Optical Dissection of Experience-Dependent Pre- and Postsynaptic Plasticity in the *Drosophila* Brain

**Graphical Abstract**

**Highlights**

- Transgenic *Drosophila* strains expressing synaptic sensors are reported
- Concurrent dual color imaging of pre- and postsynaptic activity is demonstrated
- Olfactory coding across synapses is visualized in central olfactory neurons
- Experience-dependent pre- and postsynaptic plasticity is differentiated

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**In Brief**

*Drosophila* is a favorable organism for analysis of genetic and neuronal mechanisms underlying learning and memory. Pech et al. report an optical approach to selectively monitor pre- and postsynaptic activity. They thus dissect experience-dependent pre- and postsynaptic plasticity in the intact *Drosophila* brain.
Optical Dissection of Experience-Dependent Pre- and Postsynaptic Plasticity in the *Drosophila* Brain

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SUMMARY

*Drosophila* represents a key model organism for dissecting neuronal circuits that underlie innate and adaptive behavior. However, this task is limited by a lack of tools to monitor physiological parameters of spatially distributed, central synapses in identified neurons. We generated transgenic fly strains that express functional fluorescent reporters targeted to either pre- or postsynaptic compartments. Presynaptic Ca\(^{2+}\) dynamics are monitored using synaptophysin-coupled GCaMP3, synaptic transmission is monitored using red fluorescent synaptophysin-pHTomato, and postsynaptic Ca\(^{2+}\) dynamics are visualized using GCaMP3 fused with the postsynaptic matrix protein, dHomer. Using two-photon in vivo imaging of olfactory projection neurons, odor-evoked activity across populations of synapses is visualized in the antennal lobe and the mushroom body calyx. Prolonged odor exposure causes odor-specific and differential experience-dependent changes in pre- and postsynaptic activity at both levels of olfactory processing. The approach advances the physiological analysis of synaptic connections across defined groups of neurons in intact *Drosophila*.

INTRODUCTION

Monitoring of neuronal activity is crucial for the functional analysis of distinct neuronal circuits. Optical approaches provide an advantage over electrophysiological techniques for recording spatio-temporal activity across neuronal populations without mechanical interference. With the invention of genetically encoded fluorescent reporter proteins (reviewed by Tantama et al., 2012), the corresponding sensors can be expressed reproducibly in specific neurons of interest. Therefore, transgenic model organisms, e.g., mice, *Caenorhabditis elegans*, *Danio rerio*, or *Drosophila melanogaster*, are of predominant significance in neuroscience.

*Drosophila* represents a favorable organism due to the versatility of genetic tools with which expression of transgenes, including reporter proteins, can be restricted to defined populations of neurons (Venken et al., 2011). Imaging neuronal activity in correlation with sensory stimuli or with behavioral actions using genetically encoded Ca\(^{2+}\) indicators (GECIs) represents a widely applied approach (Riemensperger et al., 2012). Typically, GECIs are expressed in the entire neuronal cytosol. Therefore, they report changes in the dynamics of cytosolic Ca\(^{2+}\) ions as integrated signals (Grienberger and Konnerth, 2012). Intracellular Ca\(^{2+}\) ions have many different sources and different physiological functions. Synaptic connections represent key elements underlying neuronal signal integration, processing, and plasticity. Therefore, techniques are required with which to restrict the analysis to either pre- or postsynaptic activity. This is of particular importance if neuronal activity is monitored in the dense neuropils of the central brain that often comprise various pre- and postsynaptic elements in close proximity. Whereas GECIs targeted specifically to presynapses have been developed for and applied in the nervous systems of some vertebrates (Dreosti et al., 2009; Li et al., 2011), no equivalent technique exists yet for the *Drosophila* brain. Here, we report the generation and characterization of transgenic *Drosophila* strains that express the Ca\(^{2+}\) sensor, GCaMP3 (Tian et al., 2009), targeted either to the presynapse or the postsynapse. In addition, we generated a fly strain that expresses the red fluorescent reporter of synaptic vesicle release, pHTomato (Li and Tsien, 2012), inserted into an intravesicular domain of Synaptophysin. In combination with the green fluorescent, synaptically targeted GECIs, vesicle release and either pre- or postsynaptic Ca\(^{2+}\) dynamics can be monitored simultaneously. We use this approach to dissect odor-evoked pre- and postsynaptic neuronal activity in olfactory projection neurons of the central brain, and we demonstrate odor-specific and differential experience-dependent changes in synaptic activity.

