKO (n= 5 brains).
(E-F') Brp-smGFPV5 in L5 neurons. WT (n= 6 brains), DKO (n= 11 brains).
(G-H') Confocal images of Brp (green- smGFPV5) in L2 neurons (magenta- myr-tdTOM) in wild type (G and G') or DKO (H and H') flies. The white dotted lines indicate the lamina neuropil. The yellow lines (G' and H') indicate the boundary between the distal and proximal lamina. Scale bar = 5µm.
(I-K) The average number of L2 Brp puncta in the distal (I) and proximal (J) halves of lamina cartridges, and the average total number of puncta per cartridge (K) in wild type (n= 75 cartridges; 5 brains) and DKO (n= 75 cartridges; 5 brains) flies. Data are represented as a mean +/- SEM.
(L and M) Cross section view of L4 processes (magenta- myr-tdTOM) in the lamina neuropil in wild type (L) and DKO (M) flies. The relative spacing of L4 processes between cartridges is similar between genotypes. Scale bar = 5µm.
(Statistical significance- *<.05, **<.005, ***<.0005)

Figure S3

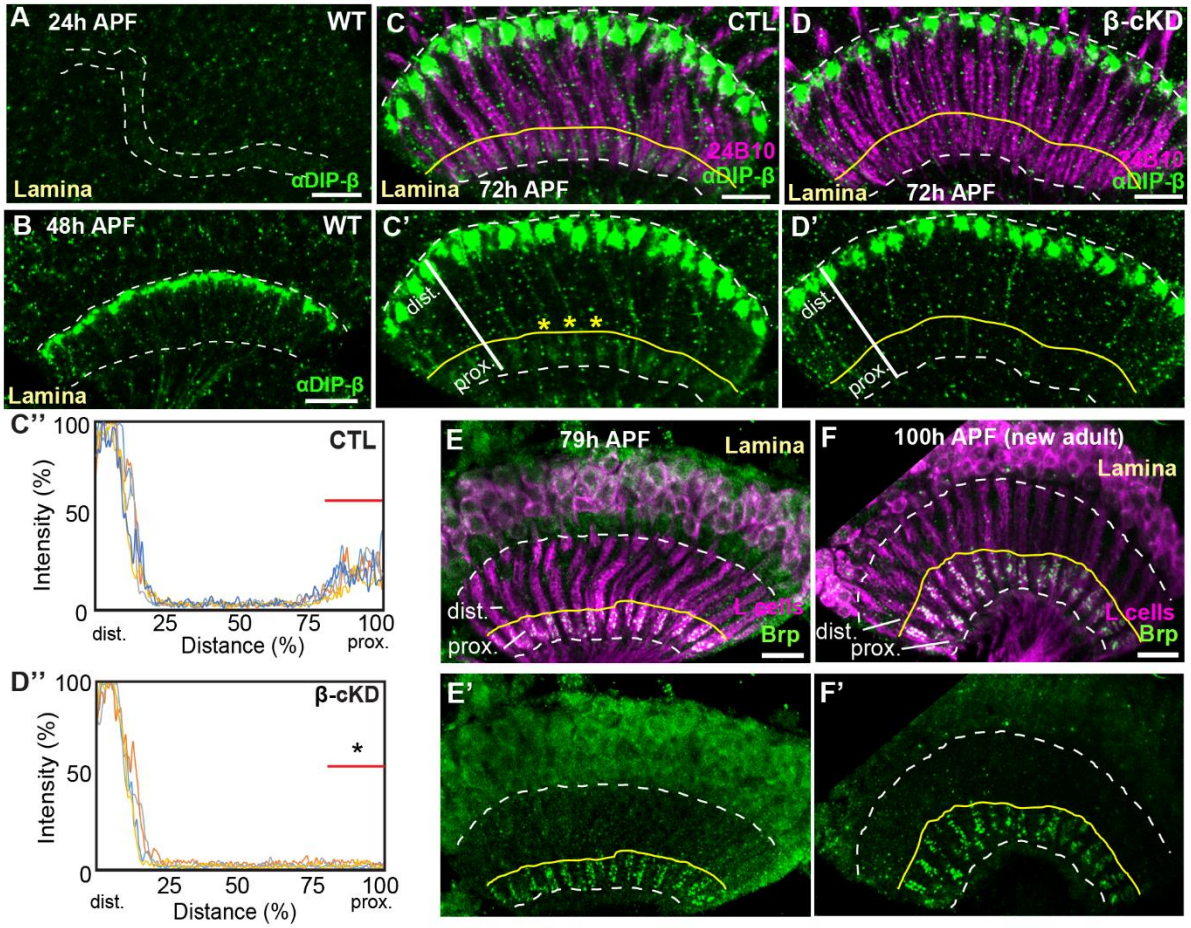

Figure S3. DIP-β is required in L4 neurons for proper synaptic connectivity (related to Figure 3)

(A and B) Confocal images showing DIP-β immunolabeling in the laminas of wild type flies at 24 (A) and 48h APF (B). DIP-β protein is detected in the distal lamina at 48h APF. This labeling likely represents expression in LaWF2 neurons. The white dotted lines indicate the lamina neuropil. Scale bar = 10µm.

