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Supplementary Materials for

Brain inflammation triggers macrophage invasion across the blood-brain barrier in *Drosophila* during pupal stages

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Figs. S1 to S8

Fig. S1 qPCR data

A) Brains of wandering third instar Drosophila larvae with pan-glial expression of PGRP-LC were subjected to quantitative real-time PCR (*PGRP-LC, Pvf2, AttacinD, Diptericin, Defensin, Metchnikowin,* (n = 4)). PGRP-LC efficiently activates expression of all AMPs tested. $p_{PGRP\text{-}LC}$ = 0.0018; p_{Pvf2} = 0.0125; p_{AttD} = 0.0011; p_{DptB} = 0.0286; p_{Def} < 0.0001; p_{Mtk} = 0.0018, (all p values were obtained using a t-test, except for DptB for which a Mann-Whitney test was used). Pupal stages could not be analyzed due to lethality. **(B)** Brains of wandering third instar larvae and different pupal stages with pan-glial expression of PGRP-LE were subjected to quantitative real-time PCR (*PGRP-LE, Pvf2, AttacinD, Diptericin, Defensin, Metchnikowin,* (n = 4)). $p_{PGRP\text{-}LE\ larvae}$ = 0.0021, $p_{PGRP\text{-}LE\ 5\text{-}12hAPF}$ < 0.0001, $p_{PGRP\text{-}LE\ 15\text{-}18hAPF}$ = 0.0245; $p_{Pvf2\ larvae}$ = 0.0007, $p_{Pvf2\ 5\text{-}12hAPF}$ = 0.0286, $p_{Pvf2\ 15\text{-}18hAPF}$ = 0.1071; $p_{AttD\ larvae}$ < 0.0001, $p_{AttD\ 5\text{-}12hAPF}$ = 0.0003, $p_{AttD\ 15\text{-}18hAPF}$ < 0.0001; $p_{DptB\ larvae}$ = 0.0286, $p_{DptB\ 5\text{-}12hAPF}$ = 0.0092, $p_{DptB\ 15\text{-}18hAPF}$ = 0.0004; $p_{Def\ larvae}$ = 0.0002, $p_{Def\ 5\text{-}12hAPF}$ < 0.0001, $p_{Def\ 15\text{-}18hAPF}$ = 0.2378; $p_{Mtk\ larvae}$ = 0.0119, $p_{Mtk\ 5\text{-}12hAPF}$ = 0.0006, $p_{Mtk\ 15\text{-}18hAPF}$ < 0.0001; (all p values were obtained using a t-test, except for DptB (larvae), and Pvf2 (young pupae) for which a Mann-Whitney test was used). **(C)** Brains of wandering third instar larvae and different pupal stages with pan-glial expression of PGRP-SA / GNBP1 were subjected to quantitative real-time PCR (*PGRP-SA, Pvf2, AttacinD, Diptericin, Defensin, Metchnikowin,* (n = 4)). $p_{PGRP\text{-}SA\ larvae}$ = 0.0035, $p_{PGRP\text{-}SA\ 5\text{-}12hAPF}$ < 0.0001, $p_{PGRP\text{-}SA\ 15\text{-}18hAPF}$ = 0.0005; $p_{Pvf2\ larvae}$ = 0.0131, $p_{Pvf2\ 5\text{-}12hAPF}$ = 0.3429, $p_{Pvf2\ 15\text{-}18hAPF}$ = 0.1781; $p_{AttD\ larvae}$ = 0.6571, $p_{AttD\ 5\text{-}12hAPF}$ = 0.0089, $p_{AttD\ 15\text{-}18hAPF}$ = 0.0018; $p_{DptB\ larvae}$ = 0.0286, $p_{DptB\ 5\text{-}12hAPF}$ = 0.1163, $p_{DptB\ 15\text{-}18hAPF}$ = 0.3946; $p_{Def\ larvae}$ > 0.9999, $p_{Def\ 5\text{-}12hAPF}$ = 0.0771, $p_{Def\ 15\text{-}18hAPF}$ = 0.2187; $p_{Mtk\ larvae}$ = 0.6933, $p_{Mtk\ 5\text{-}12hAPF}$ = 0.0354, $p_{Mtk\ 15\text{-}18hAPF}$ = 0.4857; (all p values were obtained using a t-test, except for *Pvf2* (young pupae), *AttD* (larvae), *DptB* (larvae), *DptB* (young pupae), *Def* (larvae), and *Mtk* (old pupae) for which a Mann-Whitney test was used).

Fig. S2 Comparison of different macrophage markers

The Fig. shows 22 APF old pupal brains stained for Repo (green) to label glial nuclei, HRP (cyan) to indicate neuronal membranes and anti-mCherry to detect the activity of the indicated enhancer elements. **(A)** *HmlΔ*^{dsRed}. **(B)** *srpHemo-H2A::3xmCherry*. **(C)** *srpHemo-moe::3xmCherry*. **(A'-C')** show the expression of the macrophage marker only. The asterisk in C' shows a set of 6 neurons. Scale bar is 100 µm.