RESULTS

Targeting Fluorescent Sensors to Synapses in the *Drosophila* Central Brain

Monitoring presynaptic Ca\(^{2+}\), we used a fusion construct that is functional in vertebrates, i.e., the GCaMP3 linked to the
cytoplasmic C terminus of the rat Synaptophysin protein (Dreosti et al., 2009; Li et al., 2011) (Figure 1A). Fruit flies do not endogenously express a Synaptophysin homolog. Nevertheless, this protein shows specific self-assembly in the membrane of synaptic vesicles when it is ectopically expressed (Leube, 1995). To monitor synaptic transmission concurrently, we inserted the red fluorescent pH-sensor pHTomato into the first intravesicular domain of Synaptophysin (Li and Tsien, 2012) (Figure 1B). To target the GECI to the postsynaptic densities of neurons, we linked GCaMP3 with the C terminus of the Drosophila Homer protein. Homer is a homolog of the PDZ-domain-containing Homer proteins of vertebrates, sharing equivalent subcellular localization and conserved binding motifs, and it is expressed in all neurons of the Drosophila nervous system (Xiao et al., 1998; Diagana et al., 2002) (Figure 1C). Transgenic flies were generated that express these DNA constructs under control of upstream activator sequences (UASs) (Brand and Perrimon, 1993).

To probe the efficiency of the constructs in neurons of the fly central brain, we used the well-characterized GH146-Gal4 driver line (Stocker et al., 1997), which induces gene expression in two populations of central neurons. GH146-Gal4 targets ~83 olfactory projection neurons (OPNs) that project from the antennal lobe (AL), the primary olfactory neuropil of the insect brain, to the calyx (CA) of the mushroom body (MB) and the lateral horn (LH) (Stocker et al., 1997; Wong et al., 2002) (Figure 1D). OPNs receive their main postsynaptic input in the glomeruli of the AL but also exhibit some presynaptic output in this neuropil (Ng et al., 2002; Wilson et al., 2004). The majority of their axons form the inner antennocerebral tract that terminates in the LH. Short axonal collaterals furnished with large synaptic boutons innervate the CA of the MB and provide mostly presynaptic output to intrinsic MB neurons (Kenyon cells), but they also receive some postsynaptic input (Yasuyama et al., 2002; Christiansen et al., 2011; Butcher et al., 2012). In addition, GH146-Gal4 targets large interneurons (anterior paired lateral [APL] neurons) that recurrently innervate the MB neuropil, although expression in those neurons is weak when compared to OPNs (see Figure 1E). The expression patterns of the targeted sensors indicate correct targeting of the sensor proteins to synaptic terminals in accordance with the described polarity of the neurons, as presynaptic sensors are enriched at the main output sites, CA and LH (Figures 1F and 1G), and homer-GCaMP is enriched at the main OPN input site, the AL (Figure 1H).

To detail the localization of the sensors, we analyzed the CA and the AL and calculated the ratio of immunohistochemical staining intensity in synaptic terminals to that in axons. Cytosolic GCaMP is equally present both in synaptic structures and axonal tracts. In contrast, all three synaptic sensors localize in a manner comparable to the presynaptic marker, anti-synaptotagmin (anti-syt), or the postsynaptic marker, anti-discs large (anti-DLG) (Figures 2 A and 2B). We also confirmed proper synaptic targeting by focusing on the predominantly presynaptic boutons in the CA (Figure 2C). In contrast to cytosolic GCaMP, synaptophysin-GCaMP (syp-GCaMP) and synaptophysin-pHTomato (syp-pHTomato) are strongly confined to these boutons.
Figure 2. Synaptic Localization of the Fluorescent Sensors

(A) Quantification of immunostaining intensity of the fluorescent sensors in synaptic boutons of OPNs in the mushroom body calyx compared with the intensity in adjacent axonal tracts. The ratio is compared to that of the presynaptic vesicle protein Synaptotagmin (anti-syt) and the postsynaptic protein discs-large (anti-DLG), n ≥ 10 calyces.

(B) Same as in (A) for the glomeruli of the antennal lobe (AL), n ≥ 10 ALs. Box plots indicate medians, interquartile ranges, and 10%/90% range. n.s., p > 0.05; two-sample t test; ***p < 0.001 Wilcoxon signed rank test.

(C) Immunostaining of cytosolic GCaMP, syp-GCaMP, syp-pHTomato, and homer-GCaMP in the calyx. Shown are anti-syt immunoreactivity and anti-GFP or anti-RFP immunoreactivity.

(D) Immunostaining of cytosolic GCaMP, syp-GCaMP, syp-pHTomato, and homer-GCaMP in the AL. Shown are synaptic co-stainings and anti-GFP or anti-RFP immunoreactivity.

(E–I) High-resolution STED microscopy images of a single bouton in the calyx. Shown are a merge and the individual channels of anti-GFP immunoreactivity of syp-GCaMP and either anti-syt immunoreactivity (E) or immunoreactivity against the active zone protein Bruchpilot (nc82) (F). Immunoreactivity of syp-pHTomato and either anti-syt immunoreactivity (G) or immunoreactivity against Bruchpilot (nc82) (H). Arrowheads point to regions of co-localization in clusters of vesicles.

(I) Shown are a merge, anti-DLG immunoreactivity, and anti-GFP immunoreactivity of homer-GCaMP.

(J) High-resolution detail within a glomerulus of the AL, shown as a merge, and individual anti-DLG immunoreactivity and anti-GFP immunoreactivity of homer-GCaMP. Arrowheads point to co-localization.

