The roles of dystrophin and dystrobrevin : in synaptic signaling in drosophila

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CHAPTER 5

The Dp186 Dystrophin Isoform is Required for Wild Type Synaptic Gain in the *Drosophila* Olfactory Circuit

The Dp186 Dystrophin Isoform is Required for Wild Type Synaptic Gain in the *Drosophila* Olfactory Circuit

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Abstract

The olfactory system of *Drosophila* provides an excellent model to define the neural circuits and mechanisms which control behaviors elicited by environmental stimuli. Here, we show that the Dystrophin Dp186 isoform plays an important role in maintaining information transfer through the olfactory system. Dystrophin's roles have been primarily described as those of a structural anchor required for muscle integrity. A variety of recent studies have also, however, implicated Dystrophin in both peripheral and central synaptic function likely by its acting to scaffold components of signaling pathways. We show that the Dp186 protein is expressed at both sides of the primary synapse between the antennal olfactory receptor neuron and its downstream projection neuron in the antennal lobe which subsequently relays information into the central brain. No apparent abnormalities are observed in the number or the spatial organization of the olfactory sensillae, in olfactory receptor axon targeting or projection neuron morphology in *Dp186* mutants. Dp186 is, however, required in olfactory receptor neurons for their wild type levels of output upon odorant-evoked stimulation. Mutants also display decreased odor avoidance behavior at low, but not high, odorant concentrations. Dp186 expression in either the olfactory receptor or projection neurons rescues the low concentration odor avoidance phenotype. These results suggest that Dp186 is required to maintain wild type synaptic transmission levels at both the primary olfactory synapse and at downstream synapses. Furthermore, they indicate that increasing response or transfer gain at a particular point in this circuit can compensate for reduced function elsewhere.

Keywords: Dystrophin, olfaction, olfactory receptor neurons, projection neurons, antennal lobe, electroantennalgram, behavior, *Drosophila* melanogaster

Classification: Biological Sciences, neuroscience

Introduction

The olfactory system allows the detection and discrimination of a large variety of diverse odors in the external environment. Olfactory cues are used to locate food, potential mates and predators. Significant progress has been made in unraveling the molecular mechanisms and neuronal circuits that underlie the transmission of the information conveyed by these chemosensory cues to the central brain areas responsible for adaptive behaviors (reviewed in [1]). One of the model systems that have been invaluable for these studies has been the *Drosophila* olfactory system.

The olfactory system in *Drosophila* shares many morphological and functional characteristics with the vertebrate olfactory bulb (reviewed in [2–4]). Odorant molecules are detected by G protein-coupled receptors in sensory neurons called Olfactory Receptor Neurons (ORNs) in the antenna or maxillary pap. ORN axons relay olfactory information to the antennal lobes in the brain (reviewed in [5–8]). Each ORN expresses a single odorant receptor (OR) and all the ORNs that contain a given OR project to the same synaptic region, a glomerulus, in the antennal lobe. Multiple ORNs converge to make synapses with a much smaller number of secondary Projection Neurons (PNs) which relay the olfactory information to the higher order centers of the brain, the mushroom body and the lateral horn of the protocerebrum.

While the overall organization of the olfactory bulb of *Drosophila* is very similar to that of vertebrates, both of the bilaterally symmetric antennal lobes contains ~ 43 glomeruli, rather than the ~1000 in the mouse. The relatively simple, yet conserved, properties of the fly's olfactory system together with its' amenability to genetic analyses have made it into an attractive model to identify the molecules required for the establishment, maintenance and efficacy of an olfactory circuit (reviewed [1], [4], [9–11]). Essentially complete maps of the fly's 62 *or* gene expression domains and their axonal projections to particular glomeruli have been generated [12], [13]. While much has been established regarding OR specificity and ORN axon targeting and synaptogenesis, very few of the other genes required for the establishment and function of the olfactory circuit have been identified.

In this study, we show that the Dystrophin (Dys) Dp186 protein isoform is required for wild type synaptic output levels from ORNs and for wild type olfactory avoidance behavior at low odorant concentrations. Dystrophin's best-understood role is to stabilize the muscle sarcolemma against contraction-induced damage. Its absence in humans results in Duchenne Muscular Dystrophy (DMD), a fatal genetic disorder characterized by progressive muscle degeneration (reviewed in [14]), and frequently associated cognitive deficits (reviewed in [15]). Strikingly, Dystrophin and its ortholog, Utrophin, have also known to be enriched at a variety of synapses including the neuromuscular junction (NMJ); however, their roles there are just beginning to emerge (reviewed in [16–18]). Dystrophin is highly evolutionarily conserved, with respect to its structure and tissue-specific expression, indicating that many of its basic functions are likely conserved as well.

We have previously studied the roles of the Dystrophin DLP2 isoform at the *Drosophila* model NMJ [19], [20] and the Dp186 isoform at central brain synapses [21]. The absence of the DLP2 from the postsynaptic side of the NMJ results in elevated levels of neurotransmitter release by the presynaptic motoneuron [19]. This is likely mediated by a small GTPase signaling pathway which regulates the retrograde control of transmitter release [20]. The Dystrophin Dp186 isoform plays an analogous role in the regulation of neurotransmitter release at a central synapse in the *Drosophila* [21]. Similar synaptic roles of Dystrophin in the mammalian hippocampus have also been described [22] indicating that Dystrophin's synaptic roles are likely conserved.

Here, we show that the Dystrophin isoform Dp186 is expressed in the olfactory system in higher brain olfactory centers and at both sides of the ORN/PN synapse. No apparent antennal olfactory sensillae cell fate changes or ORN axon guidance defects are observed in mutant animals. We find, however, that Dp186 is required for wild type levels of output from odor-exposed ORNs. Mutants also display decreased olfactory avoidance behavior which can be rescued by expression of Dp186 in either the ORNs or PNs. Thus, wild type Dp186 function in either the pre- or postsynaptic partner is sufficient to compensate for decreased function, caused by the lack of Dp186, of the other. These results suggest that Dp186's role in the olfactory system is to maintain wild type synaptic efficacy at multiple points in the circuit. Lastly, this study further establishes the importance of Dystrophin in synaptic transmission and identifies one of the first molecules required for regulating transduction gain of the olfactory sensory input to higher brain centers. Adequate levels of signaling through this circuit are required to elicit complex behaviors in response to environmental olfactory stimuli.

