

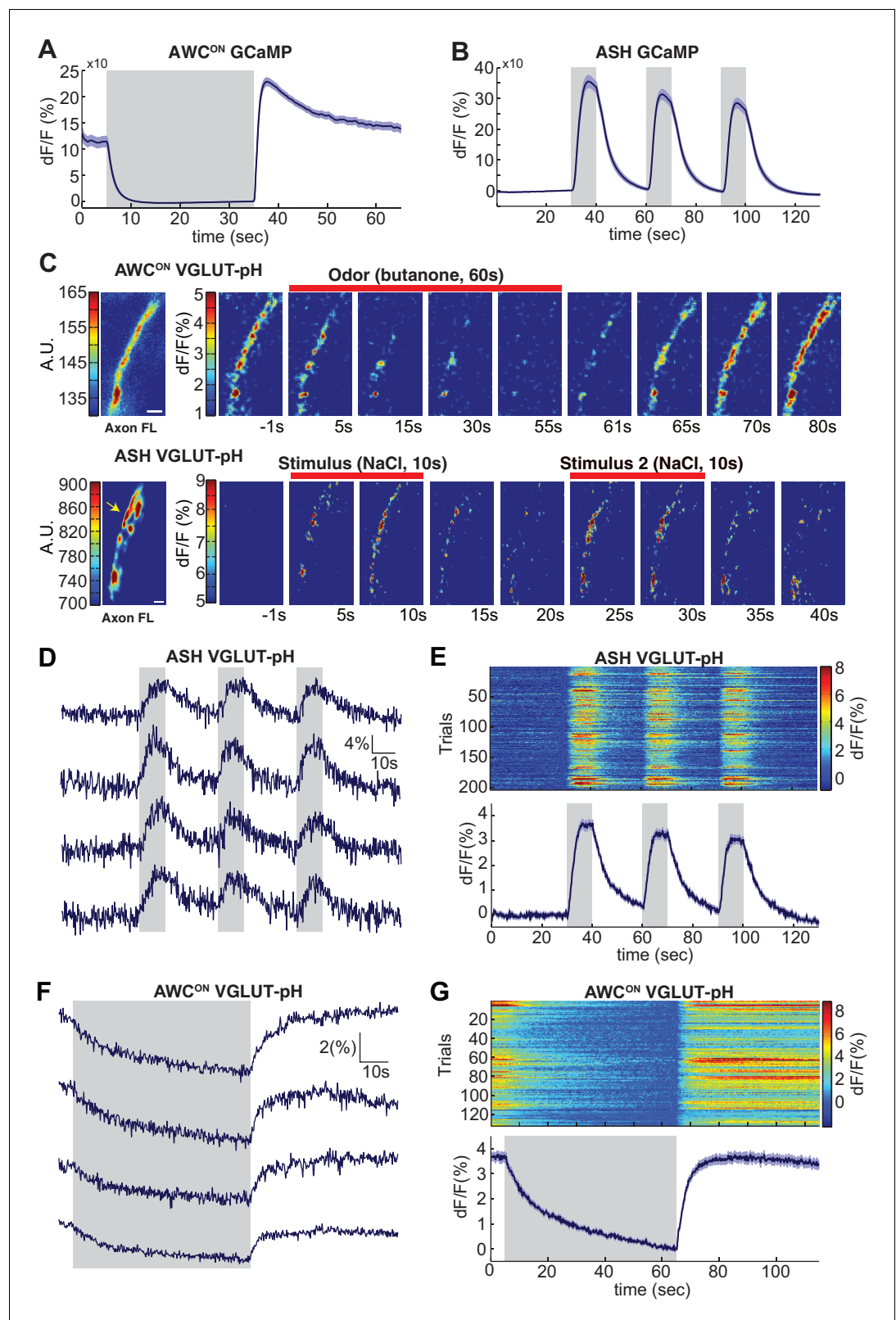


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## Figures and figure supplements

Diverse modes of synaptic signaling, regulation, and plasticity distinguish two classes of *C. elegans* glutamatergic neurons