The scale bars represent 20 μm in (C) and (D), 1 μm in (E–I), and 2 μm in (J).
whereas homer-GCaMP punctae are diffusely distributed across
the CA (Figure 2C) in accordance with the microanatomy of
calycal boutons (Yasuyama et al., 2002; Leiss et al., 2009;
Butcher et al., 2012). In addition, this staining pattern might partly
reflect postsynapses of the APL neuron in the CA. Furthermore,
we analyzed the localization of all constructs in the main OPN
input region, the AL. Cytosolic GCaMP is present throughout
neuronal ramifications within the AL, whereas the presynaptically
targeted sensors are confined to irregularly shaped and hetero-
geneously distributed punctae within the AL’s glomeruli (Fig-
ure 2D). By contrast, homer-GCaMP is localized to punctae
that are not only much more numerous but also smaller and
more densely distributed, in accordance with the fine mesh of
dendritic processes pervading the glomeruli (Figure 2D). To visu-
alize the subcellular localization of the sensors, we used high res-
olution STED microscopy (Figures 2E–2J). Within individual bou-
tons in the CA, an accumulation of syp-GCaMP or of syp-
phTomato punctae was evident in distinct regions (Figures 2G
and 2J). These punctae reflect the accumulation of synaptic ves-
icles at active zones as they co-localize with anti-syt immunore-
activity (Figures 2E and 2G). In addition, they are localized
around and partially co-localize with immunoreactivity against
the active zone protein Bruchpilot (Wagh et al., 2006) (Figures
3H and 3I). These data confirm that both synaptophysin-
coupled constructs are targeted to presynaptic vesicles. STED
microscopy analysis further revealed that homer-GCaMP punc-
tae are directly adjacent to or co-localize with anti-DLG, both in
the CA (Figure 2I) and the AL (Figure 2J). In conclusion, homer-
GCaMP localizes to substructures of the complex postsynaptic
matrix. We also confirmed the correct localization of the three sensors in motor neurons of the larval body wall. Again,
syp-GCaMP and syp-phTomato are located presynaptically in the
boutons of neuromuscular junctions and co-localize with anti-syt immunoreactivity (Figures S1A and S1B). In contrast,
homer-GCaMP is absent from presynaptic boutons but is de-
tected in dendritic structures of motor neurons within the ventral
ganglion of the larval CNS (Figure S1C).

To test whether the expression of the sensors affects the
morphology of synaptic boutons, we expressed the constructs
in larval motor neurons and determined the number and size of
boutons at NMJ6/7 in segment A3 using anti-DLG or anti-syt immu-
monostaining (Figure S1D). No differences in either the number
or size of both type Ib and type Is boutons were observed
when compared to w1118 larvae (Figure S1E). To further test
whether the sensors cause any functional impairment, we ex-
pressed the constructs ubiquitously in all cells and quantified
synaptic vesicle recycling via FM (steryl) dye uptake and release
(Kuromi and Kidokoro, 1999). FM dye is taken up during stimula-
tion and therefore serves as a measure for the size of the vesicle
population that undergoes recycling. Further stimulation in
absence of the dye releases it from these vesicles (termed FM
destaining), thereby providing an estimate for the exocytosis ki-
netics. Neither synaptic targeting of GCaMP nor syp-phTomato
expression altered FM dye uptake compared to the respective
controls (Figure S1F). FM dye destaining was also unaffected
(Figure S1G). We further tested the performance of such animals
in two behavioral assays. We found neither an overall morpho-
logical aberration of larvae (Figure S2A) nor a behavioral deficit
in a larval chemotaxis assay (Figure S2B). Moreover, the
morphology of adult animals and their brains were indistinguish-
able from those of control animals and the flies behaved normally
in a negative geotaxis assay (Figures S2C and S2D). In conclu-
sion, we did not detect any interference of the three constructs
with neuronal function.

**Functionality of the Synaptic Sensors**

To compare the functionality of the synaptic sensors in reporting
pre- or postsynaptic Ca2+ influx, or vesicle exocytosis, we ex-
pressed the DNA constructs in OPNs and used in vivo wide-field
imaging to monitor the fluorescence within the entire AL and CA
neuropils. KCl-induced neuronal depolarization caused clear in-
creases in fluorescence emission in either sensor (Figure S3).
The relative changes in fluorescence intensity of syp-GCaMP ex-
ceeded that of cytosolic GCaMP3, whereas homer-GCaMP
showed smaller signal amplitudes when compared to cytosolic
GCaMP (Figures S3A and S3B). Because all GECIs share the
same Ca2+ sensor and differ only in their subcellular localization,
the differential amplitudes reflect differential contributions from
sources of Ca2+ influx in pre- and postsynaptic compartments.
The red syp-phTomato displayed relative changes in fluores-
cence and a signal-to-noise ratio within a similar range when
compared with synaptopHluorin (Ng et al., 2002) (Figure S3C).