Fig. S3 Differential effects of *Pvf2* expression

(A) Brains of wandering third instar larvae and different pupal stages with pan-glial expression of *Pvf2* were subjected to quantitative real-time PCR (*PGRP-SA, PGRP-LC, PGRP-LE, Pvf2, AttacinD, Diptericin, Defensin, Metchnikowin,* (n = 4 – 8)), $p_{PGRP\text{-}SA\ larvae} = 0.8703$, $p_{PGRP\text{-}SA\ 5\text{-}12hAPF} = 0.2$, $p_{PGRP\text{-}SA\ 15\text{-}18hAPF} = 0.0026$; $p_{PGRP\text{-}LC\ larvae} = 0.004$, $p_{PGRP\text{-}LC\ 5\text{-}12hAPF} = 0.8286$, $p_{PGRP\text{-}LC\ 15\text{-}18hAPF} = 0.0105$; $p_{PGRP\text{-}LE\ larvae} = 0.0162$, $p_{PGRP\text{-}LE\ 5\text{-}12hAPF} = 0.0709$, $p_{PGRP\text{-}LE\ 15\text{-}18hAPF} = 0.4569$; $p_{Pvf2\ larvae} = 0.004$, $p_{Pvf2\ 5\text{-}12hAPF} = 0.0286$, $p_{Pvf2\ 15\text{-}18hAPF} < 0.0001$; $p_{AttD\ larvae} = 0.6828$, $p_{AttD\ 5\text{-}12hAPF} = 0.0286$, $p_{AttD\ 15\text{-}18hAPF} = 0.0040$; $p_{DptB\ larvae} = 0.004$, $p_{DptB\ 5\text{-}12hAPF} = 0.0286$, $p_{DptB\ 15\text{-}18hAPF} = 0.0709$; $p_{Def\ larvae} = 0.2532$, $p_{Def\ 5\text{-}12hAPF} = 0.2432$, $p_{Def\ 15\text{-}18hAPF} = 0.9253$; $p_{Mtk\ larvae} = 0.9253$, $p_{Mtk\ 5\text{-}12hAPF} = 0.0639$, $p_{Mtk\ 15\text{-}18hAPF} = 0.0249$; all p values were obtained using a Mann-Whitney test, except for PGRP-SA (larvae, old pupae), PGRP-LC (old pupae), PGRP-LE (young old pupae), Pvf2 (old pupae), DptB (old pupae), Def (larvae, young pupae), and Mtk (young and old pupae) for which a t-test was used. **(B-D)** Brains of indicated age stained for mCherry expressing macrophages and glial nuclei (anti-Repo, green). **(B,B')** Induction of *Pvf2* at the onset of pupal development (0 h APF) is sufficient to trigger invasion of macrophages (arrows), however, fewer cells are found in the CNS compared to those that express *Pvf2*

throughout development in a pan-glial manner. **(C,D)** Adult specific pan-glial expression of *Pvf2* does not cause invasion of macrophages into the brain. Scale bars are 100 µm.

Fig. S4 Invading macrophages explore the surface of the subperineurial glia

Electron microscopic images of a 12 h APF pupal brain. **(A)** Overview, the boxed area is shown in **(B,C).** A macrophage (Mø), that can be recognized by many large vesicles, sitting between perineurial (PG) glial cells is exploring the surface of a subperineurial glial cell (SPG). Neuronal nuclei (N). **(D,E)** A macrophage (colorized in purple) trapped between perineurial glia and subperineurial glia contacts the intact septate junctions (SJ) of the subperineurial glia (arrowheads in E). Scale bars are as indicated.

Fig. S5 Activation of the Toll pathway is not sufficient to trigger macrophage invasion

Pupal brains of the indicated age stained for glial nuclei (anti-Repo, green), and invading macrophages (mCherry, magenta). **(A,B)** Activation of the Toll pathway by either suppressing *cactus* expression or by overexpression of *Toll* does not result in the invasion of macrophages into the pupal brain. **(C)** Pan-neuronal expression of *PGRP-LE* does not trigger invasion of macrophages into the pupal brain. **(D)** *Gliotactin-Gal4* mediated expression of *PGRP-LC* leads to the infiltration of some macrophages into the brain (arrowheads). Scale bars are 100 µm.