(C-D'') DIP-β immunolabeling in the proximal lamina at 72h APF in control flies (UAS-RNAi only) and in flies with conditional knockdown of DIP-β in L4 cells (β-cKD, UAS-β-RNAi + L4 split-GAL4: 31C06AD, 24G07DBD).

(C-D') Confocal images of DIP-β immunolabeling in the laminas of a control fly (C and C') or a β-cKD fly (D and D'). The white lines show the region of lamina cartridges assessed in (C'' and D''). In (C'), the yellow asterisks indicate individual cartridges. Scale bar = 10µm.

(C'' and D'') Quantification of DIP-β fluorescence intensity along the long axis of lamina cartridges (see white lines in (C' and D')). Significantly reduced fluorescence intensity is

observed in the proximal lamina (80-100% distance) of β-cKD flies (the proximal lamina is marked by red bar in C'' and D'') compared to control flies (n=3 cartridges per brain, control: n=5 brains, β-cKD: n=4 brains).

(E-F') Confocal images in a longitudinal plane of lamina cartridges in wild type flies showing Brp expression (green- smGFPV5) in L cells (magenta- myr-tdTOM) at 79 and 100h APF. The dotted lines delineate the lamina neuropil. The yellow line marks the boundary between the proximal and distal lamina. n=5 brains for both time-points. Scale bar = 10μm.

(Statistical significance- *<.05, **<.005, ***<.0005)

Figure S4

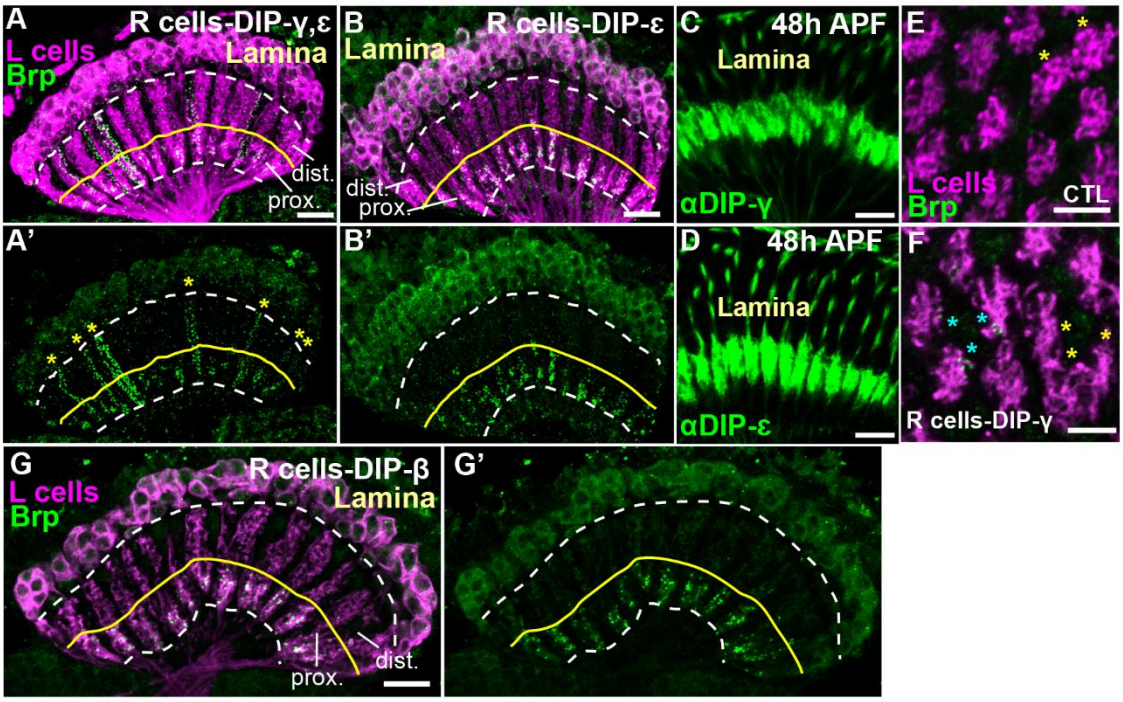

Figure S4. Confocal analysis of DIP mis-expression (related to Figure 4)