**Two-Photon Imaging of Odor-Induced Synaptic Activity**

We next tested whether the synaptically targeted sensors can be
used to monitor synaptic activity induced by physiological, sen-
sory stimuli in largely intact animals. Odor-induced activity
of presynaptic boutons of OPNs in the CA was monitored using
two-photon microscopy. The baseline fluorescence of both syn-
aptohysin-coupled sensors clearly demarcated individual
presynaptic boutons (Figures S4A and S4F). The animals were
stimulated with methyl cyclohexanol (MCH) and 3-octanol
(3Oct) while monitoring either Ca2+ transients or synaptic vesicle
release. Syp-GCaMP reliably reported presynaptic Ca2+ influx,
and syp-phTomato reliably reported increased vesicle exocy-
tosis, both across many synapses and at the subcellular level
(Figures S4A–S4J; Movie S1). The odor-induced fluorescence
change of both sensors was characterized by a sharp peak
upon odor onset, followed by decay nearly as soon as during
odor stimulation (Figures S4D and S4J), and some boutons
re-sponded specifically to odor offset (Figures S4B, S4C,
and S4H). Odor on- and offset Ca2+ transients in mutually exclusive
OPNs might be attributed to a release of inhibition via recurrent
interneurons in the AL (Silbering et al., 2008) and/or direct feed-
back from the LH and the MB lobes to the calycal boutons. The
spatiotemporal, combinatorial nature of odor representations
across OPNs (Wilson, 2013) is, therefore, reflected also at
the level of presynaptic activity. As syp-phTomato displayed
increased fluorescence at few and distinct regions at the borders
of individual boutons (Figure S4J), it provides a tool to visualize
transmission events that are spatially confined to individual
active zones in a given focal plane.

To record postsynaptic odor representations using homer-
GCaMP, we focused on the AL. Baseline fluorescence clearly
demarcated individual glomeruli (Figure S4K). Homer-GCaMP
reported odor-induced Ca2+ transients reliably in postsynapses.
within overlapping subsets of glomeruli (Figures S4K–S4M). In addition to Ca$^{2+}$ transients induced by odor onset, homer-GCaMP also reported spontaneous Ca$^{2+}$ activity (Movie S2) and Ca$^{2+}$ transients induced by odor offset signaling (e.g., glomerulus DL1 shown in Figures S4L and S4M). These complex activation patterns of distinct glomeruli were stereotypic across individuals in terms of the amplitudes and kinetics of odor-evoked responses (e.g., glomerulus DL4 shown in Figure S4N).

**Concurrent Imaging of Presynaptic Ca$^{2+}$ Dynamics and Synaptic Transmission**

Next, we tested whether syp-GCaMP and syp-pHTomato can be used to monitor two physiological parameters simultaneously. We co-expressed both constructs in OPNs and focused again on presynaptic boutons in the CA. Immunohistochemical staining confirmed that both constructs were targeted to the same boutons (Figure 3A). The fluorescence emitted by the two sensors co-localized completely in vivo as well, although baseline fluorescence intensities differed slightly across distinct boutons (Figure 3B). We analyzed comparatively the fluorescence emission changes of both reporters in response to the odors, MCH and 3Oct, in individual presynaptic boutons (Figures 3C and 3D). Syp-GCaMP and syp-pHTomato showed odor-induced increases in fluorescence emission in equivalent boutons. The exact kinetics of fluorescence changes, however, differed between the two emission wavelengths, as expected for the different properties of the sensors and the parameters measured (Figures 3C and 3D).

We focused next on OPN presynapses in the AL, where the stereotypic neuronal connectivity across individuals allowed the comparison of identical neurons with respect to their odor-response profiles, i.e., identifiable glomeruli. Both presynaptic constructs co-localize in the AL, which was confirmed with immunohistochemical labeling and was also visible in vivo (Figures 3E and 3F). Both reporters showed odor-induced increases in fluorescence emission, as shown for glomerulus DL4 (Figure 3G). Again, the overall odor-induced glomerular activity patterns, the relative amplitudes, and the time courses were a close match in presynaptic Ca$^{2+}$ and synaptic transmission (Figure 3H). Baseline fluctuations of syp-pHTomato emission differed between glomeruli, indicating that different OPNs exhibit different spontaneous vesicle release rates. The VA1 glomerulus, for example, showed comparably high spontaneous vesicle exocytosis that was reduced during odor presentations (Figure 3H). In order to characterize the relation between presynaptic Ca$^{2+}$ and vesicle release, we analyzed MCH-induced signals in the DL4 glomerulus in detail. The relation between syp-pHTomato and syp-GCaMP signals could most accurately be described by the sigmoid function $y = a_1 + (a_2 - a_1)/(1 + 10^{(\log x_0 - x) \cdot p})$ (Figure 3I). The obtained function also described the fluorescence signals induced by the different odors in the same glomerulus (Figure 3I) and reflected different phases of stimulus-induced activity. The slope of the bottom asymptote indicates basal fluctuations of the pHTomato signal, e.g., spontaneous transmitter release. The fact that there is a top asymptote reflects that transmitter release persists on top of elevated activity. This indicates that we optically recorded synaptic activity within the dynamic ranges of the sensors and that the odor responses recorded were below a range in which vesicle pool size and recruitment become limiting factors.