**Donovan Ventimiglia and Cornelia I Bargmann**



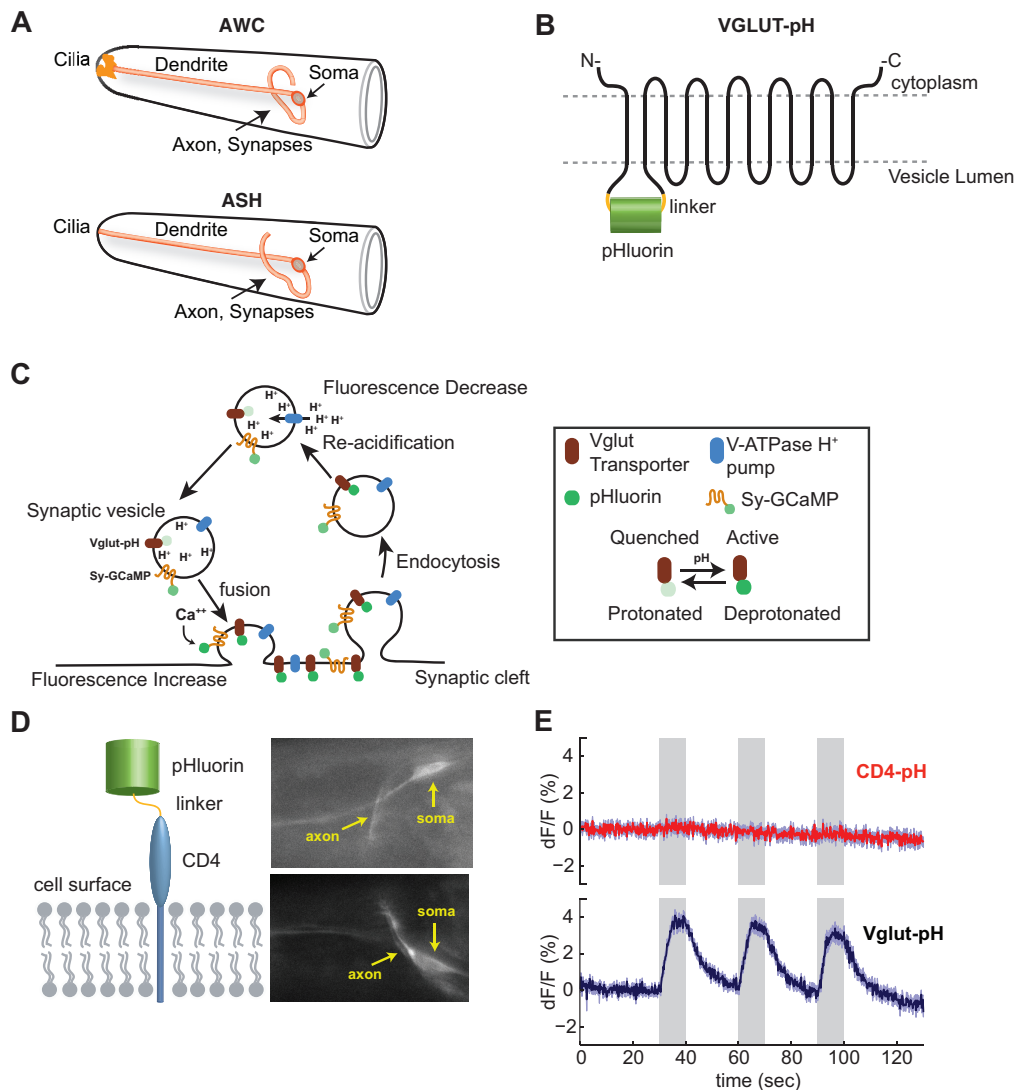
**Figure 1.** Sensory stimuli evoke VGLUT-pH signals in AWC<sup>ON</sup> and ASH neurons. (A) AWC<sup>ON</sup> GCaMP5A responses in the cell body upon butanone stimulation (n = 47, 16 animals, 2–3 trials each). (B) ASH GCaMP3 responses in the cell body upon 500 mM NaCl stimulation (n = 39, 13 animals, three trials each). Gray shaded

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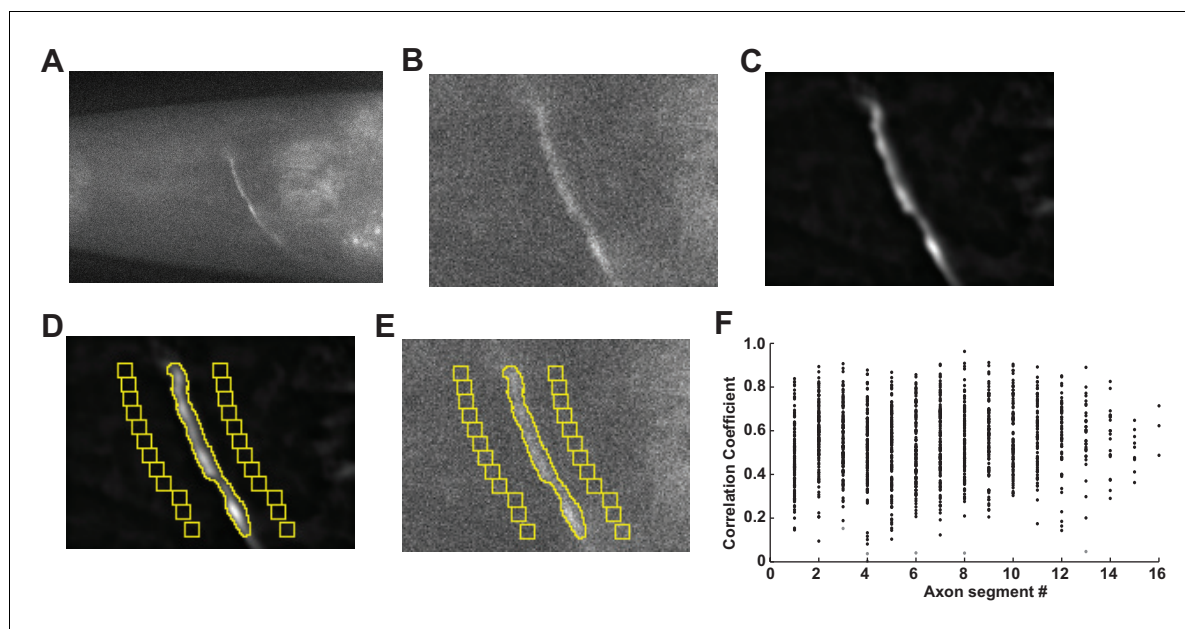
areas mark stimulus period. (C) Individual AWC<sup>ON</sup> and ASH VGLUT-pH responses. Left: Fluorescence intensity of VGLUT-pH along the axon prior to stimulation (a.u. arbitrary units). White scale bars = 2  $\mu$ m. Right: Images of VGLUT-pH fluorescence changes upon butanone (AWC<sup>ON</sup>) or NaCl (ASH) stimulation at  $t = 0$  (red bars), presented as change in fluorescence versus reference  $F$  ( $F$  at  $-t = 55$  s for AWC<sup>ON</sup>,  $t = -1$  s for ASH, see Materials and methods). Recordings were smoothed using a running average (three frames, three pixels in  $x$  and  $y$ ). (D,F) Single trials of ASH (D) and AWC<sup>ON</sup> (F) VGLUT-pH responses from four individuals. Top trace in each panel is from the axon in (C). (E,G) Population ASH (E) and AWC<sup>ON</sup> (G) VGLUT-pH responses. Top panel: Heat map of individual trials, three trial per animal, presented in sequential order. Bottom panel: Mean response from all trials. AWC<sup>ON</sup>: 132 trials from 44 animals, three trials each. ASH: 204 trials from 68 animals, three trials each. Blue shading around traces indicates S.E.M.