(A-B') Confocal images (longitudinal view of lamina cartridges, 1-2 day old adults) show the distribution of Brp (green- smFPV5) expressed in L cells (magenta- myr-tdTOM) in the laminas of flies mis-expressing DIPs-γ and ε (A and A') or DIP-ε alone (B and B') in R cells. The white dotted line outlines the lamina and the yellow line separates the proximal (prox.) and distal lamina (dist.). Scale bar = 10μm.

(A and A') Mis-expression of DIPs-γ and ε causes L cells to form streams of ectopic synapses within the distal regions of cartridges (yellow stars in A'). n= 9 brains.
(B and B') Mis-expression of DIP-ε only was not sufficient to induce ectopic L cell synapses in the distal lamina. n= 5 brains.
(C and D) Immunolabeling of DIPs-γ (green, C) (n=2 brains) and ε (green, D) (n=2 brains) in mis-expression experiments (DIPs-γ and ε in R cells) at 48h APF. Both proteins are strongly expressed in R cell terminals within the lamina neuropil. Scale bar = 10μm.
(E and F) Confocal images of cross-sections through the laminas of control flies (E, GMR-GAL4; n= 3 brains) and flies mis-expressing DIP-γ in R cells (F, n= 13 brains). The morphology and spacing of L cell processes (magenta- myr-tdTOM) shows fusing in F. Scale bar= 5μm.
(G-G') Mis-expression of DIP-β in R cells was not sufficient to induce ectopic L cell synapses in the distal lamina. n= 7 brains. Scale bar = 10μm
(Statistical significance- *<.05, **<.005, ***<.0005)