**Concurrent Imaging of Postsynaptic Ca$^{2+}$ and Presynaptic Vesicle Release**

Next, we combined the syp-pHTomato with homer-GCaMP and co-expressed both sensors in OPNs, again focusing on the AL. Homer-GCaMP and syp-pHTomato were spatially segregated from each other. Only very rarely was co-localization detected (Figure 4A), although both constructs were targeted to the same set of glomeruli. The separated localization at only marginally overlapping structures was also observed with the in vivo baseline fluorescence and became most obvious when focusing on subglomerular structures in individual glomeruli, e.g., DL4 (Figures 4B and 4C). OPNs innervating DL4 respond to both MCH and 3Oct, with both postsynaptic Ca$^{2+}$ increase and presynaptic vesicle exocytosis (Figure 4D). When all glomeruli within this focal plane were analyzed, the odor-induced combinatorial activity pattern, the relative amplitudes, and duration of glomerular activation of postsynaptic Ca$^{2+}$ and presynaptic transmission were a close match in most cases (Figures 4D and 4E). Similarly as with presynaptic Ca$^{2+}$ transients, the relation between postsynaptic Ca$^{2+}$ and presynaptic vesicle release within the same neurons is best described by a sigmoid function (Figure 4F).

To further substantiate that syp-pHTomato reliably reports vesicle exocytosis independently of co-expressed Ca$^{2+}$ sensors, we expressed syp-pHTomato and homer-GCaMP together with temperature-dependent shibirets (Kitamoto, 2001) to disrupt vesicle-recycling reversibly. Switching the temperature at the fly’s brain to the restrictive temperature of shibirets diminished odor-induced syp-pHTomato signals while leaving homer-GCaMP signals unaffected (Figure S5). These experiments demonstrate that homer-GCaMP and syp-pHTomato can be used concurrently to differentiate, spatially and functionally, pre- and postsynaptic signaling within the same neurons, even in such dense aggregations of synapses as a glomerulus of the AL.

**Differential Experience-Dependent Synaptic Plasticity in the AL**

We asked whether the targeted sensors can be used to detect experience-dependent, physiological plasticity at synapses of the central brain. As a first step, we used artificial, chronic deprivation of synaptic transmission, which has been shown to induce structural changes at OPN synapses (Kremer et al., 2010). We restricted the artificially induced block of synaptic transmission to the flies’ post-eclosion stage and used heat-sensitive shibirets to transiently prevent vesicle recycling of OPNs for 5 days after eclosion. Subsequently, flies recovered at the permissive temperature and we then imaged the responses of the synthetically targeted sensors to two monomolecular odors and two complex blends of fruit in the AL and the CA (Figures 5 and 6) at the permissive temperature. In the AL, we compared odor responses in glomeruli DC1, DM3, and DL5, which respond to MCH, 3Oct, and apple and banana, respectively, using either a dual color combination of pre- or postsynaptic Ca$^{2+}$ sensors and syp-pHTomato (Figures 5A
Figure 3. Concurrent Imaging of Presynaptic Ca\textsuperscript{2+} and Vesicle Release

(A) Co-expression of syp-GCaMP and syp-pHTomato in calycal boutons of OPNs. Blue, anti-synaptotagmin; magenta, syp-pHTomato detected by anti-RFP; green, syp-GCaMP detected by anti-GFP. The inset shows a magnification of one bouton; white color indicates co-localization.

(B) In vivo fluorescence of syp-pHTomato and syp-GCaMP in OPNs within the calyx.

(C) Odor-induced fluorescence change of syp-GCaMP (green) and syp-pHTomato (magenta) in one individual bouton (indicated by orange circle in B). Each row of the heatmaps represents one bouton, each column one 200-ms time frame.

(D) Odor-induced fluorescence change of syp-GCaMP (left) and syp-pHTomato (right) in 20 individual boutons. Each row of the heatmaps represents one bouton, each column one 200-ms time frame.

(E) Co-expressed syp-GCaMP and syp-pHTomato in the AL. The inset shows a magnification within one glomerulus; white color indicates co-localization.

(F) In vivo fluorescence of syp-pHTomato and syp-GCaMP. Glomerulus DL4 is indicated by the orange circle.

(G) Odor-induced fluorescence change of syp-GCaMP (green) and syp-pHTomato (magenta) in DL4.

(H) Fluorescence changes over time of syp-GCaMP (left) and syp-pHTomato (right) in nine glomeruli. Each row represents one glomerulus, each column one 250-ms time frame.