Fig. S6 The blood-brain barrier is maintained during early pupal stages

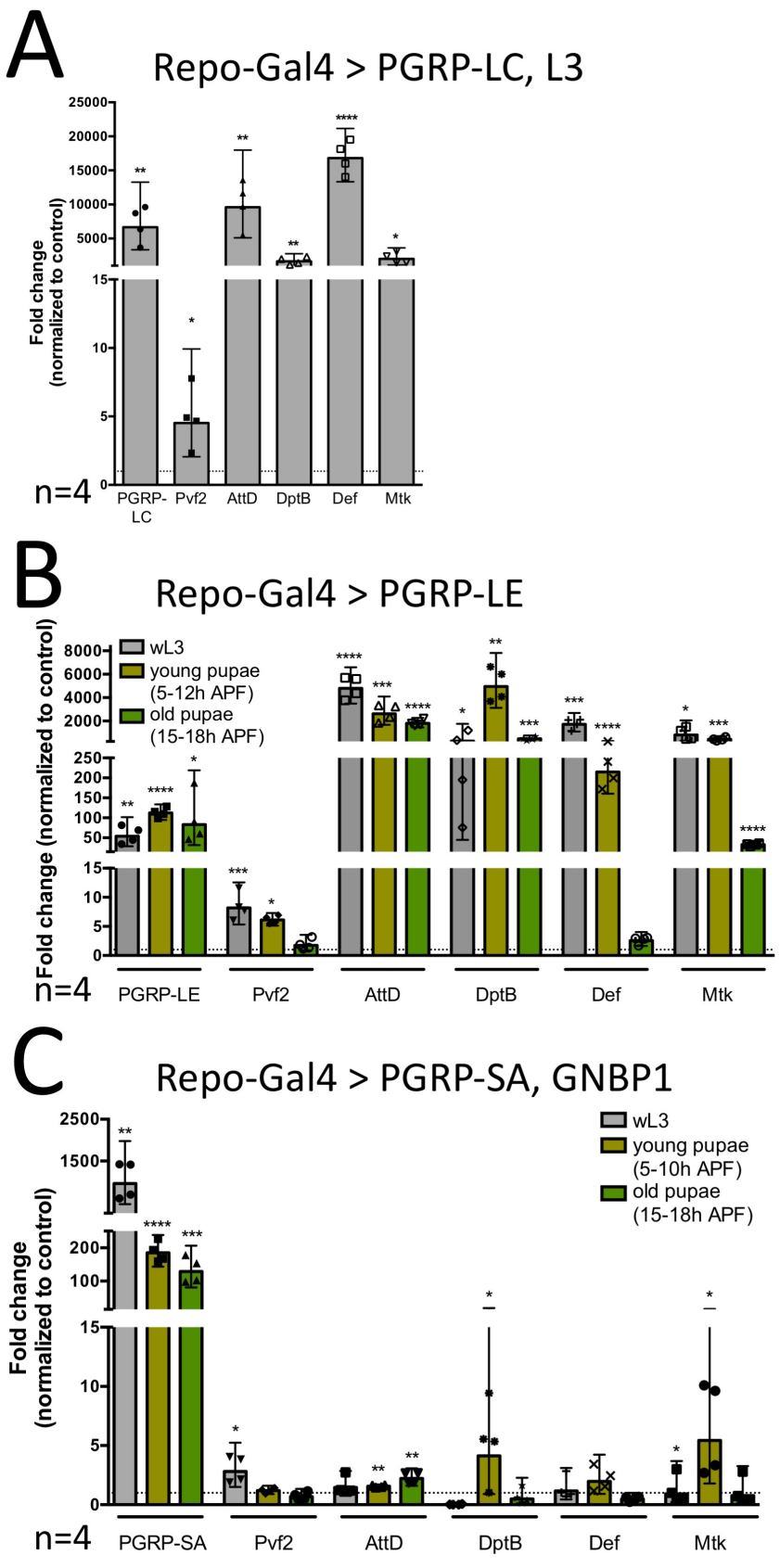
Quantification of control brains *[repo-Gal4, UAS-GFPdsRNA]* for the BBB integrity. **(A,B)** Penetration of fluorescein-labelled 70 kDa dextran during different developmental stages. L3: wandering third instar larva, 5 h APF, 24 h APF and 48 h APF. **(B)** Quantification of changes in fluorescence uptake after 40 min at different developmental time points as indicated. No significant changes can be detected. **(C,D)** Penetration of Texas-red-labelled 10 kDa dextran during the same time points as in **(A)**.**(D)** Quantification of fluorescence changes between the developmental stages indicated (L3 vs 5 h APF: $p = 0.0159$, 5h APF vs 24 h APF: $p = 0.0317$, 24 h APF vs 48 h $p = 0.3095$).

Fig. S7 The blood-brain barrier is maintained during early pupal stages

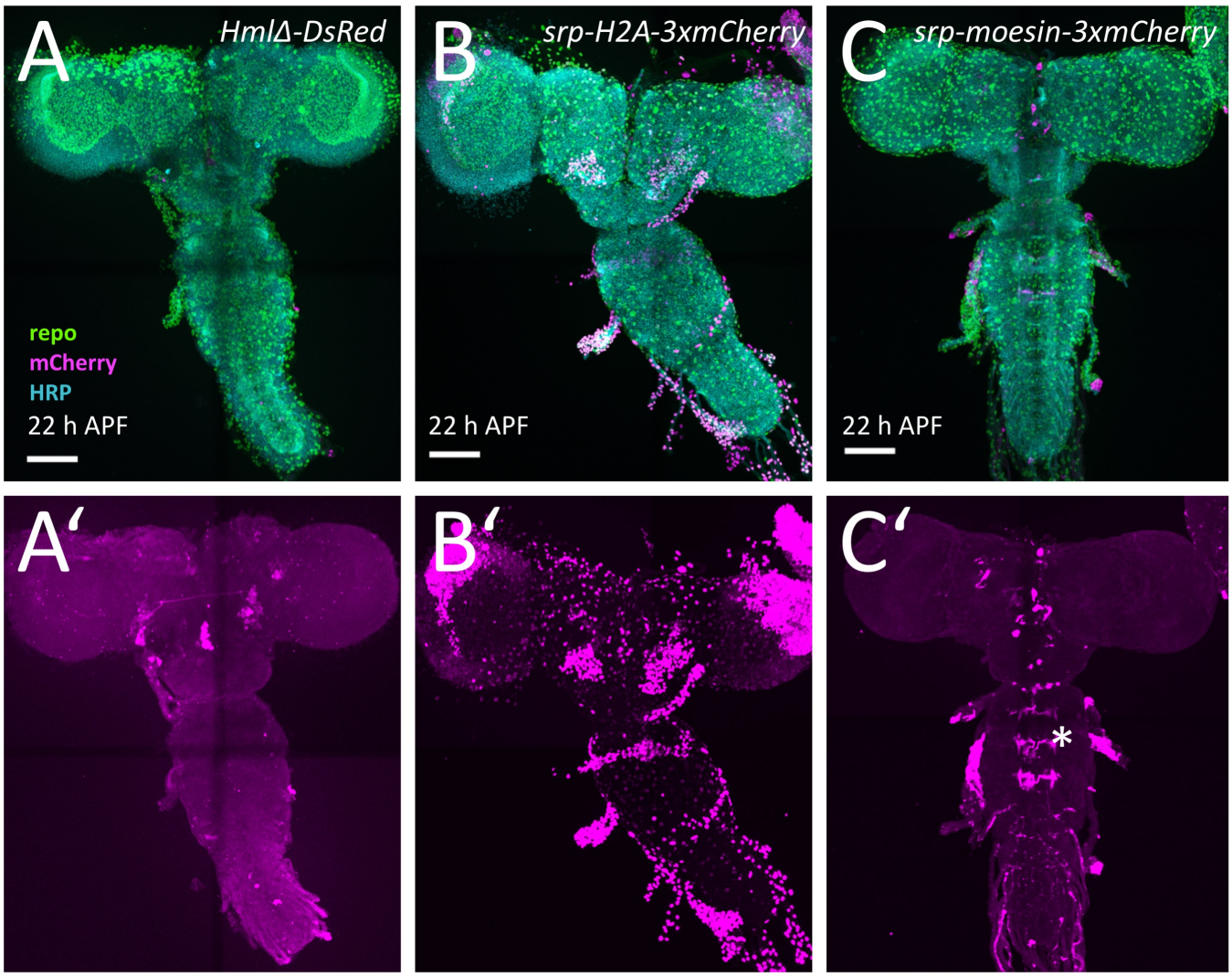
Pupal brains of the indicated age stained for GFP (green), the septate junction component NrxIV (magenta) and neuronal cell membranes (anti-HRP, cyan). *LexAop-GFP* is expressed in subperineurial glial cells using *GMR54C07-LexA*. Note that subperineurial glial cells are always present at the brain surface. Occasional holes can be detected at variable positions indicating preparation artefacts. Scale bars are 100 µm.