Materials and Methods

Drosophila stocks

The *Drosophila* stocks, w^{1118}, SG18.1-GAL4, GH146-GAL4, repo-GAL4, MZ19-GAL4, UAS-nSyb and UAS-mCD8GFP that were used in this study were obtained from the Bloomington *Drosophila* Stock Center and are described at http://flybase.org. We confirmed the specificity of the SG18.1-GAL4 and GH146-GAL4 drivers by examining animals expressing UAS-mCD8-GFP under their control. The *Dp186* mutant lines $Dys^{Dp186\ 166.3}$ and $Dys^{Dp186\ 30.3}$ were described [21]. UAS-Denmark [23] was a gift from B. Hassan. The Or-GAL4 and Or-mCD8-GFP lines [12], [13] that were used to investigate specific ORN trajectories and innervation patterns were kindly provided by T. Hummel, L. Luo , L. Vosshall and the Bloomington *Drosophila* Stock Center.

RNA *in situ* hybridization and Immunohistochemistry

RNA *in situ* hybridization of Dp186 sense or anti-sense RNA probes to cryo-sections (10 µm thick) of one day old w^{1118} fly heads were performed at 52 °C as described in Vosshall et al. [24]. Samples were visualized and photographed on a Zeiss Axioplan microscope equipped with a Zeiss Axiocam digital camera.

One to two day old *Drosophila* brains were fixed for immunohistochemistry in PLP fixative (4% paraformaldehyde, 0.25% sodium periodate, 75 mM lysine-HCl and 37 mM sodium phosphate pH 7.4) for 1 hour and treated as described [25]. The nc82 mAb [26]; Developmental studies Hybridoma Bank), anti-Dp186 [19], anti- GFP (Roche Diagnostics) were used as primary antibodies. Secondary antibodies used were Alexa 488 and Alexa 568 (Invitrogen) and Cy3-conjugated goat anti-mouse (Jackson Laboratories). Samples were visualized and photographed by confocal microscopy (Leica SL100).

Semi-quantitative RT-PCR

Semi-quantitative reverse transcription (RT-PCR) on total RNA derived from 100 adult antennae or 10 larvae of the w^{1118} or $Dys^{Dp186\ 166.3}$ genotypes were performed as described [19].

Electroantennogram (EAG)

EAG responses of adult flies were recorded as previously described [27–30]. Briefly, one to three day old flies were mounted inside a truncated micropipette tip with the anterior portion of the head protruding from the end of the tip. A fine-tip micro glass electrode, filled with Ringer's solution, was inserted into the eye and served as a reference electrode. The recording electrode

was placed on the frontal surface of the anterior aspect of the antenna. The recording electrode was connected to a DC amplifier (10x Synech). The main airflow was supplied onto the antennae through a glass tube. When a stable baseline was obtained, EAG recordings were started after odor stimulation was administrated by injecting a puff of purified air (0.5 s at 0.5L/min airflow) through an odor cartridge using an electronically controlled stimulus flow controller (SFC-2, Syntech). All odorants used were dissolved in paraffin oil at the given concentration. The following odorants were obtained from Sigma (catalog numbers in parentheses): Benzaldehyde (≥ 99.5%), trans-2-Hexen-1-al (≥ 98 %), 3-octanol (no 74870), Ethylbutyrate (E15701), Ethyl acetate (C2513) and Hexanal (21520). *Cis*-vaccenyl acetate (cVA) was obtained from the Pherobank and 4-methyl cyclohexanol (99% COS-TRANS mixture) was obtained from FLUKA.

Odor avoidance behavioral test

Flies (75 to 100 individuals) were aged for 2 to 5 days post-eclosion were loaded into the upper arm of a T-maze apparatus [31] and exposed to ambient room air drawn through a blank odor (heavy mineral oil, Mallinckrodt) at a rate of 650 mL min^{-1} for 90 sec to allow for acclimation. Following this exposure, flies were lowered to a choice point and permitted to freely move between two tubes. One tube presented an airflow of benzaldehyde (Aldrich) diluted in heavy mineral oil as indicated in the figure and the other presented a blank odor, both at a rate of 650 mL min^{-1}. After 120 sec, the choice point was closed and flies were counted in each tube. A performance index was calculated as the normalized percentage of flies avoiding the odor versus the blank. A score of 0 indicates a 50:50 distribution and a score of 100 represents 100% avoidance. All experiments were performed at 25 ± 0.5ºC in 80 ± 5% humidity under dim red light.

Statistical analyses

GraphPad Prism V.5 was used for plotting non-linear regression graphs of the EAG response curves (non-linear fit curve). The data of the EAG experiments were analyzed by repeated-measures ANOVA from the SPSS statistical analysis program V. 15.0. One-way ANOVA and Bonferroni's post-test were used for analyzing the EAG rescue experiment.

Results

The Dystrophin isoform Dp186 is expressed at several regions in the olfactory system including the primary ORN synapse

The *Drosophila Dystrophin* (*Dys*) gene encodes multiple protein isoforms, some of which are expressed in the muscle and at the NMJ [19], [32]. Other isoforms, among them Dp186, are expressed in the nervous system [21]. In the adult CNS, Dp186 protein is present at a number of synapse-rich regions of the olfactory system, such as the calyx of the mushroom bodies (MBs), the lateral horn of protocerebrum (LP) and the antennal lobes (ALs) (**Figs. 1A, C, G** and **I**). To investigate whether Dp186 in the ALs is expressed in the presynaptic ORNs we co-labeled ALs in animals expressing mCD8-GFP, a transmembrane GFP fusion protein [33] often used to visualize axon trajectories, in all ORNs with anti-Dp186 and anti-GFP. Dp186 protein colocalized with GFP at the ORN synapses in the ALs (**Fig.1E**). We also observed localization of Dp186 in the PN dendrites, as shown by the overlapping in expression between Dp186 and the dendrites of the DA1, VA1d and DC3 glomeruli visualized by their expression of the Denmark dendritic marker (**Fig1. H**). We did not observe colocalization of Dp186 and a glial cell marker (**Fig.1K**).