DOI: <https://doi.org/10.7554/eLife.31234.002>



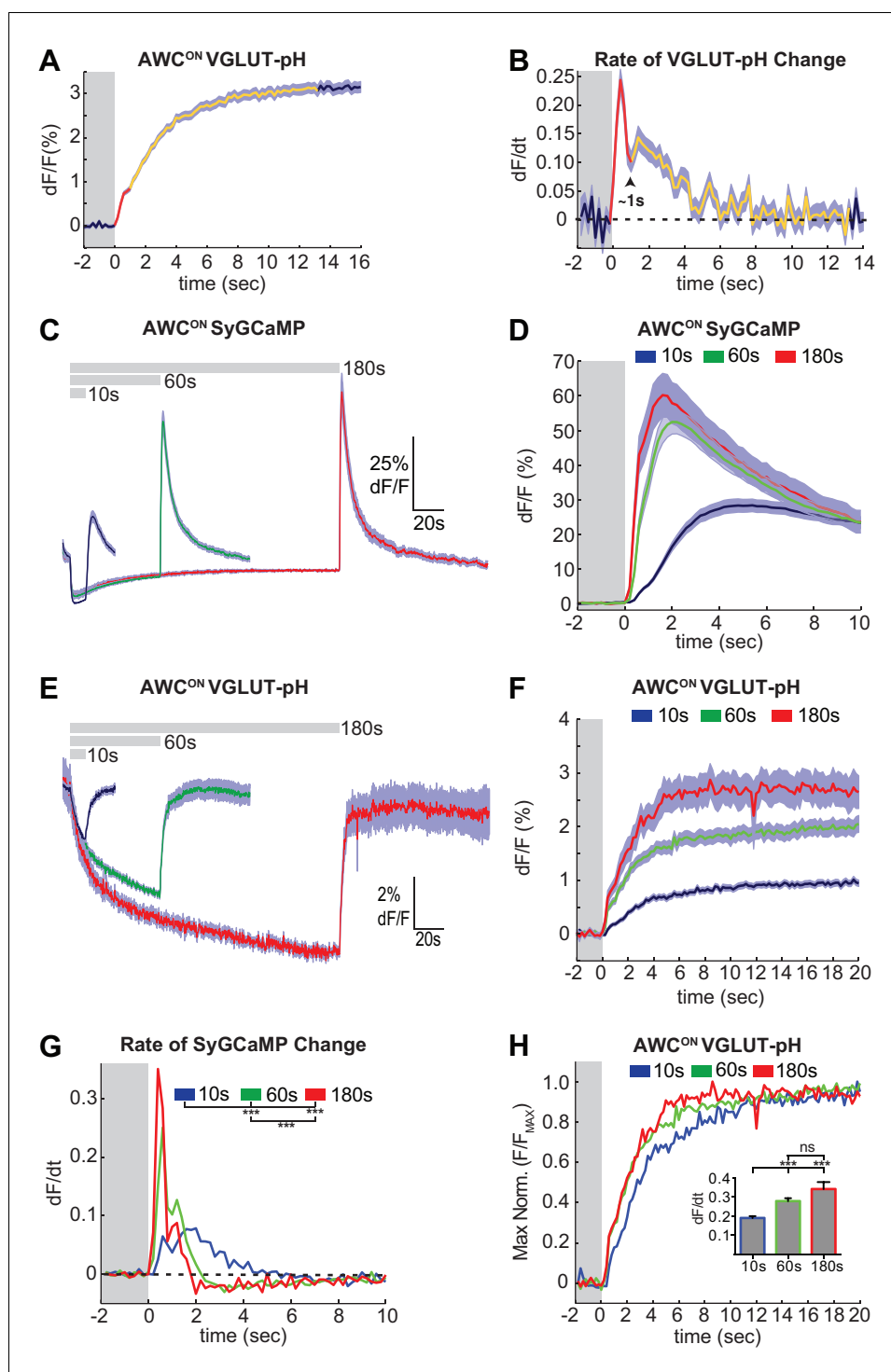
**Figure 1—figure supplement 1.** Anatomy of AWC<sup>ON</sup> and ASH and illustration of synaptic reporters. (A) Anatomical diagram of AWC and ASH neurons. Redrawn from [www.wormatlas.com](http://www.wormatlas.com). (B) Diagram of VGLUT-pH reporter, with pH-sensitive fluorophore inserted into a luminal domain of the protein. (C) Diagram of simplified synaptic vesicle cycle, incorporating synaptic activity reporters used here. VGLUT-pH reports pH changes of the synaptic vesicle lumen associated with exocytosis. syGCaMP, a fusion to the synaptic vesicle protein synaptogyrin, reports changes in calcium concentration at the cytosolic face of synaptic vesicles. Although illustrated together, these reagents are used separately. (D) LEFT: Diagram of CD4-pH construct used to target pHluorin to the extracellular surface of ASH. RIGHT: Two animals expressing CD4-pH in ASH under the *sra-6* promoter. ASH axon and cell body (soma) indicated. Images are from recordings in (E) and are averages of the first five frames. (E) TOP: Average response of ASH CD4-pH ( $n = 33$  trials, 11 animals, three trials each). No response was detected. BOTTOM: Average response of ASH VGLUT-pH control ( $n = 39$  trials, 13 animals, three trials each). Gray areas mark stimulus periods (500 mM NaCl). Shading = S.E.M.