Fig S8. *Pseudomonas aeruginosa* infection

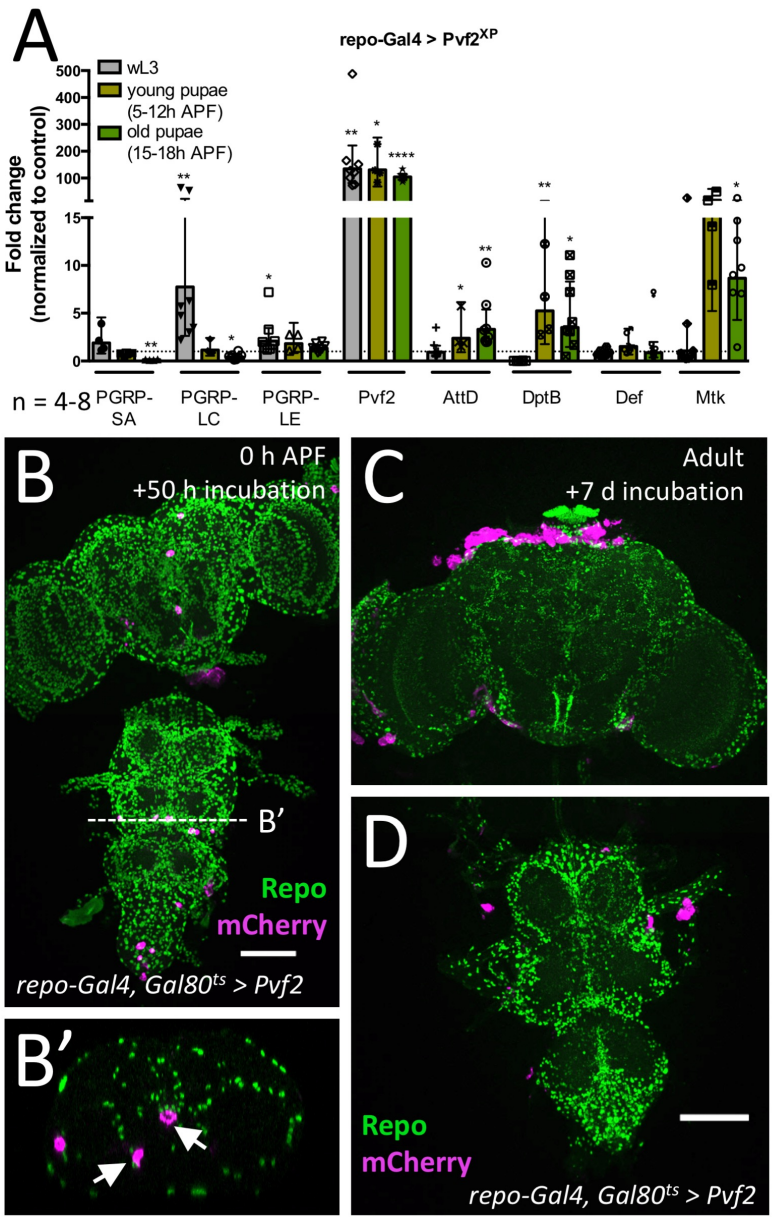
A-F) Confocal images (top and orthogonal views) showing *Pseudomonas aeruginosa* (in green) attached to the BBB (*mdr65-tomato* in magenta) (A-C) or inside the brain (D-F) upon *ex vivo* infections of late 3rd instar larval brain. Lower right images representing close-up of the dotted boxes showing attached or infiltrated bacteria. DAPI is in blue. **G)** Wandering third instar Drosophila larvae injected with mock or *Pseudomonas aeruginosa* were dissected 4-5 h after infection and the brains were subjected to quantitative real-time PCR (qPCR; *Pvf2, PGRP-LE, PGRP-SA, Tl, Dl, Dif, Rel, AttacinD, Diptericin, Defensin* (n=5); *PGRP-LC* (n=2); *Metchnikowin* (n=3)). Expression of the genes *Pvf2, Dif* were upregulated and *AttacinD* and *Defensin* were downregulated. Mann-Whitney tests were performed for each gene between Non-infected and infected CNS. $p_{Pvf2\ \text{non-infected vs infected}}=0.0317$; $p_{PGRP\text{-}LE\ \text{non-infected vs infected}} > 0.9999$; $p_{PGRP\text{-}LC\ \text{non-infected vs infected}} = 0.6667$; $p_{PGRP\text{-}SA\ \text{non-infected vs infected}} = 0.2222$; $p_{Toll\ (Tl)\ \text{non-infected vs infected}} = 0.3095$; $p_{Dorsal\ (Dl)\ \text{non-infected vs infected}} = 0.6905$; $p_{Dif\ \text{non-infected vs infected}} = 0.0079$; $p_{Relish\ (Rel)\ \text{non-infected vs infected}} = 0.6905$; $p_{AttacinD\ \text{non-infected vs infected}} = 0.0238$; $p_{Diptericin\ \text{non-infected vs infected}} = 0.4206$; $p_{Defensin\ \text{non-infected vs infected}} = 0.0079$; $p_{Metchnikowin\ \text{non-infected vs infected}} = 0.4000$.