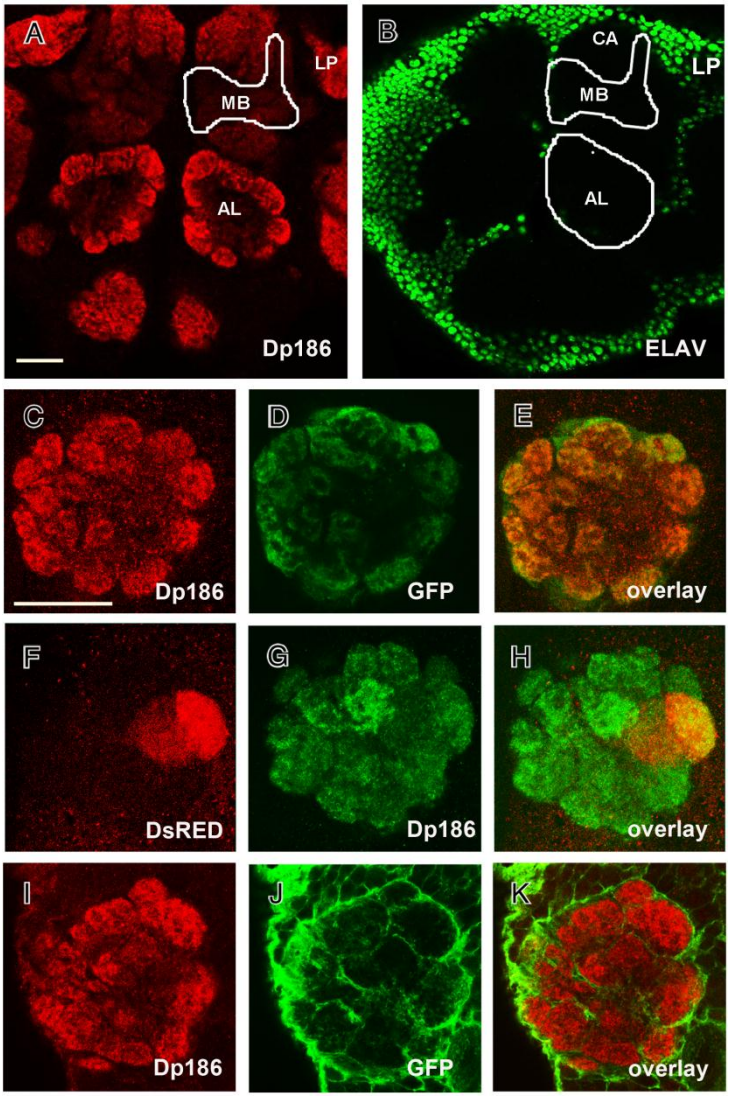

Figure 1. Dp186 protein is localized to the primary ORN presynaptic terminals and the PN dendrites of the *Drosophila* olfactory bulb, the AL. Adult *Drosophila* brains are shown in (**A**) and (**B**) and adult antennal lobes in (**C**) through (KAnti-Dp186 antibody was used in panels (**A**), (**C**), (**G**), and (**I**) and anti-GFP in panels (**B**), (**D**) and (**J**). Confocal images are shown of wild type whole-mount brains labeled with either anti-DP186 in (**A**) or the pan-neuronal nuclear marker anti-Elav in (**B**). The Dp186 protein is highly enriched at the antennal lobe (AL), the area around mushroom body (MB), the calyx (CA) and the lateral horn of the protocerebrum (LP). Dp186 protein in the AL in red (**C**) and GFP expressed at the ORN presynaptic terminals in green (**D**) (genotype UAS-nSybGFP/SG18.1- GAL4) are shown. The overlay is shown in (**E**). Dp186 colocalizes with the presynaptic marker in many glomeruli. Dp186 protein in green (**G**) and the Denmark DsRed postsynaptic dendritic marker expressed in the PN dendrites of the VA1d and DC3 glomeruli in red (**F**) (genotype UAS-Denmark-DsRed/MZ19-Gal4) are shown. The overlay is shown in (**H**). Dp186 colocalizes with the postsynaptic marker in those glomeruli that express Denmark-DsRed. Dp186 in red (I) and GFP expressed in glial cells in green (J) (genotype UAS-mCD8GFP/Repo-Gal4, pan glial driver)are shown. The overlay is shown in (K). Scale bars = 25 µm.

Dp186 mRNA is expressed by the ORNs and both pre- and postsynaptic Dp186 protein is enriched at the AL synapse.

To further confirm that Dp186 is expressed by the ORNs we performed RNA in situ hybridizations on antennal sections using antisense (**Fig. 2A**) and control sense (**Fig. 2B**) Dp186-specific riboprobes. Dp186 mRNA is widely expressed in the antenna, likely in most ORN cell bodies. The high density of Dp186 mRNA expressing cell bodies in the brain (not shown) precluded us from confirming that PNs express Dp186 mRNA by hybridization. Secondly, we performed semi-quantitative RT-PCR on wild type versus *Dp186* mutant antennal RNA. The results, a RT-PCR product of the anticipated size from wild type antennal mRNA, but not from mRNA derived from the *Dys* $^{DP186\ 166.3}$ mutant, indicate the presence of Dp186 mRNA in the antenna (**Fig. 2C**).

We then asked whether Dp186 expressed in either ORNs or PNs localizes to the AL synapse. Ectopically expression of Dp186 in all ORNs (**Fig. 2F**) or all PNs (data not shown) in the *Dp186* mutant background resulted in accumulation of Dp186 at the synapse. These results support the colocalization experiments above which indicate that Dp186 accumulates at both sides of the wild type synapse. We were unable to detect Dp186 protein in the ORN cell bodies in antennal sections from wild type individuals. We were, however, able to visualize it in other regions of the brain present in adjacent sections (data not shown) indicating that the fixing and embedding conditions did not interfere with our ability to detect Dp186 in the antenna. Together these results indicate that the PNs and ORNs express Dp186 and most of that expressed by the ORNs is transported to or retained at the presynaptic side of the ORN/PN synapse.