(I) The gray and black lines indicate the time course of fluorescence change in syp-GCaMP as a function of fluorescence change in syp-pHTomato during odor stimulation in DL4. The red line shows the sigmoid fit with the adjusted R\textsuperscript{2} values for MCH and 3Oct. Heatmaps display the mean, traces the mean, and SEM of three stimulations.

d, dorsal; l, lateral; p, posterior; scale bars: 20 μm; in insets: 2 μm.
Odor-induced synaptic signaling was compared to siblings that were raised in parallel at the restrictive temperature but that did not express shibire78. As a further control, we raised animals with or without shibire78 expression at the permissive temperature (Figure 5C). The 5-day deprivation of synaptic output did not lead to any alteration in presynaptic Ca2+ signaling of OPNs in the AL (Figure 5D) but reduced drastically the amplitudes of postsynaptic Ca2+ transients (Figure 5E). Vesicle exocytosis was not altered under any experimental condition, although we found that animals that expressed shibire78 showed a slight, but not significant, reduced transmission when raised at either the restrictive or the permissive temperature (Figure 5F). Although presynaptic Ca2+ signaling was not altered in the AL, when focusing on individual boutons of the main OPN output site in the CA, syp-GCaMP reported significantly reduced presynaptic Ca2+ signaling evoked by all four odorants used (Figures 6A–6C). This indicates that presynaptic Ca2+ dynamics within spatially separated domains in different neuropils, but in the same neurons, can be modulated independently. In contrast, altered vesicle exocytosis monitored using syp-pHTomato was not detectable in the CA under any experimental condition (Figure 6D). A more-detailed analysis of boutons in the CA revealed that the prolonged deprivation of synaptic transmission from OPNs led to increased bouton sizes, which is in accordance with the findings of Kremer et al. (2010) (Figure 6E). We did not find any difference in the relative

Figure 4. Concurrent Imaging of Postsynaptic Ca2+ and Vesicle Release

(A) Co-expression of homer-GCaMP and syp-pHTomato in OPNs in the AL. Blue, anti-syt; magenta, syp-pHTomato detected by anti-RFP; green, homer-GCaMP detected by anti-GFP. The inset shows a magnification of one glomerulus; white color indicates regions of co-localization.

(B) Individual and merged in vivo syp-pHTomato and homer-GCaMP fluorescence in OPNs in the AL.

(C) DL4 is indicated in orange and magnified.

(D) Odor-induced fluorescence change of homer-GCaMP (green) and syp-pHTomato (magenta) in DL4 (mean and SEM of three stimulations).

(E) Dynamics of fluorescence changes over time of homer-GCaMP (left) and syp-pHTomato (right) in ten glomeruli. Each row of the heatmaps represents one glomerulus, each column one 250-ms time frame. Pixel values represent the mean of three stimulations.

(F) Relation of dynamic fluorescence changes of homer-GCaMP and syp-pHTomato evoked by MCH and 3Oct. The red line shows a sigmoid fit across data points in DL4, with the adjusted R² values displayed for MCH and 3Oct.

The scale bars represent 20 μm in (A) and (B) and 2 μm in the insets (A) and (C). See also Figure S5.
amount of syp-GCaMP fluorescence per bouton, which suggests that the total number of vesicles per bouton was not altered (Figure 6E).

The differential changes in synaptic activity of OPNs induced by artificial deprivation mimic those induced by a natural experience. We exposed the animals for 5 days during early...
adulthood to a multimodal, including olfactory, cue by raising them on a piece of apple. This treatment typically causes behavioral adaptation and reduced responsiveness to the exposed odor (Sachse et al., 2007; Das et al., 2011). Subsequently, we compared odor responses of the respective sensors in these animals with odor responses in animals that were raised without apple (Figure 7A). We focused again on glomeruli DC1, DM3, and DL5 of the AL, using a dual color combination of either pre- or postsynaptic Ca\textsuperscript{2+} sensors and syp-pHTomato. As was the case in the artificial deprivation experiment, we did not observe any experience-dependent change in presynaptic Ca\textsuperscript{2+} signaling in the AL (Figure 7B). The amplitudes of postsynaptic Ca\textsuperscript{2+} transients, however, were clearly reduced in the apple-responsive glomerulus DL5 only in those animals that were raised on apple. In contrast to the deprivation experiment, this decrease in postsynaptic signaling induced by exposure to the apple odor was glomerulus specific. Syp-pHTomato signals of OPNs in the AL were also unaltered (Figure 7D).