Winkler et al., Supplementary Figure 1



Winkler et al., Supplementary Figure 2



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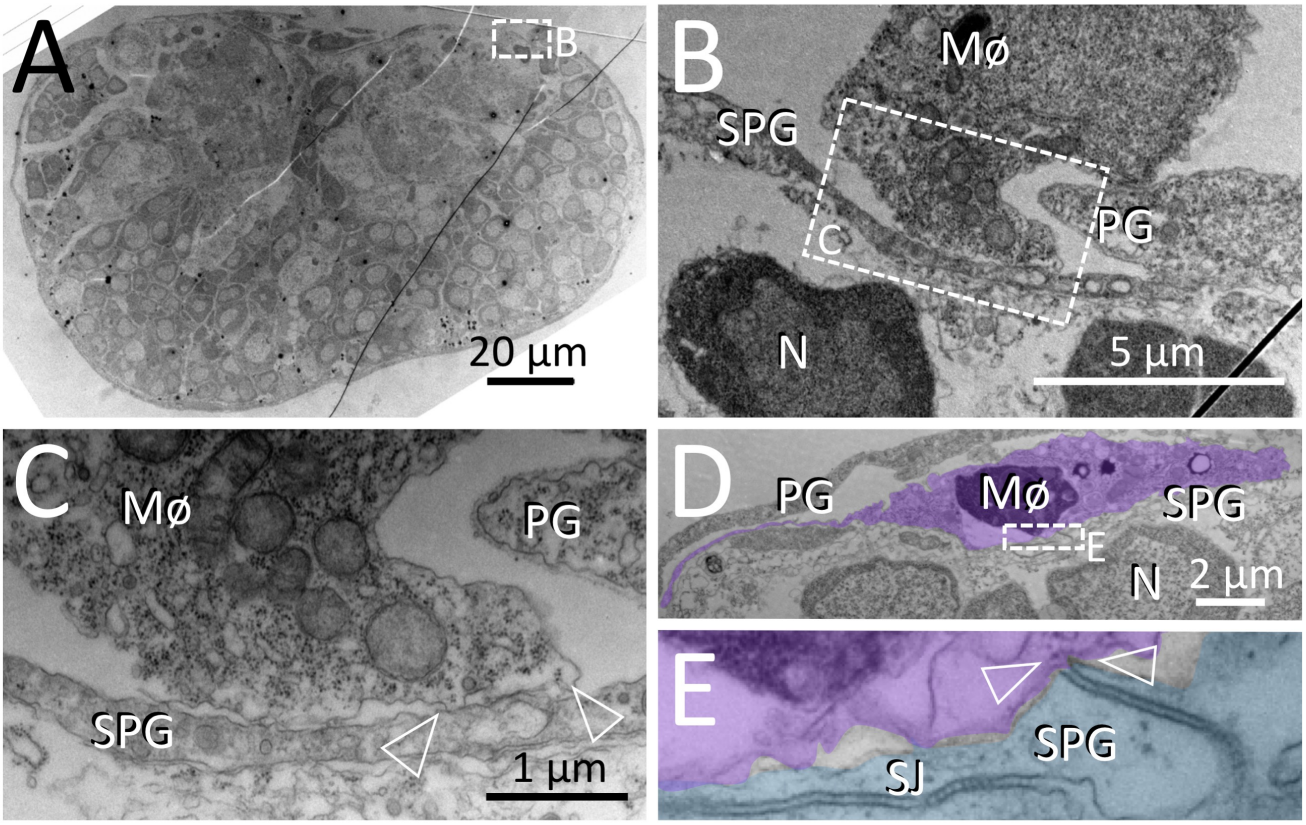