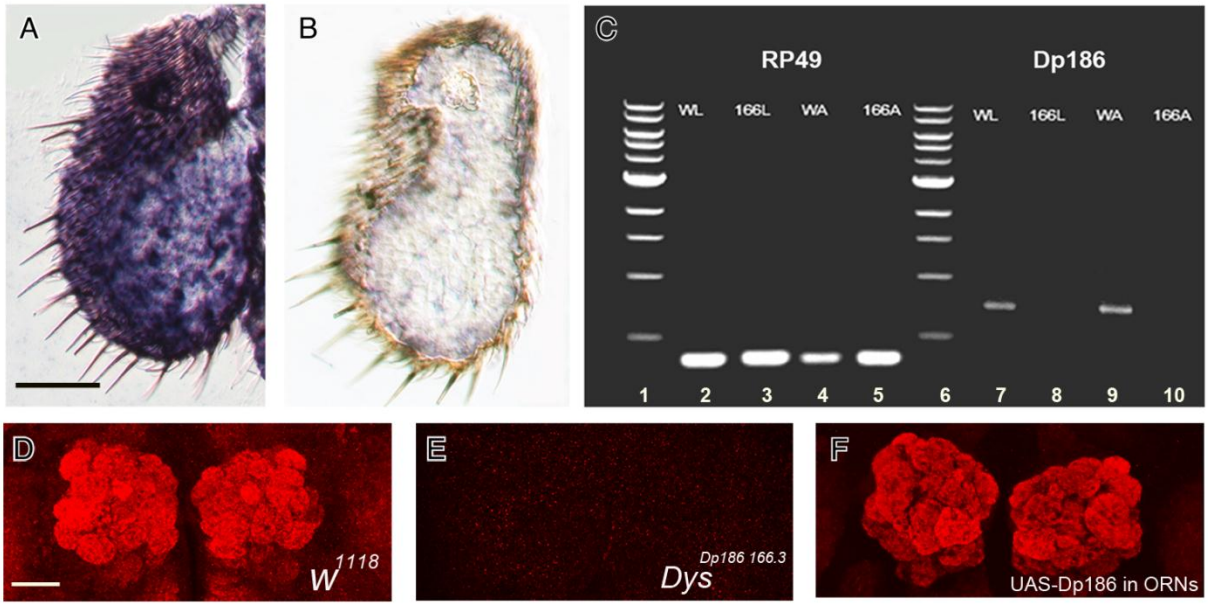

Figure 2: Dp186 mRNA is expressed in the antenna and ectopically expressed Dp186 in the ORNs becomes efficiently localized to presynaptic terminals in the antennal lobes. Expression patterns of Dp186 mRNA expression were determined by *in situ* hybridization of frozen adult wild type antennal frontal sections; anti-sense (**A**) and control sense (**B**) digoxigenin-labeled Dp186 RNA probes were used. Many presumptive ORNs express Dp186. Semi-quantitative RT-PCR analysis of RNA derived from wild type and *Dys*$^{Dp186\ 166.3}$ mutant animals (**C**) confirms that Dp186 is expressed in the antenna. PCR fragments were generated by RT-PCR of mRNA derived from wild type larvae (WL), *Dys*$^{Dp186\ 166.3}$ mutant larvae (166L), wild type antennae (WA) and *Dys*$^{Dp186\ 166.3}$ mutant antennae (166A) using primers for RP49 (ribosomal control, lanes 2-5) and Dp186 (lanes 7-10) (Materials and Methods). Confocal images of whole mount adult ALs are shown (**D**-**F**). DP186 protein in red is shown in the ALs of wild type (**D**), the *Dys*$^{Dp186\ 166.3}$ mutant (**E**) and after overexpression of UAS-Dp186 in the *Dys*$^{Dp186\ 166.3}$ mutant background (**F**) (genotype is UAS-Dp186/SG18.1; *Dys*$^{Dp186\ 166.3}$). Scale bar = 50 µm for A and B. Scale bar = 25 µm for D-F.

ORNs show a reduced synaptic activity when Dp186 is absent

We first measured the odor-evoked output of the ORNs by performing electroantennograms (EAGs) on control and *Dp186* mutant antennae. EAGs are extracellular recordings which can be used to characterize the electrical output response of the entire *Drosophila* antennae in response to various odorants [34]. Quantitative differences in amplitude of the EAGs in response to different odorant concentrations have been reported for a number of different *Drosophila* mutants [35], [36].

We observed a significant reduction in *Dp186* mutant ORN output relative to controls after stimulation with the odorants benzaldehyde, 3-octanol, ethyl butyrate, ethyl acetate, hexanal and cis-vaccenyl acetate (**Fig. 3**). The effect of the Dp186 mutation, however, is only observed at high concentrations of E_2-Hexenal and no significant differences from wild type were observed for the *Dp186* mutant presented with methylcyclohexanol at any concentration (**Fig. 3**). We conclude therefore that *Dp186* mutant flies exhibit reduced ORN outputs in response to a number of different odorants, including benzaldehyde, but that some ORN classes may be relatively unaffected by the absence of Dp186.

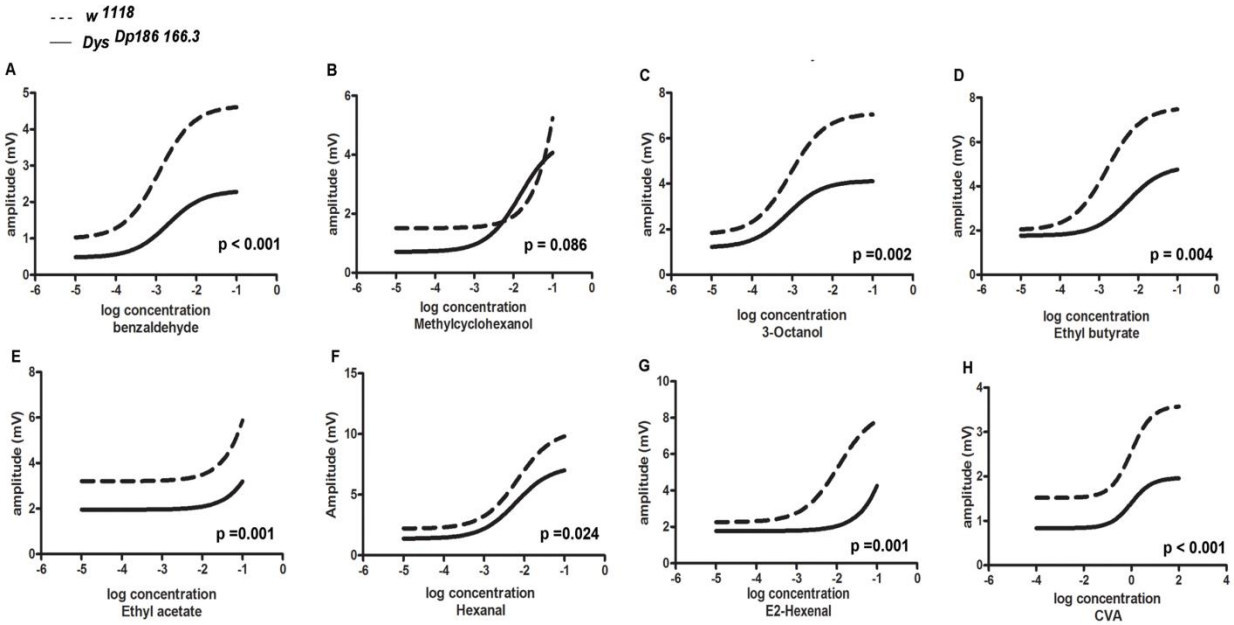

Figure 3. EAG analyses shows decreased synaptic activity of the ORNs for most odors tested. The following odorants were applied for EAG recordings of both the wild type (w1118) control and the mutant $Dys^{Dp186\ 166.3}$ antennae: benzaldehyde (**A**), methylcyclohexanol (**B**), 3-octanol (**C**), Ethyl butyrate (**D**), Ethyl acetate (**E**), Hexanal (**F**), E2 -Hexenal (**G**) and Cis-vaccenyl acetate (CVA) (**H**). Concentration of 0.001%, 0.01%, 0.1%, 1% and 10% (vol./vol.) were used to determine the dose-response curves. 17 to 22 individuals were tested with each odorant at each given concentration. The P values for the statistical significance of difference are indicated in each panel.