Figure S5

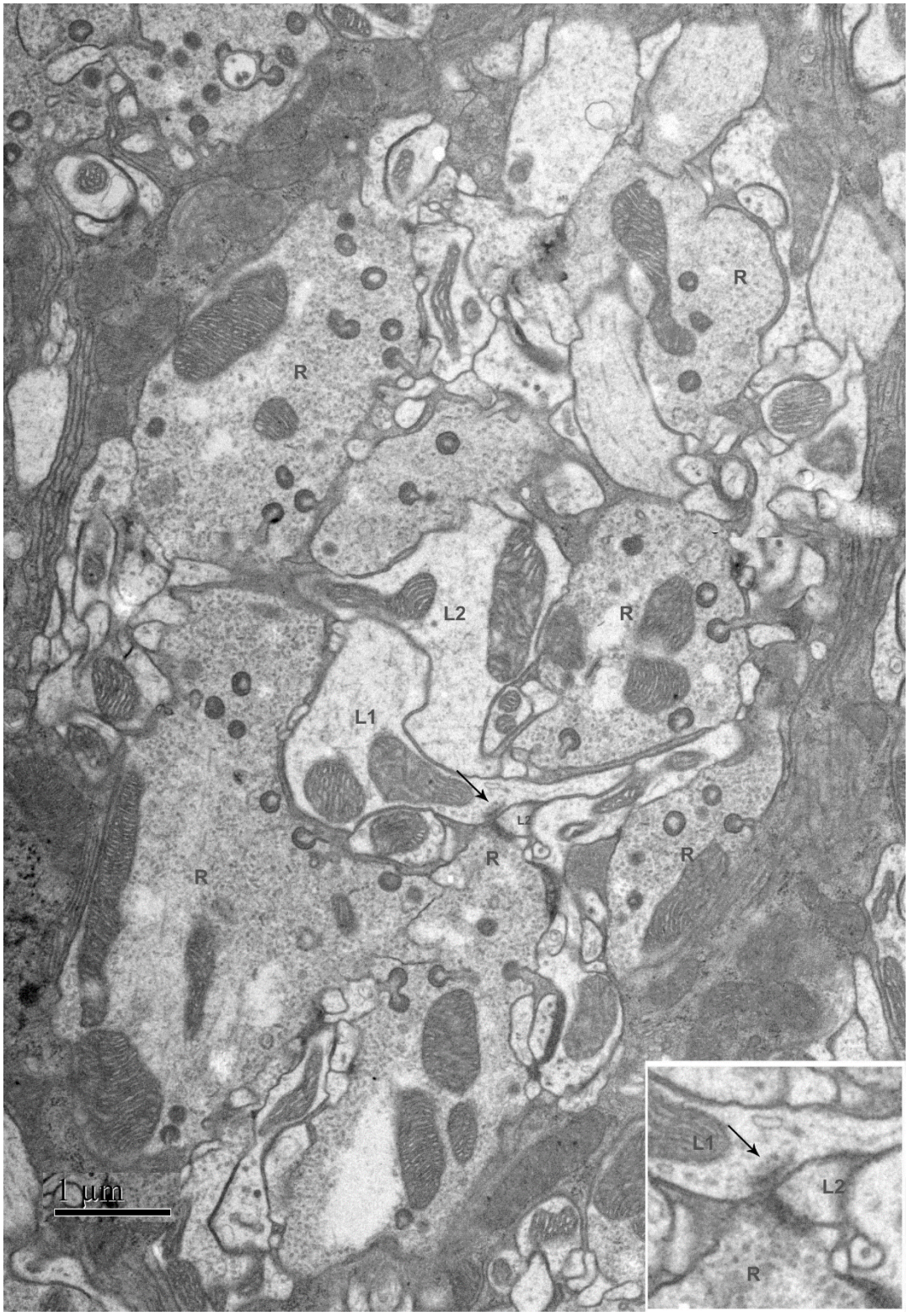

Figure S5. EM analysis of DIP mis-expression (related to Figure 4)

Cross section through a lamina cartridge (60nm section) from a fly mis-expressing DIPs-γ and ε in R cells imaged by TEM. 6 R cell profiles surrounding the axon profiles of L1 and L2 neurons are identified, demonstrating that the general arrangement of profiles within cartridges is not perturbed under these conditions, allowing each profile to be identified from its position. Arrow at pre-synaptic site indicates a putative L1 to R cell synapse (also shown in Figure 3M). Scale bar= 1µm.

Table S1

Layer	R1	R2	R3	R4	R5	R6	R1-6	L1	L2	L3	L4a	L4b	L5	L1-5	Unidentified
1	0	0						0	0	0		0	0		0
11	3	3	B					0	0	1		0	0		1
21	4	4	0					0	0	0		1	0		14
31	2	2	2					0	1	1		0	0		18
41	1	3	0	B		B		0	0	0		0	0		18
51	2	2	3	1	B	1		0	1	0		2	0		15
61	3	4	0	1	0	4		0	2	1		0	0		14
71	3	1	1	3	3	8		2	1	1		2	0		25
81	4	4	1	5	6	4		1	0	1	B	1	0		20
91	6	5	3	2	2	3		0	0	0	0	0	0		17
101	1	2	4	3	2	2		1	0	0	0	1	0		7
111	3	3	0	3	2	4		0	1	1	0	1	0		3
121	4	0	2	4	3	1		0	2	0	0	0	0		4
131	4	5	3	3	3	3		0	0	2	0	0	0		6
141	4	2	1	3	4	2		1	1	1	0	0	0		10
151	3	5	E	1	3	1		0	0	2	0	0	1		4
161	5	5		2	3	5		0	0	2	0	0	2		5
171	1	2		6	2	4		0	1	0	0	0	0		3
181	1	3		3	3	6		1	5	0	1	0	0		3
191	3	3		3	2	1		0	2	0	1	0	2		4
201	0	2		3	3	3		1	3	0	1	0	0		0
211	3	2		5	1	2		0	2	0	2	0	0		1
221	1	1		2	3	1		0	2	0	3	0	0		2
231	2	3		2	3	2		1	3	0	0	0	0		0
241	0	E		3	3	4		1	0	0	3	0	0		1
251	E			1	3	0		0	2	0	0	0	0		2
261				5	1	E		1	1	0	0	0	0		0
271				1	E			1	2	0	0	0	0		0
281				E				0	1	0	0	0	0		0
291								1	0	0	0	0	0		0
301								0	2	0	0	1	0		0
311								0	1	0	0	0	0		0
	63	66	20	65	55	61	**330**	12	36	13	11	9	5	**86**	**197**
Sum	##														

Table S1. **EM analysis of DIP mis-expression** (**related to Figure 4)**

Numbers and distributions of R and L cell pre-synaptic sites identified by EM within a cartridge from a fly mis-expressing DIPs-γ and ε in R cells. The first row indicates pre-synaptic cell types. The left most column (Layer) shows the positions of synapses within the cartridge from distal

(Layer 1) to proximal (Layer 311). Each layer reports data for x10 60nm sections, Layer 1 thus comprising sections 1-10. If processes could not be traced throughout the cartridge B indicates the section where it was first identified, and E indicates the last section in which it was identified.