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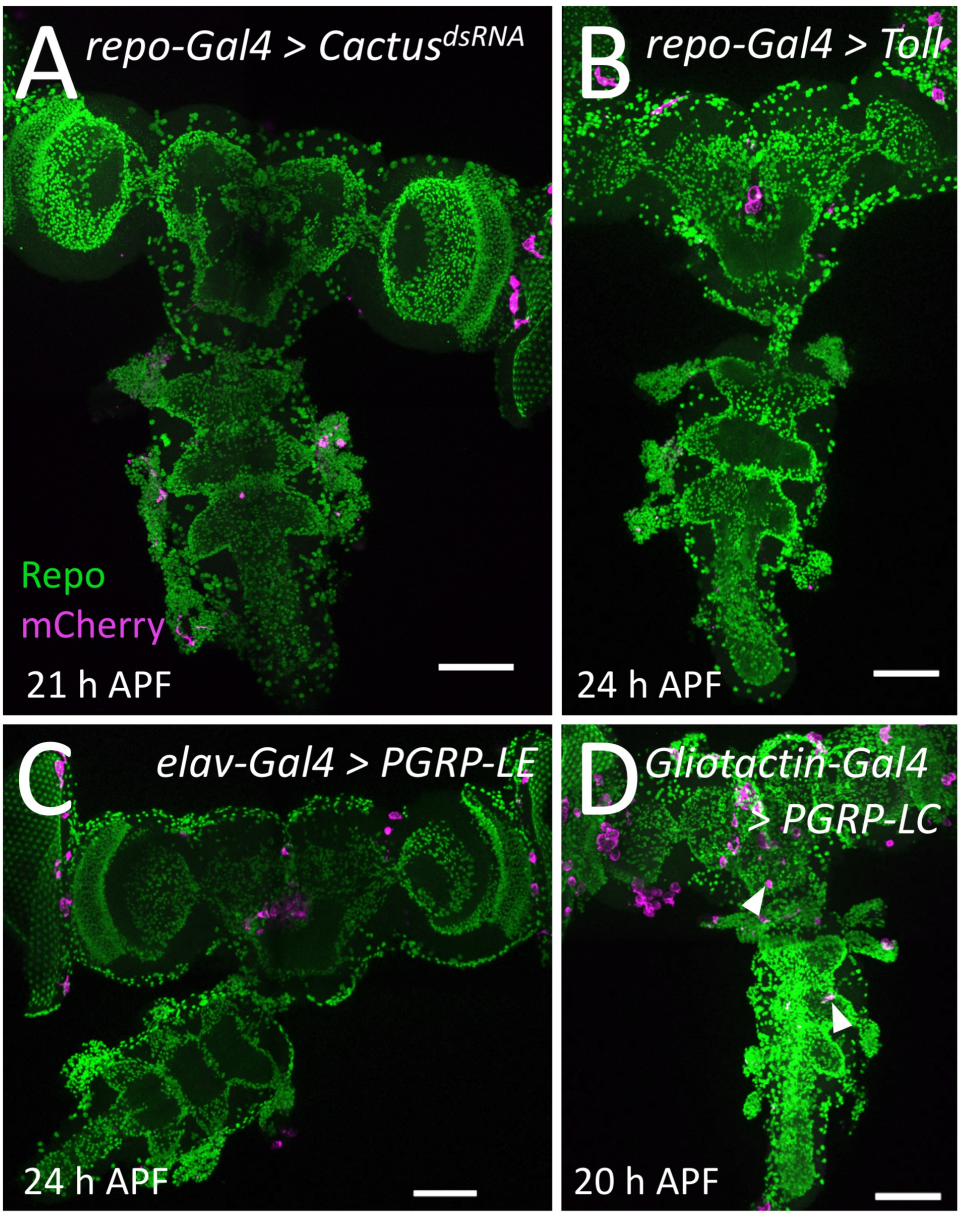

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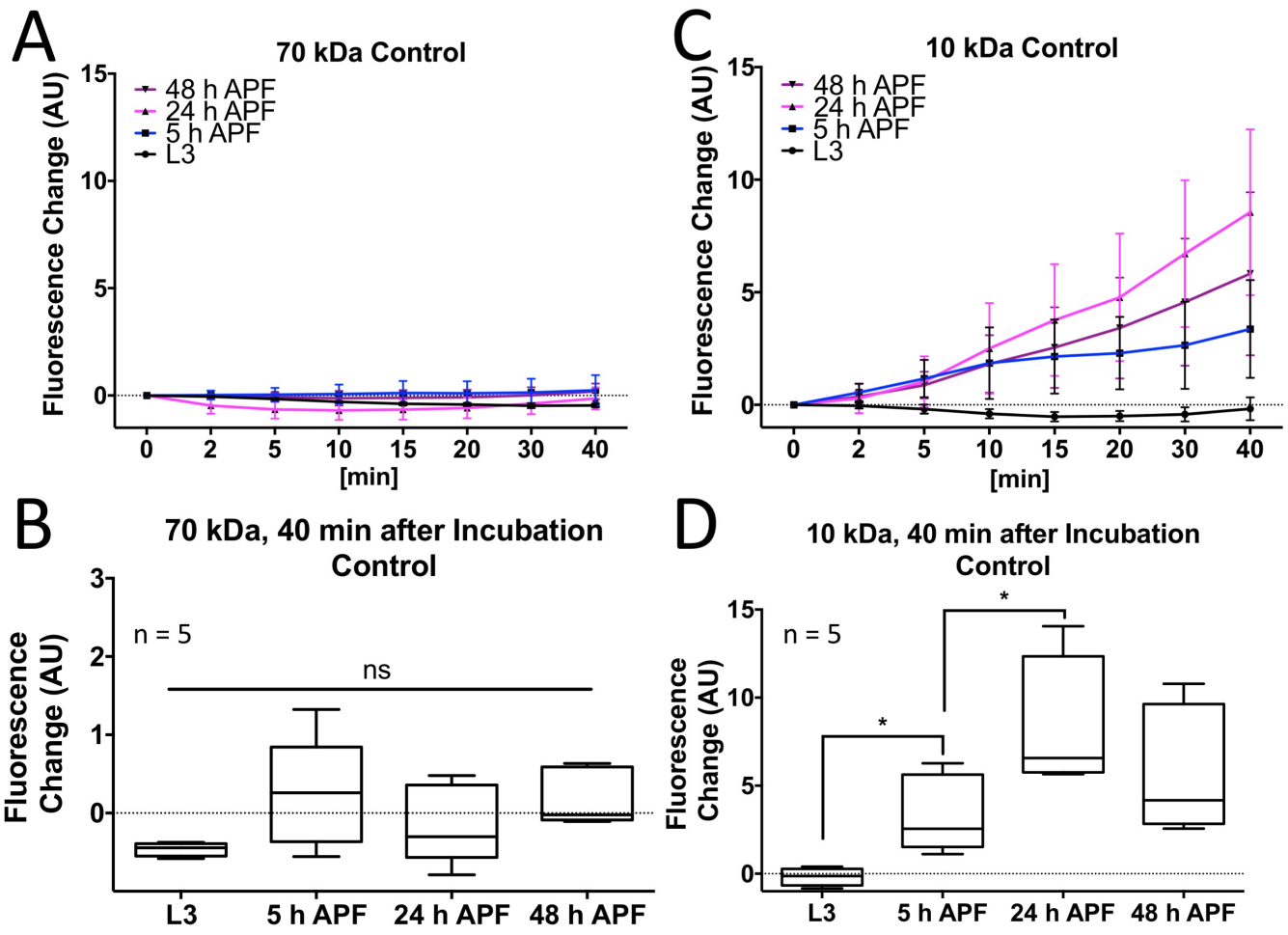