Dp186 is required in the ORNs for their normal output

We next performed synapse side-specific rescues to determine where Dp186 is required for wild type ORN output in response to benzaldehyde. Expression of Dp186 in ORNs, but not in the PNs, rescues the *Dp186* mutant reduced ORN output phenotype (**Fig. 4**). We conclude that *Dp186* is required in the ORNs for wild type levels of synaptic output. Therefore, unlike the cases of Dystrophin DLP2 at the NMJ [19] or Dp186 at the central interneuronal synapse [21], the presence or absence Dp186 in the postsynaptic PNs is unlikely to affect ORN neurotransmitter release levels.

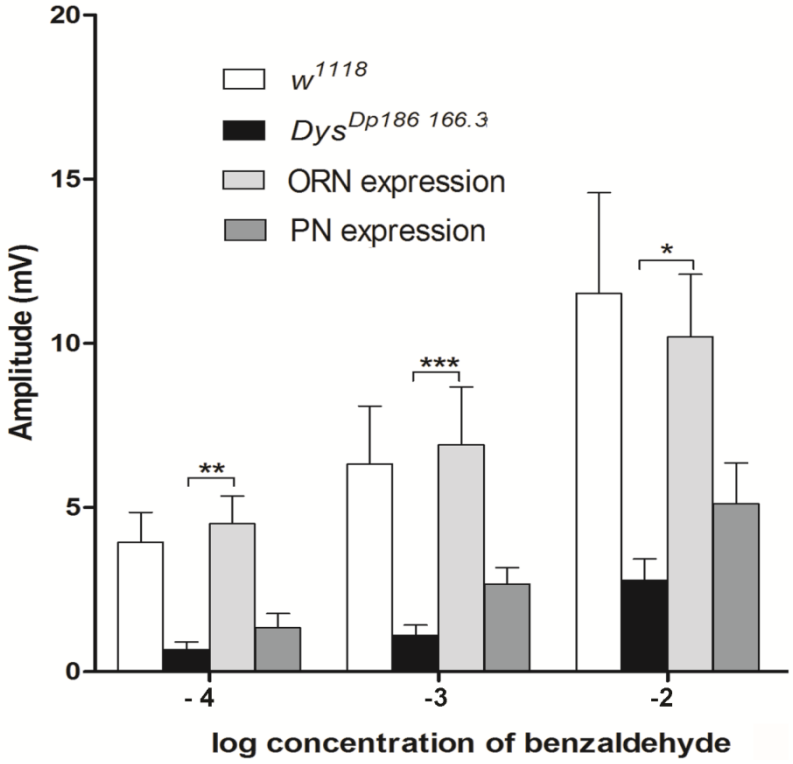

Figure 4. Dp186 is required in the ORNs and not the PNs for normal EAG responses to benzaldehyde stimulation. The average amplitudes (+/- S.E.M.) of the responses after stimulation by different concentrations of benzaldehyde by wild type (w^{1118}) adult flies, *Dp186* mutants and when Dp186 protein was re-expressed in the $Dys^{Dp186\ 166.3}$ mutant in all olfactory receptor neurons (ORN expression) (genotype: SG18.1-Gal4/UAS-Dp186; $Dys^{Dp186\ 166.3}$) or in the projection neurons (PN expression) (genotype: GH146-Gal4/UAS-Dp186; $Dys^{Dp186\ 166.3}$)*I* are shown. The numbers of individuals tested were: 5 (w^{1118}), 10 (*Dp186* mutant) and 9 each for the ORN and PN expression for each concentration. The p values $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ are indicated by *, ** and ***, respectively. Expression of Dp186 in the ORNs, but not the PNs, restores mutant ORN output to wild type levels.

The number and the locations of olfactory sensillae are not altered in *Dp186* mutants

To address the possibility that decreased electrical output from the Dp186-deficient antenna is due to changes in the cell fates or positions of the ORNs themselves, we evaluated whether the number or the spatial distribution of the antennal sensillae is altered in the *Dp186* mutant. There are three types of antennal sensillae that can be identified by their distinct morphology; the basiconic, trichoid or coeloconic sensillae [37–39]. Representative photographs of the distribution of the coeloconic sensillae in $Dys^{Dp186\ 166.3}$ and $Dys^{Dp186\ 30.3}$ individuals indicate no apparent changes, relative to controls, in their localization (**Sup. Fig. 1A-C**). Similarly, the distributions of the basiconic and trichoid sensillae did not differ between the mutants and wild type controls (data not shown). Furthermore, we did not observe any significant quantitative differences in the numbers of sensillae in the three classes between the mutants and controls (**Sup. Fig 1D**). We conclude that the absence of Dp186 apparently does not significantly alter sensillae cell fate or localization.

Dp186 is required for wild type odor avoidance behavior at low odorant concentrations

We next examined whether Dp186 is required for a wild type olfaction-evoked behavior We tested whether *Dp186* mutant flies display an avoidance response respond to odor stimuli that are normally perceived as repulsive [31]. We presented wild type controls and the $Dys^{Dp186\ 166.3}$ and $Dys^{Dp186\ 30.3}$ mutants with the repulsive olfactory cue benzaldehyde and determined their performance index (PI) in a T-maze conditioning apparatus [31] with exposure to relatively low levels (6 x 10-4 dilution) of odorant. The PI of the avoidance behavior of the *Dp186* mutant flies was significantly reduced compared to the control flies suggesting that the odor avoidance response is abnormal in the *Dp186* mutant (**Fig. 5**). This effect was not observed when the *Dp186* mutants were exposed to higher levels (1.2 x 10-3 dilution) of benzaldehyde (data not shown), indicating that the effects of Dp186-deficiency can be overridden by increased stimulus levels. Reduced avoidance behavior was also observed at high, but not low, dilutions of another repellent, 3-octanol (data not shown), indicating that this effect is not a benzaldehyde- or ORN class-specific.