DOI: <https://doi.org/10.7554/eLife.31234.003>



**Figure 1—figure supplement 2.** VGLUT-pH data acquisition. (A) Single plane of AWC<sup>ON</sup> VGLUT-pH recording after drift-registration correction. (B) Zoomed view around the axon in (A). (C) Mean intensity projection after the recording was processed by rolling background subtraction and 3D-averaging. (D) Selection of axon and background ROIs. (E) Intensity measurements are then taken from the raw drift-corrected recording. (F) ROI segments along the axon correlated with overall mean activity of the axon. Each point represents the correlation coefficient of an axon segment with the mean signal of the axon during a standard recording (see Materials and methods). Data from 120 AWC<sup>ON</sup> VGLUT-pH axons stimulated with 11.2  $\mu$ M butanone. Each axon had 8–16 ROI segments.  $p < 0.000001$  for all correlation coefficients except the points labeled in light gray, which were not significant  $p > 0.05$ .

DOI: <https://doi.org/10.7554/eLife.31234.004>



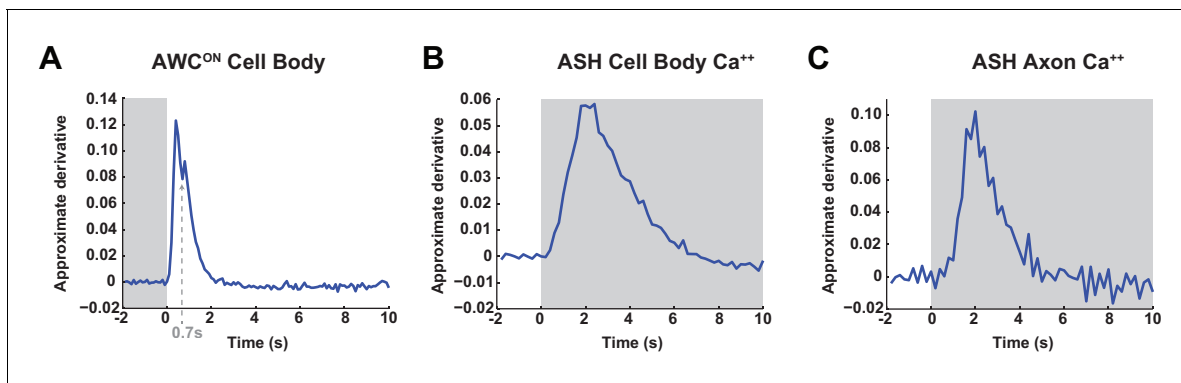
**Figure 2.** Two kinetic phases of VGLUT-pH responses and calcium influx in AWC<sup>ON</sup>. (A) Mean AWC<sup>ON</sup> Vglut-pH signal upon odor removal (60 s stimulus, n = 219 trials, 1–3 trials per animal). Trace is colored according to transitions in time derivative in (B). (B) Mean time derivative of AWC<sup>ON</sup> VGLUT-pH signals in (A). (C) AWC<sup>ON</sup> synaptic calcium responses to butanone pulses measured with syGCaMP; traces aligned to odor addition. 10 s pulses: n = 42 (7 animals, six trials each). 60 s pulses: n = 21 (7 animals, three trials each). 3 min pulses: n = 7 (7 animals, one trial each). (D) syGCaMP responses from (C) aligned to odor removal. (E) AWC<sup>ON</sup> VGLUT-pH responses to butanone pulses; traces aligned to odor addition. 10 s pulses: n = 120 (20 animals, six trials each). 60 s pulses: n = 59 (21 animals, 2–3 trials each). 3 min pulses: n = 14 (14 animals, one trial each). (F) VGLUT-pH

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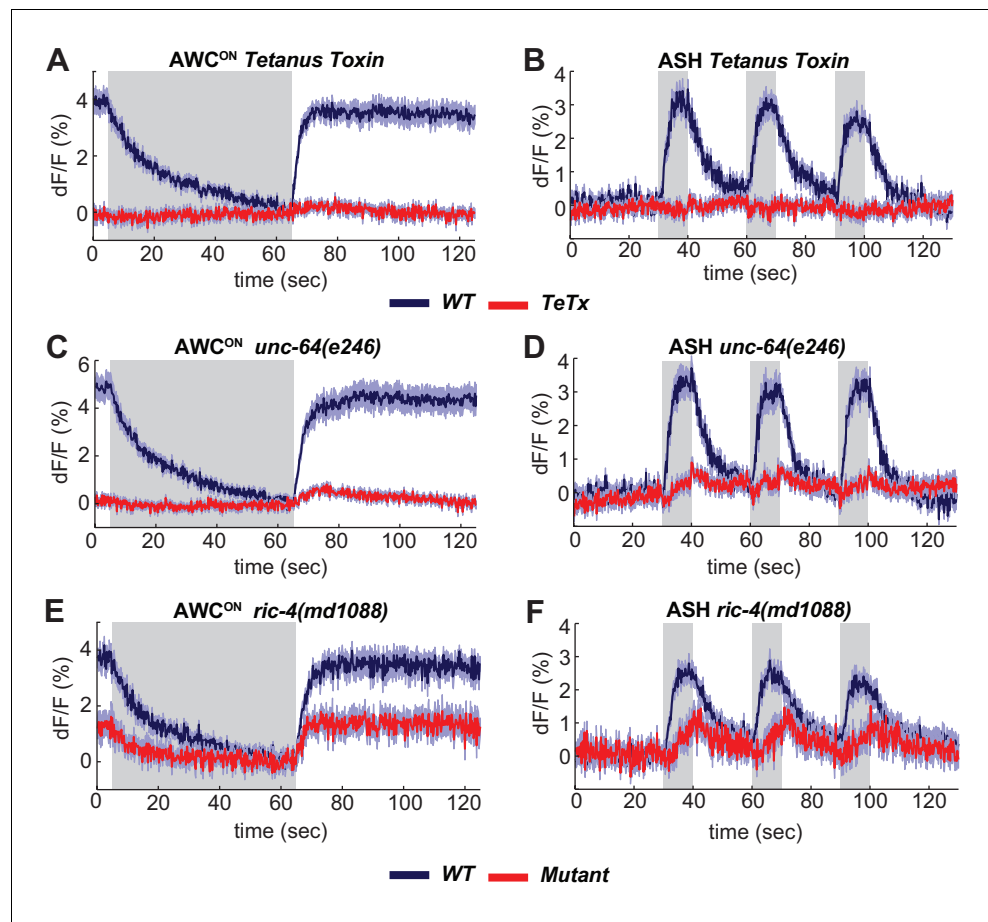
responses from (E) aligned to odor removal. (G) Mean time derivative of AWC<sup>ON</sup> syGCaMP signals in (D) shows different peak rates 1 s after odor removal (\*\*p<0.0001, one-way ANOVA with Tukey's correction). (H) Average VGLUT-pH odor removal responses from (F) after normalizing response magnitude. Inset: Average peak time derivative of AWC<sup>ON</sup> VGLUT-pH signals 1 s after odor removal. \*\*\*p<0.0001, ns (p=0.14), One-way ANOVA with Tukey's correction. For time derivative plots each individual trial was smoothed with a running average (three frames) before taking the derivative. Units are change in dF/F (%) per 200 ms. Gray areas in A,B,D,F-H mark odor stimulus periods. Shading indicates S.E.M.