DISCUSSION

Approaches to the detection of physiological parameters underlying synaptic functioning in real time, in vivo, and across many synapses are important for understanding the principles governing the ways in which neural circuits accomplish signal integration, learning, and memory. Presynaptically targeted GECIs
have been successfully used to monitor presynaptic Ca\(^{2+}\) signaling in vertebrate systems, (e.g., Dreosti et al., 2009; Zhao et al., 2011a; Li et al., 2011; Akerboom et al., 2012; Walker et al., 2013). Furthermore, synaptic transmission can be visualized using pH-sensitive fluorophores targeted to the lumen of synaptic vesicles (Miesenböck et al., 1998; Granseth et al., 2006; Zhu et al., 2009; Li and Tsien, 2012). Whereas Ca\(^{2+}\) dynamics are frequently monitored in the dendrites and postsynaptic spines of mammalian cultured neurons or brain sections (Denk et al., 1996; Oertner, 2002), specific sensors tagged to the postsynaptic density have not been described. So far, postsynaptic Ca\(^{2+}\) dynamics have been measured solely in muscle cells, at the neuromuscular junction of larval Drosophila. For this purpose, GECIs were introduced between the transmembrane domain of CD8 and the DLG-interacting domain of the K\(^{+}\) channel Shaker (Guerrero et al., 2005; Peled et al., 2014). Whereas Drosophila represents a key model organism to dissect the neuronal circuits that undergo stimulus processing and behavior (Venken et al., 2011), there is still a lack of efficient techniques to visualize synaptic activity in the brain. We generated transgenic fly lines to overcome these limitations.

First, presynaptic Ca\(^{2+}\) is detected using a synaptophysin-coupled GCaMP3 construct. Ca\(^{2+}\) has multiple functions in presynaptic terminals and also multiple locations, comprising high Ca\(^{2+}\) concentration nano- and microdomains around voltage-gated channels and more-global, intraterminal Ca\(^{2+}\) (Neher and Sakaba, 2008), and all contribute to the signals monitored using targeted GECIs. In vertebrate neurons, the molar quantity of Ca\(^{2+}\) and its spatial diffusion is highly variable among neurons and even among boutons of the same axon. In neocortical presynaptic boutons of different mammalian tissues, for example, action-potential-induced and volume-averaged Ca\(^{2+}\) changes cover an estimated range of peak concentrations from 300 nM to 1,000 nM (e.g., Koester and Sakmann, 2000; Dreosti et al., 2009; Vaithianathan and Matthews, 2014). The dynamic range (~100 nM—~10,000 nM) and K\(_D\) (~600 nM) of GCaMP3 (Tian et al., 2009) is within this concentration range. It should be noted, however, that the peak Ca\(^{2+}\) concentration at the active zone of some specialized synapses may exceed 10 \(\mu\)M (e.g., Bollmann et al., 2000). A very local saturation of GCaMP3 can, therefore, under respective experimental conditions, not be excluded. GCaMP3 shows a relatively strong baseline fluorescence compared to other recently reported GCaMP variants (Akerboom et al., 2012; Chen et al., 2013; Zhao et al., 2011b), which is crucial for the detection of the small and often dispersed structures in vivo (see also Mao et al., 2008). Second, we apply the red pHTomato coupled to Synaptophsynin (Li and Tsien, 2012) to the central brain of Drosophila. This sensor is equally effective regarding presynaptic localization, relative changes in fluorescence, and signal-to-noise ratio when compared to synaptopHluorin that has been used in Drosophila (Ng et al., 2002). In response to physiological stimuli, amplitudes of fluorescence changes and signal to noise are small, however, due to the stochastic nature of exocytosis and endocytosis events and the small percentage of ready releasable vesicles (Sankaranarayanan et al., 2000; Denker et al., 2011). However, in combination with green fluorescent Ca\(^{2+}\) sensors, this sensor is helpful in identifying and differentiating pre- and postsynaptic compartments and in analyzing the physiological properties of synaptic function by correlating with physiological and behavioral responses.
synaptic vesicle release to synaptic Ca\textsuperscript{2+} influx. Third, we report homer-GCaMP as a postsynaptic Ca\textsuperscript{2+} sensor encompassing Ca\textsuperscript{2+} sources that can potentially derive from ligand-gated ionotropic receptors, voltage-gated Ca\textsuperscript{2+} channels of the postsynaptic membrane, or internal Ca\textsuperscript{2+} stores.

The olfactory pathway of the Drosophila central brain provides an advantageous test system due to the combinatorial nature of odor representations across anatomically and functionally well-characterized populations of neurons (Wilson, 2013). We demonstrate that the monitoring of pre- and postsynaptic Ca\textsuperscript{2+} dynamics is readily feasible, as is that of synaptic transmission across populations of synapses and within individual synaptic boutons. Experience-dependent plasticity of synaptic signaling in the AL has been proposed as a contributor to decreasing behavioral responsiveness following prolonged odor exposure (Sachse et al., 2007; Das et al., 2011), but tools to confirm this assumption have been lacking. Here, we show that the targeted sensors are appropriate to bridge that gap. The targeted sensors allow one not only to detect synaptic plasticity but also to tease apart experimentally the underlying signaling at the pre- and postsynapse within a complex circuit. The decrease in postsynaptic activity in OPNs caused by prolonged odor stimulation can be mimicked by artificially silencing OPN output. This indicates that olfactory adaptation is accompanied by a decrease in OPN activity, which might be mediated by plasticity of inhibitory local interneurons, as suggested by Das et al. (2011). This reduction of the postsynaptic Ca\textsuperscript{2+} in the AL is reflected by a reduction of presynaptic Ca\textsuperscript{2+} signaling in the CA, but not in the AL. Post- and presynaptic Ca\textsuperscript{2+} influxes in OPNs are, therefore, modulated independently. A large number of non-associative, associative, and complex learning paradigms have been described in Drosophila. The establishment of a method to monitor physiological parameters of synaptic activity in vivo in a largely intact animal provides a possibility to uncover synaptic plasticity underlying neuronal circuit processing and experience-dependent, adaptive behavior.