The abnormal olfactory avoidance behavior displayed by the *Dp186* mutant could be the result of the observed decreased ORN outputs from *Dp186* mutant ORNs or caused by ORN axon guidance defects which preclude proper synaptogenesis. To evaluate the latter possibility, we examined the innervation patterns of the benzaldehyde-responsive ORNs in the *Dp186* mutant. Or7a, Or42b, Or46a, Or67a, Or67b, Or82a were previously identified as benzaldehyde-responsive ORs [6], [8], [40] OR axon trajectories and their synapses on the ALs were visualized in wild type and *Dp186* mutant brains by use of *Or* promoter-GFP or *Or* promoter–GAL4 transgenes and UAS-mCD8-GFP reporters [12], [13] We did not observe any axon guidance or innervation defects in the benzaldehyde-responsive ORN axon trajectories, nor for a number of other ORNs (**Sup. Fig. 2**). Finally, we examined the morphologies of the PN dendrites by expressing Denmark in all (GH146-GAL4) or a subset (MZ19-GAL4) of the PNs in the wild type or *Dp186* mutant backgrounds. No apparent differences were seen between mutants and controls (data not shown). Together, these data indicate that the ORN axon trajectories and the locations and overall morphology of the primary olfactory synapses are wild type in the *Dp186* mutant.

Finally, we addressed where Dp186 is required for wild type olfactory avoidance behavior by performing rescue experiments in the *Dp186* mutant background. The abnormal olfactory behavior was rescued when Dp186 expression was restored in all neurons in the *Dp186* mutant background (**Fig. 5**). Moreover and surprisingly, restoration of Dp186 expression in either the PNs or ORNs restored wild type odor avoidance PI (**Fig. 5**). Flies lacking the DLP2 Dystrophin isoform, which is not expressed in the central nervous system [19] exhibited wild type avoidance (**Fig. 5**), indicating that the phenotype is due to the absence of Dp186 and not another Dystrophin isoform. Thus, although the most proximal olfactory system defect in the *Dp186* mutant appears to be reduced ORN output, the odor avoidance behavioral phenotype can be rescued by restoring Dp186 either to the ORN's or to the downstream PNs. These results indicate that reduced ORN output can be rescued by Dp186-dependent increases in transmission gain at downstream points in the pathway.

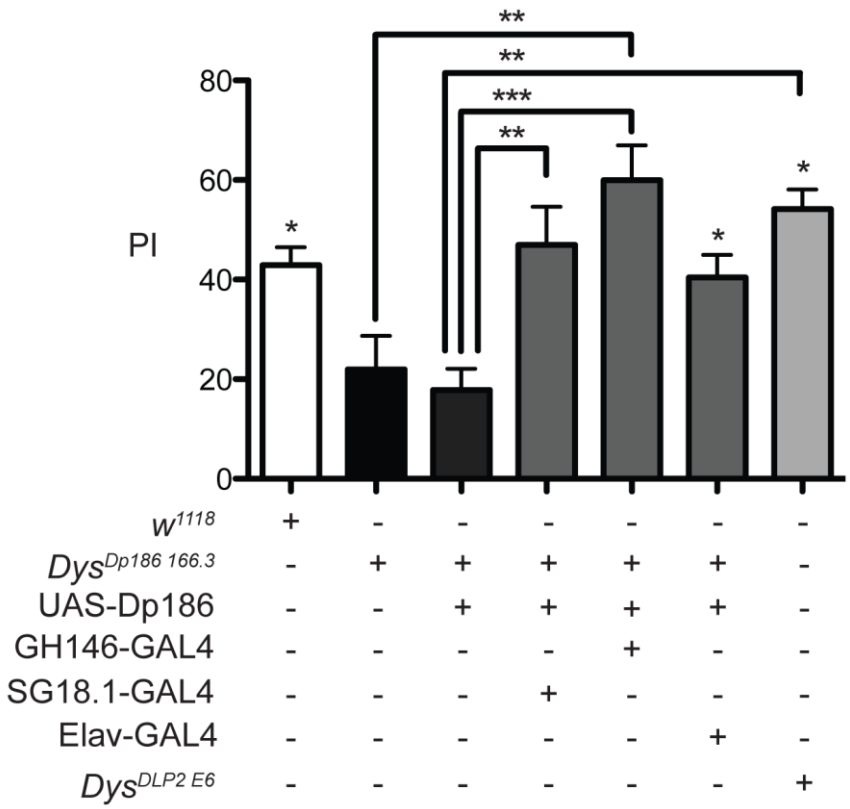

Figure 5: The *Dys*$^{Dp186\ 166.3}$ mutant exhibit decreased benzaldehyde odor avoidance behavior. Odor avoidance was assayed using the T-maze paradigm with groups of 75-100 flies (Materials and Methods). A performance index (PI) was calculated as the normalized percentage of flies choosing to avoid the odor-containing arm. The avoidance response of the *Dys*$^{Dp186\ 166.3}$ mutant was significantly reduced as compared to the wild type w^{1118} controls (ANOVA, $F_{(5,56)} = 5.778$, $P < .0001$; SNK, $P < 0.05$). Pan-neuronal expression of Dp186 using the Elav-GAL4 driver in a *Dys*$^{Dp186\ 166.3}$ background resulted in a rescue of the behavioral response to wild type levels. In addition, expressing Dp186 protein either pre- or post-synaptically using the SG18.1-GAL4 or GH146-GAL4 lines restored odor avoidance behavior to wild type levels. Odor avoidance was wild type in flies lacking the DLP2 Dystrophin isoform (*Dys*$^{DLP2\ E6}$). All rescued crosses demonstrated statistically-significant differences from both the *Dys*$^{Dp186\ 166.3}$ and UAS-Dp186 only controls (SNK, $P < 0.05$). Single asterisks indicate statistical difference between those bars and both *Dys*$^{Dp186\ 166.3}$ and UAS-Dp186 lines. All other significance is indicated with individual labels. Columns indicate mean ± SEM; n = 6-13/bar.

Discussion

In this study, we have established a requirement for the Dystrophin Dp186 isoform in the efficient transduction of olfactory sensory input to the *Drosophila* antennal lobe and for wild type olfactory avoidance behaviors regulated by this circuit. Overall electrical output from the primary ORNs in the AL is significantly reduced when Dp186 is absent. No cell fate changes are apparent in the *Dp186* mutant antennal sensillae. Nor are there apparent defects in ORN projections to and synaptic contacts with the PNs in the AL glomeruli or in overall PN morphology. Together, these data suggest that the efficacy of transmission is affected without obvious structural changes to the ORN/PN synapse. Similarly, the *Drosophila* Dystrophin-deficient NMJ [19] and the mammalian NMJ lacking Dystrophin [41] display only relatively subtle structural changes. Thus, the effects of a lack of Dystrophin at the synapse are significantly different from those observed when it is missing from the mammalian sarcomere where it is required to stabilize the muscle against exercise-induced degeneration (reviewed in [42]).