DOI: <https://doi.org/10.7554/eLife.31234.006>



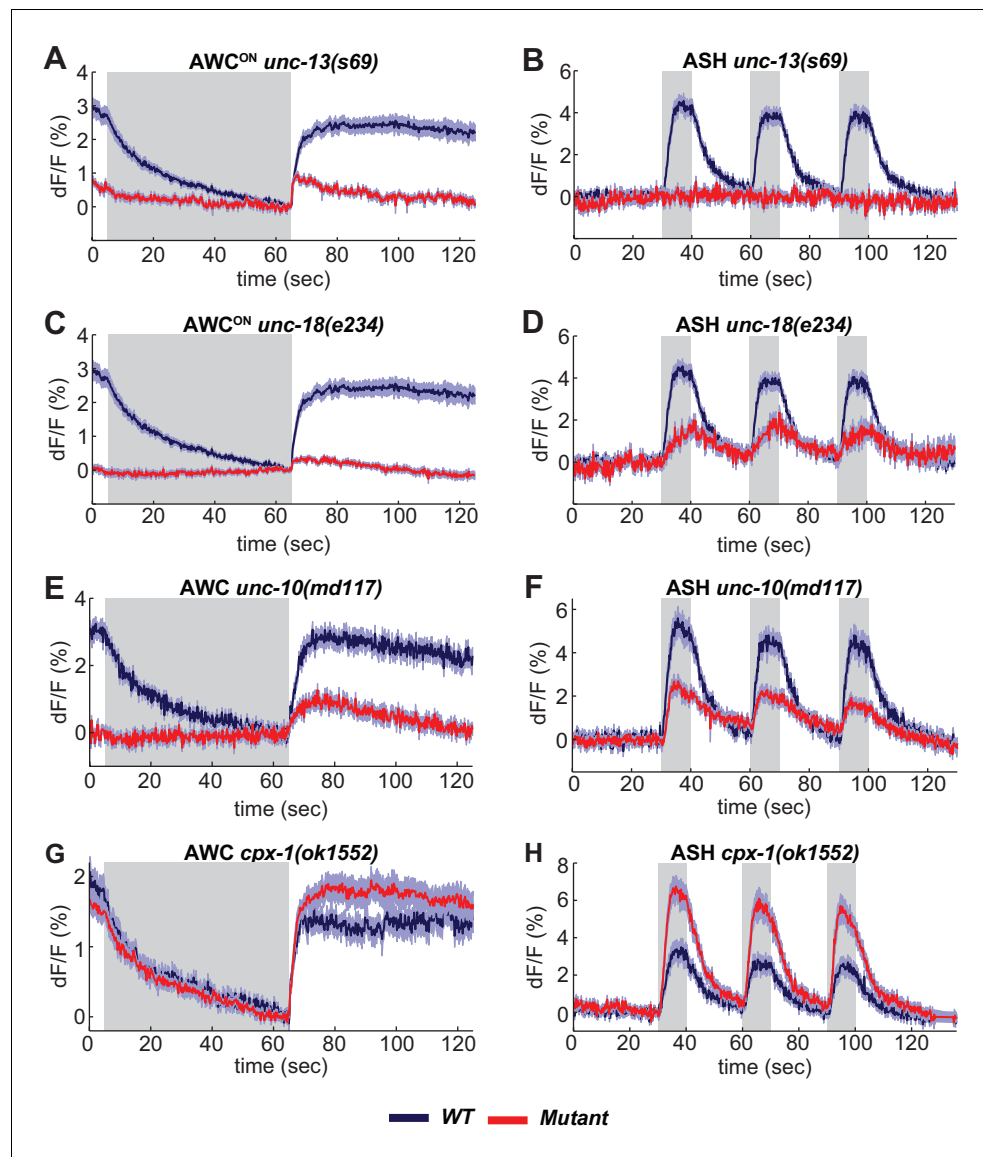
**Figure 2—figure supplement 1.** Time derivative of AWC<sup>ON</sup> and ASH GCaMP signals in the cell body and ASH GCaMP signals in the axon. (A) A transition between two positive influx rates may occur ~0.7 s after odor removal, similar to the transition observed in synaptic calcium by syGCaMP recordings in **Figure 2G**. Average from 47 trials from 16 wild-type animals. (B,C) Time derivative of the mean ASH GCaMP3 signal at the cell body (B) and axon (C) during a 500 mM NaCl stimulus. No heterogeneity of influx rates was detectable with this sensor. Data from **Figure 1B**. Gray areas mark stimulus periods.