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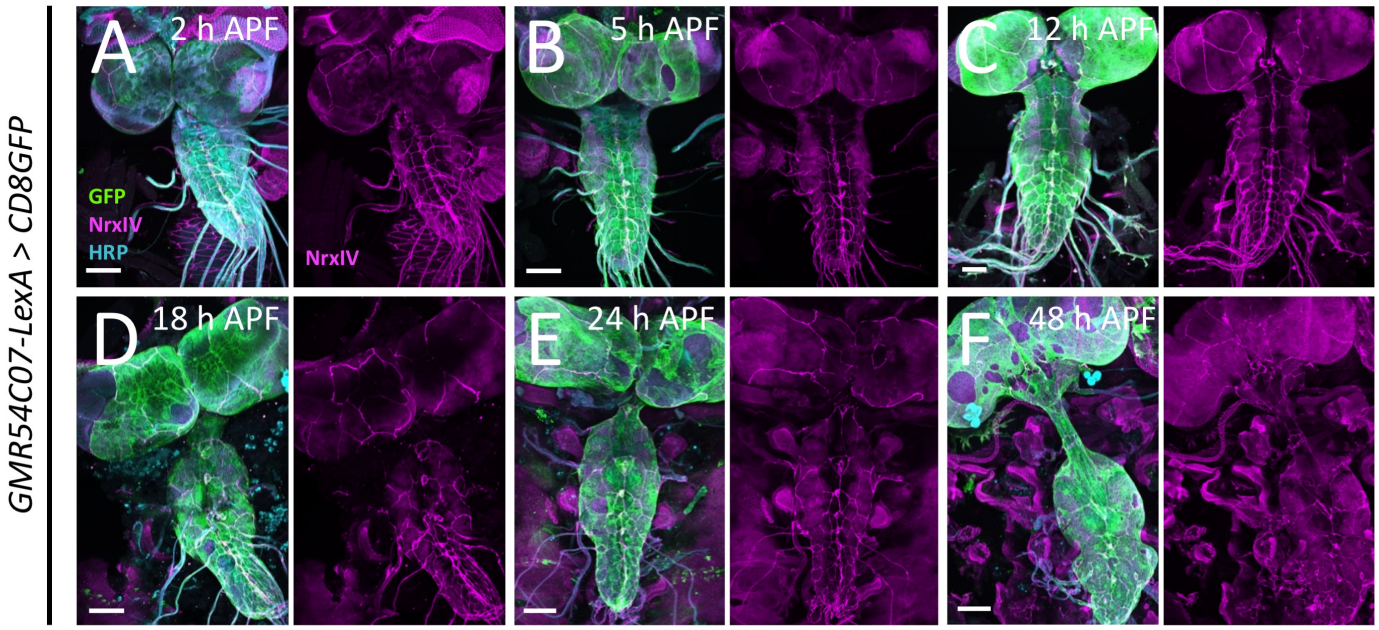