We find that Dp186 produced by the ORNs in the antenna becomes largely localized to their presynaptic terminals in the ALs. Furthermore, when Dp186 is overexpressed in the ORNs the protein becomes localized to the AL glomeruli. We also observe overlap in the expression domains of Dp186 with a marker for the postsynaptic dendrites of the PNs and thus conclude that Dp186 is present at both sides of the ORN/PN synapse.

We show that ORN output is significantly reduced, as assayed by EAGs, in the *Dp186* mutant. We tested 8 different odorants and found a significant reduction in ORN output in the *Dp186* mutant for all but one. Since the EAG measures total output from all ORNs responding to a test odor, we cannot conclude presently whether individual odor-responsive mutant ORNs have reduced output or whether a subset are silenced. Similar effects seen with the different odorants, however, indicate that it is more likely that there is attenuation of odor-evoked output at the level of the single ORN.

Mutant responses to odorants were clearly reduced when benzaldehyde and 3-octanol were administered. When these two odorants were employed in an odor avoidance paradigm [31], the performance index of *Dp186* mutant flies was found to be significantly lower than in the wild type control animals. Abnormal odor avoidance behavior was observed at low odorant concentrations but not at higher concentrations indicating that information transmission through the circuit is reduced but not eliminated by the absence of Dp186. Expression of Dp186 in the ORNs, but not the PNs, in the *Dp186* mutant background restored ORN output, as assayed by EAGs, to wild type levels. In contrast, odor avoidance behavior was fully restored to wild type levels when Dp186 was expressed in either the mutant ORNs or PNs.

These results suggest that DP186's maintains the efficacy of synaptic transmission at both the ORN/PN synapse in the AL and at points downstream including, perhaps, the PN/higher brain center synapse. The expression of Dp186 observed at both the ORN/PN synapse and in central brain regions associated with olfaction support this hypothesis. When Dp186 is absent from the ORNs, but expressed in the PNs, overall transmission to the central brain is apparently sufficiently restored to allow wild type behavioral response levels, even though ORN output levels are observed to remain compromised. Conversely, the restoration of Dp186 to ORNs in the mutant background allows them to sufficiently depolarize the PNs to, despite the lack of Dp186 in the PNs and higher order brain centers, elicit a wild type behavioral response levels.

The results presented in this study most clearly define Dp186's role in the ORN where, analogously to what has been reported for mammalian and *C. elegans* dystrophin species at the neuromuscular junction and central brain synapses [43–45], it might regulate ORN receptor clustering or dynamics. Its presence in the higher order olfactory centers and PNs and the rescue of the odor avoidance phenotype by expression of Dp186 in the PN clearly indicate, however, that Dp186 also acts in neurons which are postsynaptic to the ORNs. We previously showed that the Dystrophin DLP2 and Dp186 isoforms are present and function at the postsynaptic sides of the neuromuscular junction and at an identified interneuronal synapse, respectively [19], [21]. The absence of these isoforms from the postsynaptic compartment, the muscle versus the motoneuron, leads to increased presynaptic neurotransmitter release. The role of Dp186 in the PNs appears to, however, be different since we observed the expression of Dp186 solely in the ORNS was sufficient to restore their outputs to wild type levels despite the absence of Dp186 from the PNs.

In conclusion, Dp186 is required to maintain wild type synaptic gain at minimally two segments of a neural circuit which translates odor reception by the ORN to behavior. Few proteins have been identified to date that are required for modulation of synaptic transmission in the olfactory

system of vertebrates or invertebrates. Dystrophin isoforms and other members of the DGC have been reported to be expressed in the mammalian olfactory bulb [45–49] indicating that what we have found in the fly may well be conserved. The studies presented provide a fruitful starting point to further unravel the molecular mechanisms involved in olfactory circuit function and, more generally, in neural circuits which couple sensory input to complex behaviors.