DOI: <https://doi.org/10.7554/eLife.31234.007>



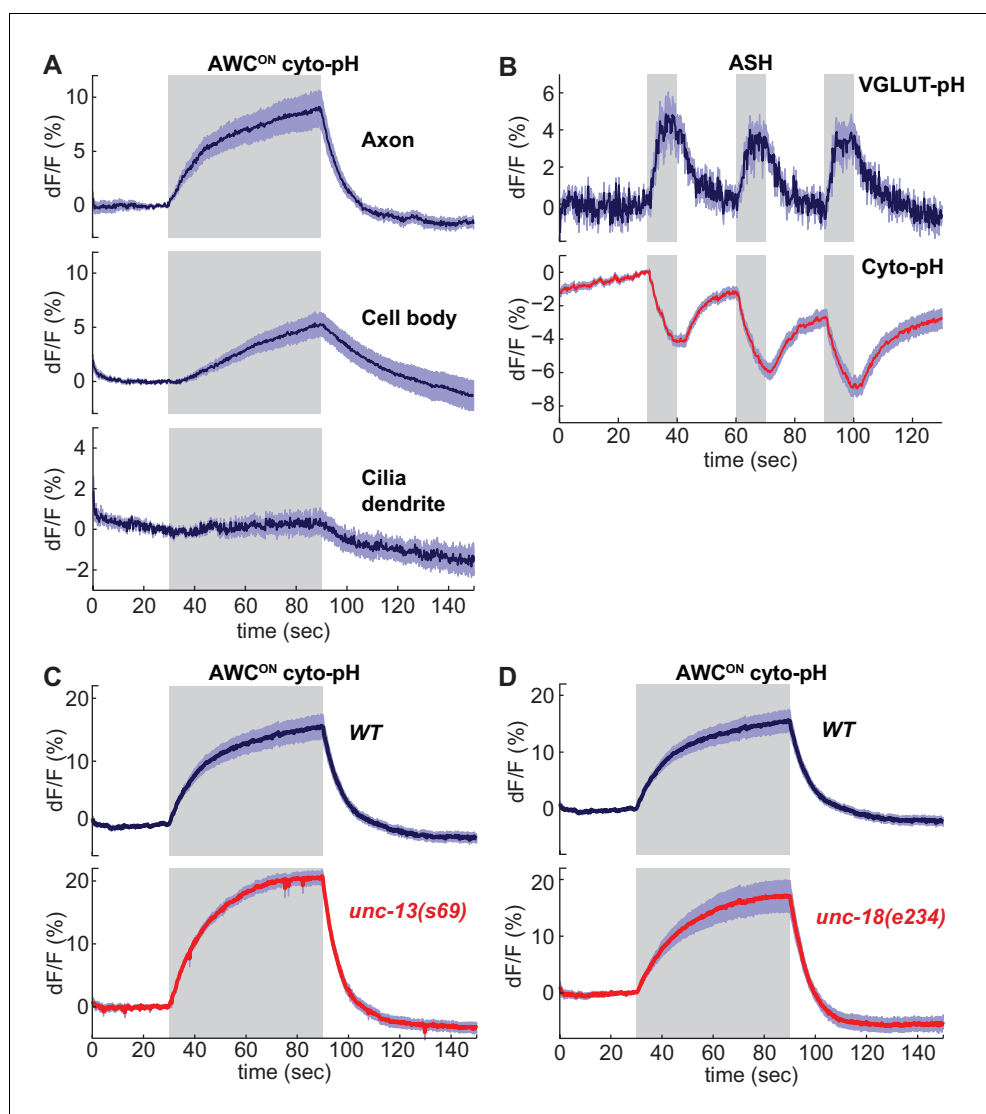
**Figure 3.** The SNARE complex is required for VGLUT-pH responses in AWC<sup>ON</sup> and ASH. (A,B) VGLUT-pH responses are eliminated by cell-specific expression of TeTx light chain. (A) AWC<sup>ON</sup> *str-2* promoter driving TeTx n = 25 (9 animals, 2–3 trials each). AWC<sup>ON</sup> wt (TeTx-array negative animals tested in parallel) n = 28 (10 animals, 2–3 trials each). (B) ASH *sra-6* promoter driving TeTx n = 33 (11 animals, three trials each). ASH wt (TeTx-array negative animals tested in parallel) n = 24 (8 animals, three trials each). (C,D) VGLUT-pH responses in *unc-64(e246)* (partial loss of function) syntaxin mutants. (C) AWC<sup>ON</sup> *unc-64(e246)* n = 30 (10 animals, three trials each). AWC<sup>ON</sup> wt n = 23 (9 animals, 2–3 trials each). (D) ASH *unc-64(e246)* n = 24 (8 animals, three trials each). ASH wt n = 18 (6 animals, three trials each). (E, F) VGLUT-pH responses in *ric-4(md1088)* (partial loss of function) SNAP-25 mutants. (E) AWC<sup>ON</sup> *ric-4(md1088)* n = 12 (4 animals, three trials each). AWC<sup>ON</sup> wt n = 15 (5 animals, three trials each). (F) ASH *ric-4(md1088)* n = 16 (6 animals, 2–3 trials each). ASH wt n = 32 (11 animals, 2–3 trials each). Mutations are described in **Supplementary file 2**. All differences are significant (p<0.0001, unpaired t-test), as detailed in **Supplementary file 3A**. Gray areas mark stimulus periods. Shading indicates S.E.M.