**EXPERIMENTAL PROCEDURES**

**Generation of DNA Constructs and Transgenic Flies**
The DNA of synaptophysin-GCaMP3 was obtained from S. Voglmaier and inserted into the pUAST vector. The DNA of pH Tomato was amplified from w1118 flies and inserted with Y. Li, cloned into the intravesicular domain of Synaptophysin, and inserted into pUAST. cDNA of Homer was amplified from w1118 flies and inserted with a C-terminal-linked GCaMP3 into pUAST. A detailed description of procedures is provided in the Supplemental Experimental Procedures, along with a list of transgenic fly stocks used.

**Immunohistochemistry**
Brains of 3- to 6-day-old female flies or larval fillets of third instar larvae were prepared and immunostained as described in detail in the Supplemental Experimental Procedures.

**Confocal Imaging**
Stacks of confocal images were generated using a confocal microscope (Leica) with a 20×/NA = 0.7 objective for whole brain scans and a 63×/NA = 1.4 objective for neuropils or larval motor neurons. A detailed description of image acquisition and analysis is provided in the Supplemental Experimental Procedures.

**Two-Color STED Microscopy**
Immunohistochemically stained brains were embedded in a polymer resin, cut into 50–60 nm sections, and imaged using a STED microscope (Leica) equipped with a 100×/NA = 1.4 oil immersion objective. Sample preparation was performed as described in Revelo et al. (2014), with modifications described in the Supplemental Experimental Procedures.

**Wide-Field Imaging**
Brains of female transgenic flies (5 days old) were imaged at 5 Hz through a window in the head capsule using a fluorescence microscope (Zeiss) equipped with a 20×/NA = 1 water-immersion objective. KCl was injected into the Ringer’s solution covering the brain (final concentration ~0.05 M). Imaging and quantification of fluorescence changes are described in detail in the Supplemental Experimental Procedures.

**Two-Photon Imaging**
Stimulus-induced fluorescence changes in brain regions of female transgenic flies (3–6 days old) were imaged through a window in the head capsule at a frame rate of 4 or 5 Hz using a LSM 7MP two-photon microscope (Zeiss) equipped with a mode-locked Ti-sapphire laser (Coherent) and a 20×/NA = 1 water-immersion objective. All sensors were excited at 950 nm, and a dichroic mirror was combined with a 500- to 550-nm and a 575- to 610-nm BP filter to record GCaMP and pH Tomato simultaneously. Imaging procedures, preparation, stimulus delivery, temperature control under the microscope, and image analysis is provided in the Supplemental Experimental Procedures.

**Deprivation Experiments and Apple-Exposure Experiment**
Detailed experimental protocols are provided in the Supplemental Experimental Procedures.

**Behavioral Assays**
Groups of ~20 third instar larvae were scored according to their chemotaxis toward an odor. Geotaxis was quantified in mixed populations of 20 flies, 4–6 days old, as described by Benzer (1967) with modifications described by Inagaki et al. (2010). Details for both assays are provided in the Supplemental Experimental Procedures.

**FM Dye Experiments**
Flies of third instar larvae were loaded with dye in high-potassium medium (see Supplemental Experimental Procedures for details) for 30 s, washed, and subsequently stimulated several times with 20-Hz trains of 100 mA for 10 s. Images of boutons at the neuromuscular junction were recorded and analyzed as described in the Supplemental Experimental Procedures.

**Statistical Analysis**
Data were tested for normal distribution using the Kolmogorov-Smirnov test. For comparing multiple groups, a one-way ANOVA was used. In the case of significance, the data were subjected to post hoc pairwise comparison using the two-sample Student’s t test and p values were Bonferroni corrected. To test for difference to a fixed value, the Wilcoxon signed rank test was used. For determining the relation of ∆F/F0 values obtained from two wavelength channels, different models of the least square fit were compared by means of R\textsuperscript{2} values, the Akaike information criterion (Akaike, 1974), and the Bayesian information criterion (Schwarz, 1978).

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures, six figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.02.065.

**AUTHOR CONTRIBUTIONS**
A.F. and U.P. designed the study, interpreted the results, and wrote the manuscript. U.P. performed all experiments and analyzed the data, except for STED microscopy and FM dye experiments. N.H.R. and U.P. performed and
analyzed STED experiments, supervised by S.O.R. K.J.S. and S.O.R. performed and analyzed FM dye experiments. A.F. supervised the entire study.

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