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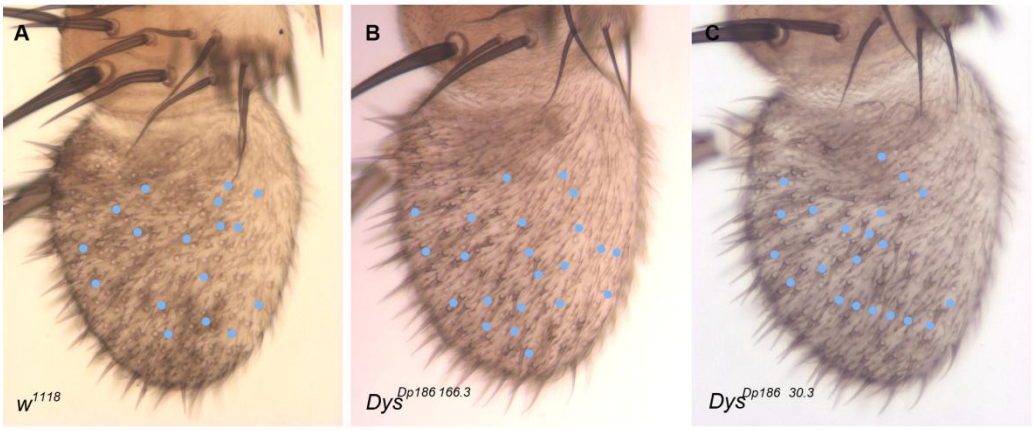
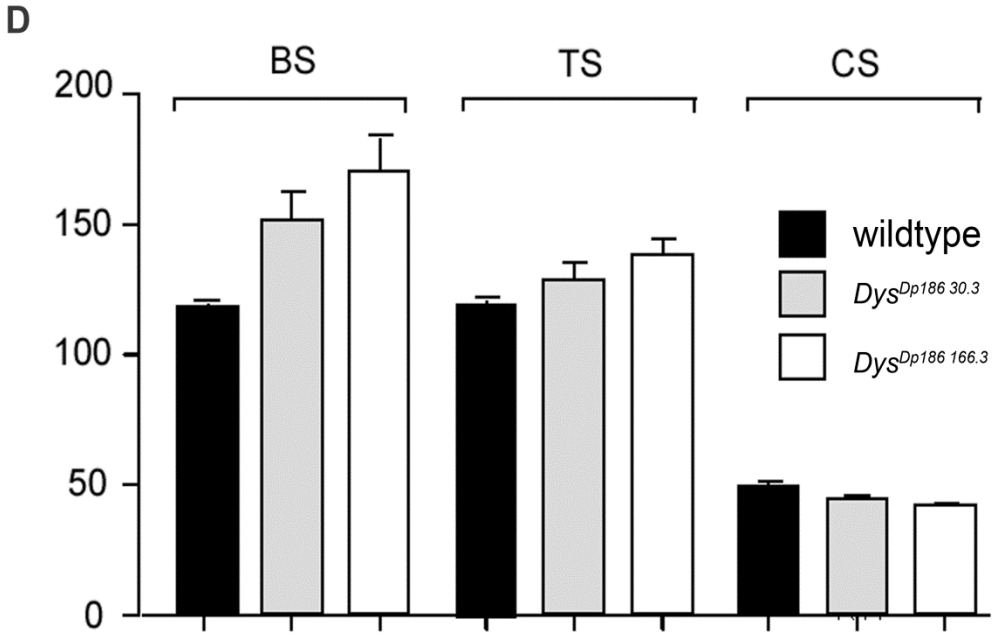
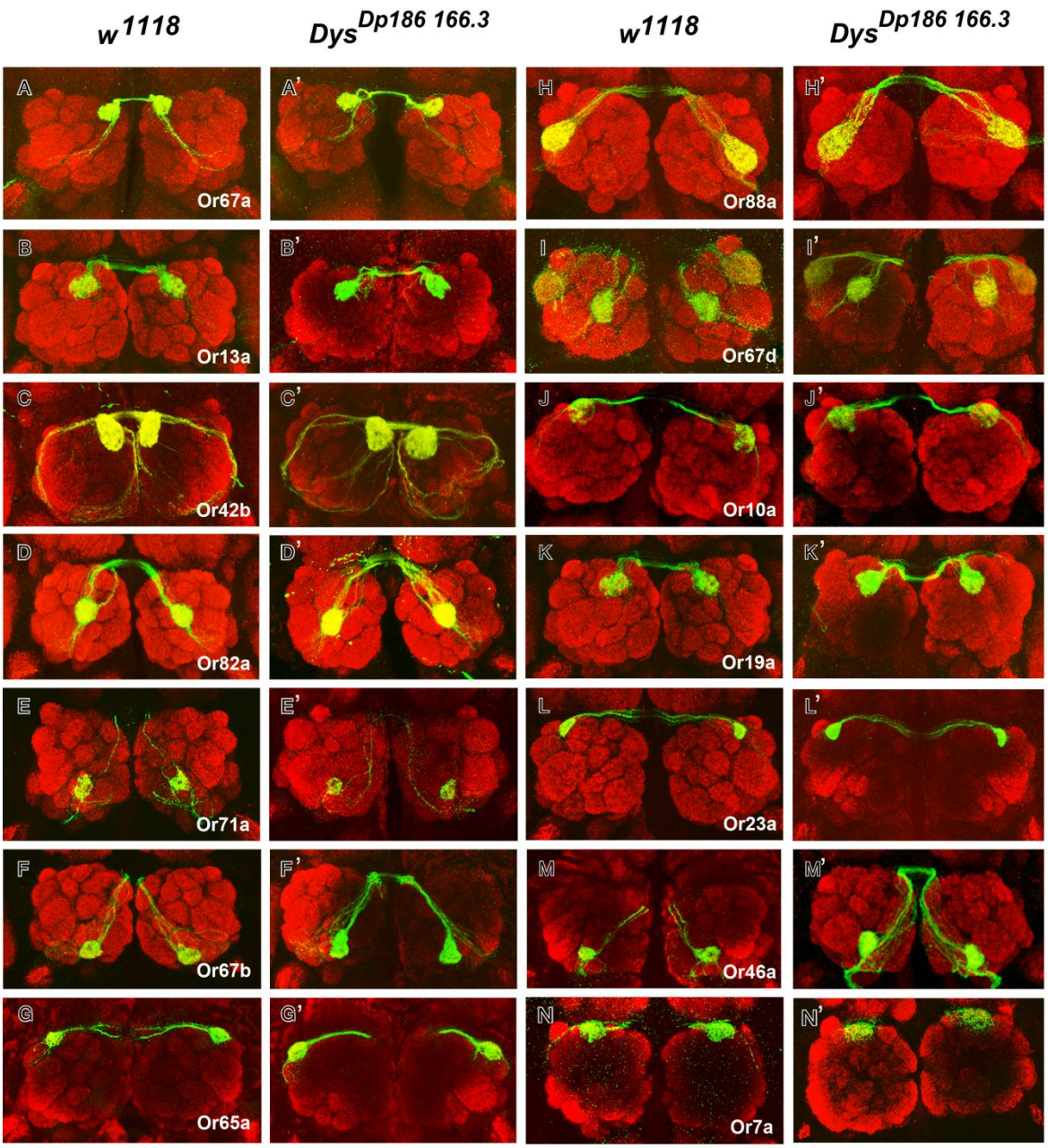
Supplemental Figures





Supplemental Figure 1. The spatial distribution and numbers of sensillae appear wild type in *Dys*$^{Dp186\ 166.3}$ and *Dys*$^{Dp186\ 30.3}$ mutant antennae. Adult antennae were mounted in Faure's mountant and the patterns of basiconic (BS), trichoid (TS) and coeloconic (CS) sensillae were analyzed as described [39]. Representative coeloconic sensillae are highlighted by blue dots in wild type (**A**), *Dys*$^{Dp186\ 166.3}$ (**B**) and *Dys*$^{Dp186\ 30.3}$ antennae (**C**). The numbers of the three types of sensillae were quantified in the wild type and the *Dys*$^{Dp186\ 30.3}$ and *Dys*$^{Dp186\ 166.3}$ mutants (**D**). No statistically significant differences were found between the wild type and the mutant individuals.



Supplemental Figure 2: The ORN projections of the benzaldehyde- and other odorant-responsive ORNs at the antennal lobes are wild type in *Dys*^{Dp186} mutants. The ALs of the indicated *Or* promoter-fusion or *Or* promoter-GAL4 –driven mCD8-GFP reporter lines in the wild type (**A-N**) and the *Dys*^{Dp186} mutant (**A'-N'**) backgrounds were stained with anti-GFP (in green) to visualize the ORN projections and co-labeled with synaptic marker anti-Bruchpilot (mAb nc82; in red) to stain the AL glomeruli. All ORNs that are responsive to benzaldehyde for which Or promoter-GAL4 or Or promoter-mCD8-GFP transgenes were available (Or7a, Or42b, Or46a, Or67a, Or67b, Or82a) and a number of other ORNs indicated in the Figure were examined. Dorsal is up and lateral to the right in all panels.