DOI: <https://doi.org/10.7554/eLife.31234.009>



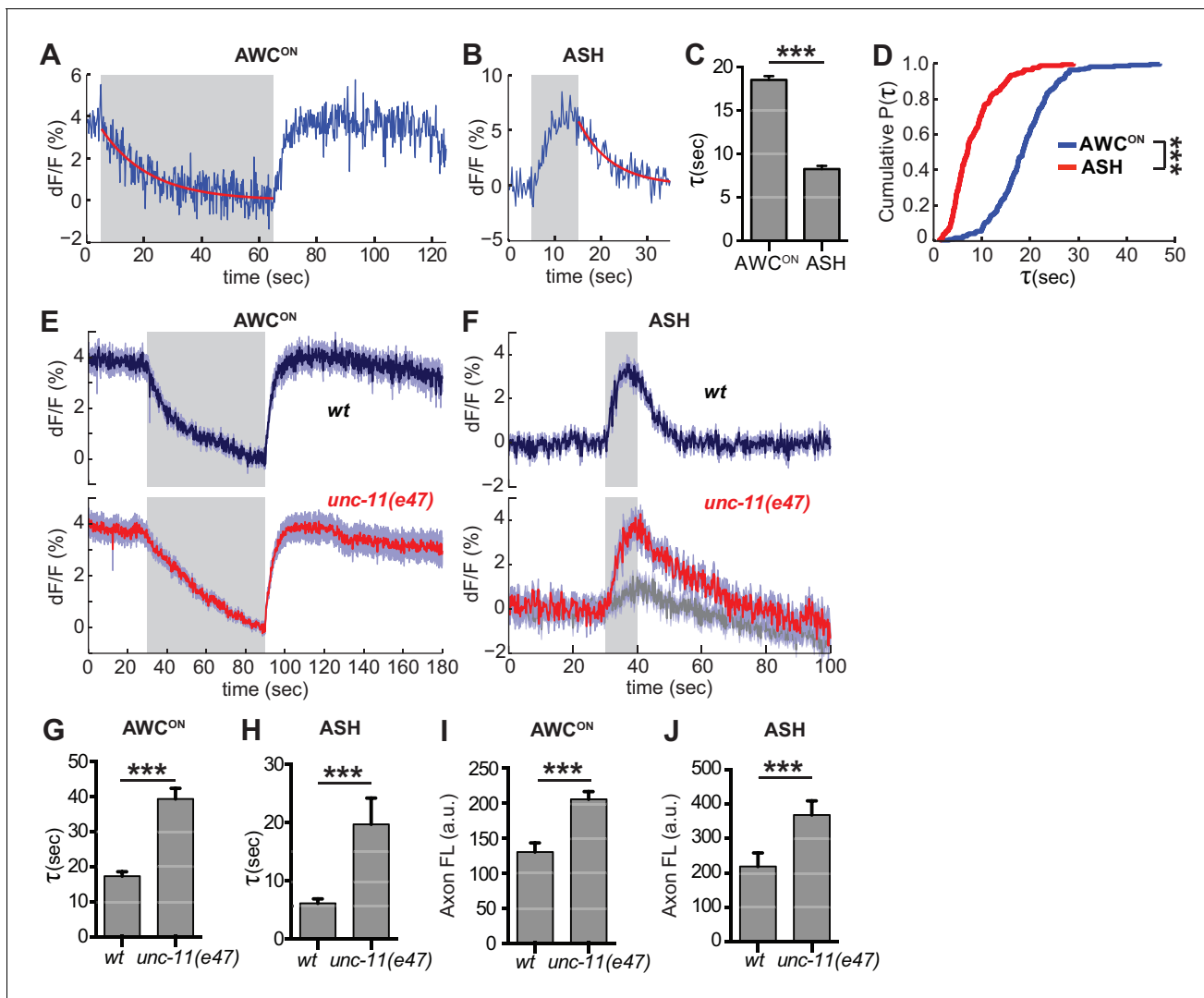
**Figure 4.** SNARE regulators differentially affect AWC<sup>ON</sup> and ASH. (A,B) VGLUT-pH responses in *unc-13(s69)* null mutants. (A) AWC<sup>ON</sup> responses in *unc-13(s69)*, *n* = 25 (9 animals, 2–3 trials each) and *wt*, *n* = 33 (11 animals, three trials each). (B) ASH responses in *unc-13(s69)*, *n* = 18 (7 animals, 1–3 trials each) and *wt*, *n* = 42 (14 animals, three trials each). (C,D) VGLUT-pH responses in *unc-18(e234)* mutants. (C) AWC<sup>ON</sup> responses in *unc-18(e234)*, *n* = 25 (9 animals, 1–3 trials each) and *wt*, *n* = 33 (11 animals, three trials each). (D) ASH responses in *unc-18(e234)*, *n* = 22 (8 animals, 1–3 trial each) and *wt*, *n* = 42 (14 animals, three trials each). (E,F) VGLUT-pH responses in *unc-10(md117)* mutants. (E) AWC<sup>ON</sup> responses in *unc-10(md117)*, *n* = 23 (8 animals, 1–3 trials each), and *wt*, *n* = 27 (9 animals, three trials each). (F) ASH responses in *unc-10(md117)*, *n* = 33 (12 animals, 2–3 trials each), and *wt*, *n* = 24 (8 animals, three trials each). (G,H) VGLUT-pH responses in *cpx-1(ok1552)* mutants. (G) AWC<sup>ON</sup> responses in *cpx-1(ok1552)*, *n* = 36 (12 animals, three trials each), and *wt*, *n* = 17 (6 animals, 2–3 trials each). (H) ASH responses in *cpx-1(ok1552)*, *n* = 38 (13 animals, 2–3 trials each), and *wt*, *n* = 33 (11 animals, three trials each). WT and mutants are significantly different in panels A–F and H, as detailed in **Supplementary file 3B**. Gray areas mark stimulus periods. Shading indicates S.E.M.