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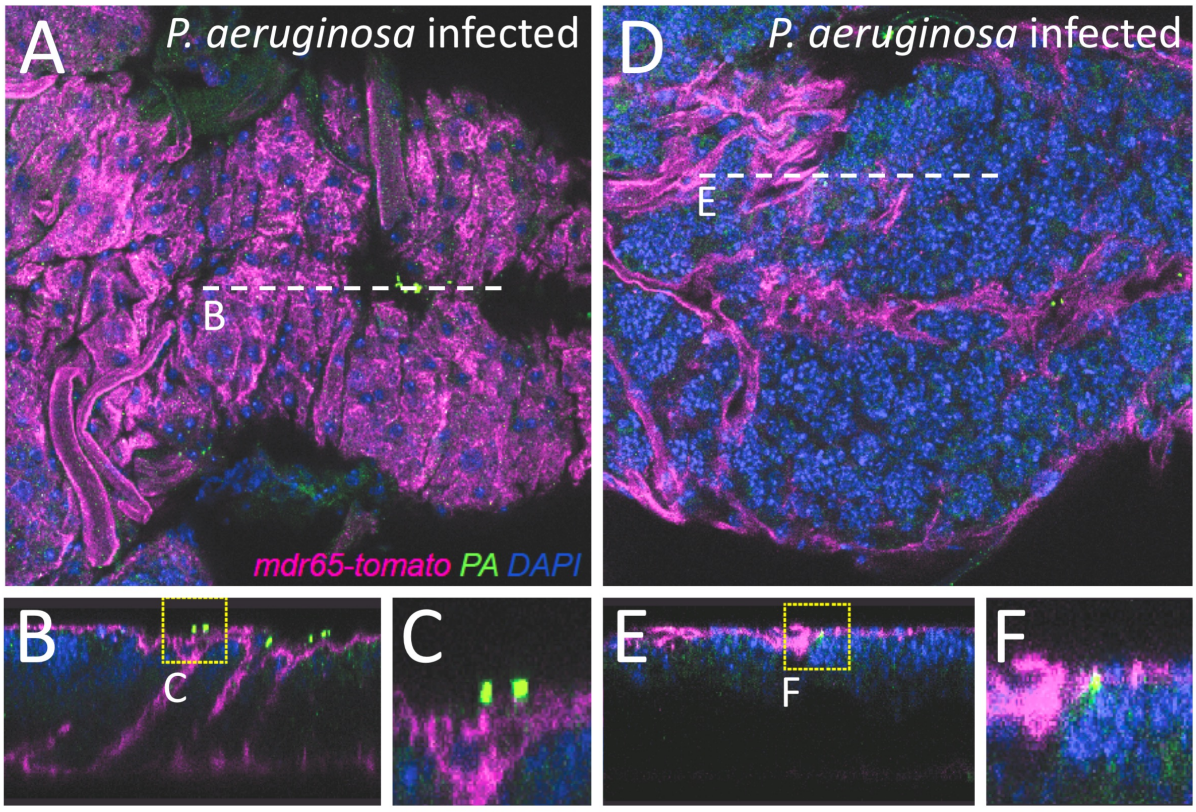

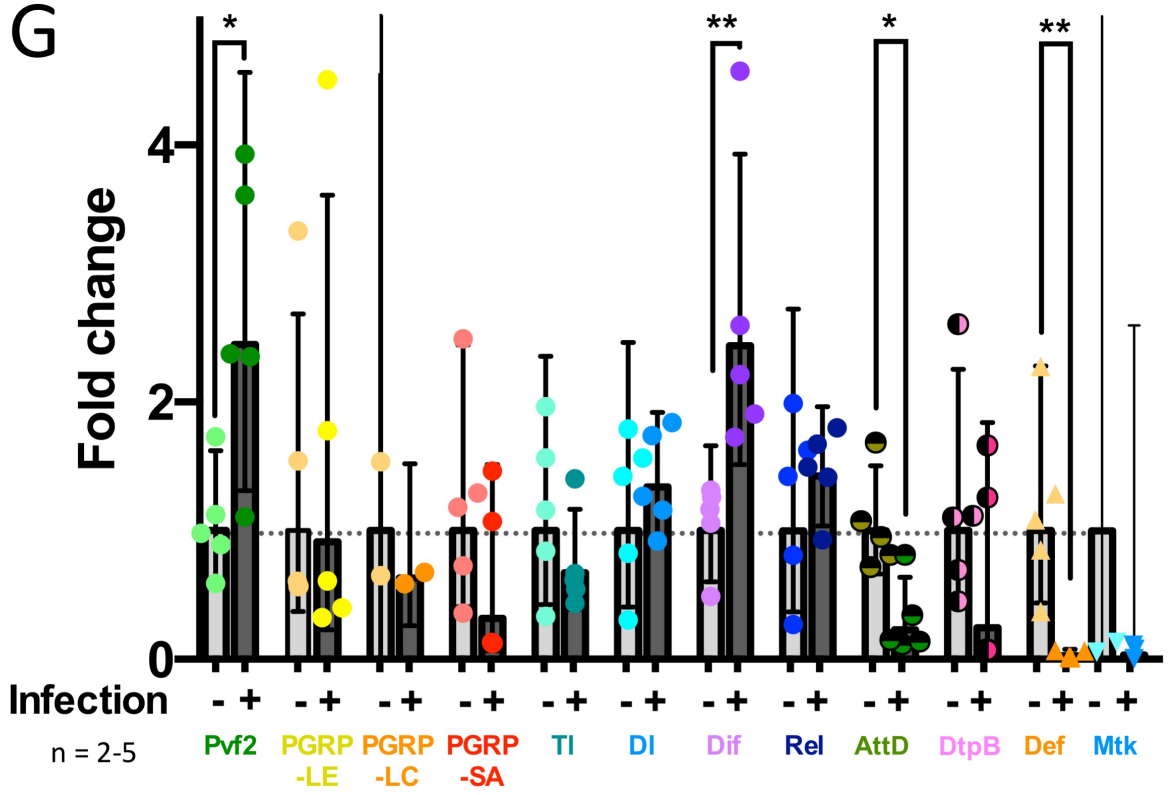

Winkler et al., Supplementary Figure 8