DOI: <https://doi.org/10.7554/eLife.31234.011>



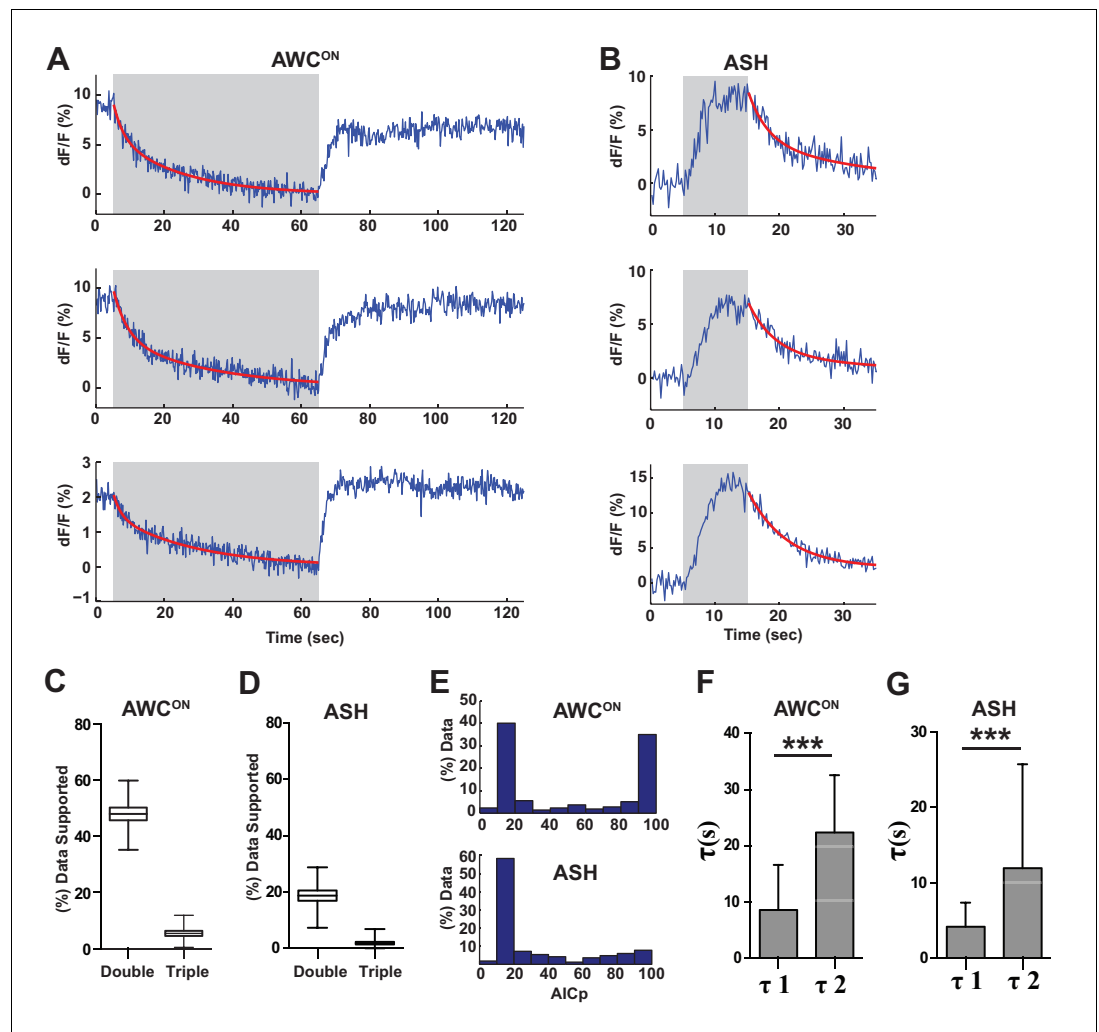
**Figure 5.** Sensory stimuli evoke cytoplasmic pH changes in AWC<sup>ON</sup> and ASH. (A) Average cyto-pH signals at different subcellular sites of AWC<sup>ON</sup>. Axon and cell body,  $n = 9$  (3 animals, three trials each), Cilia-dendrite  $n = 6$  (2 animals, three trials each). (B) Average ASH VGLUT-pH response (top) and cyto-pH response (bottom), tested in parallel under the same stimulus conditions. VGLUT-pH  $n = 9$  (3 animals, three trials each). cyto-pH  $n = 27$  (9 animals, three trials each). (C,D) Average AWC<sup>ON</sup> cyto-pH responses in (C) *unc-13(s69)* and (D) *unc-18(e234)* mutants. Mutants and wild-type controls were measured on the same days; neither mutant was significantly different from wild-type ( $p > 0.12$ , one-way ANOVA with Tukey's correction). *unc-13(s69)*  $n = 15$  (5 animals, three trials each). *unc-18(e234)*  $n = 11$  (4 animals, 2–3 trials each). wt  $n = 18$  (6 animals, three trials each). Gray areas mark stimulus periods. Shading indicates S.E.M.

DOI: <https://doi.org/10.7554/eLife.31234.013>



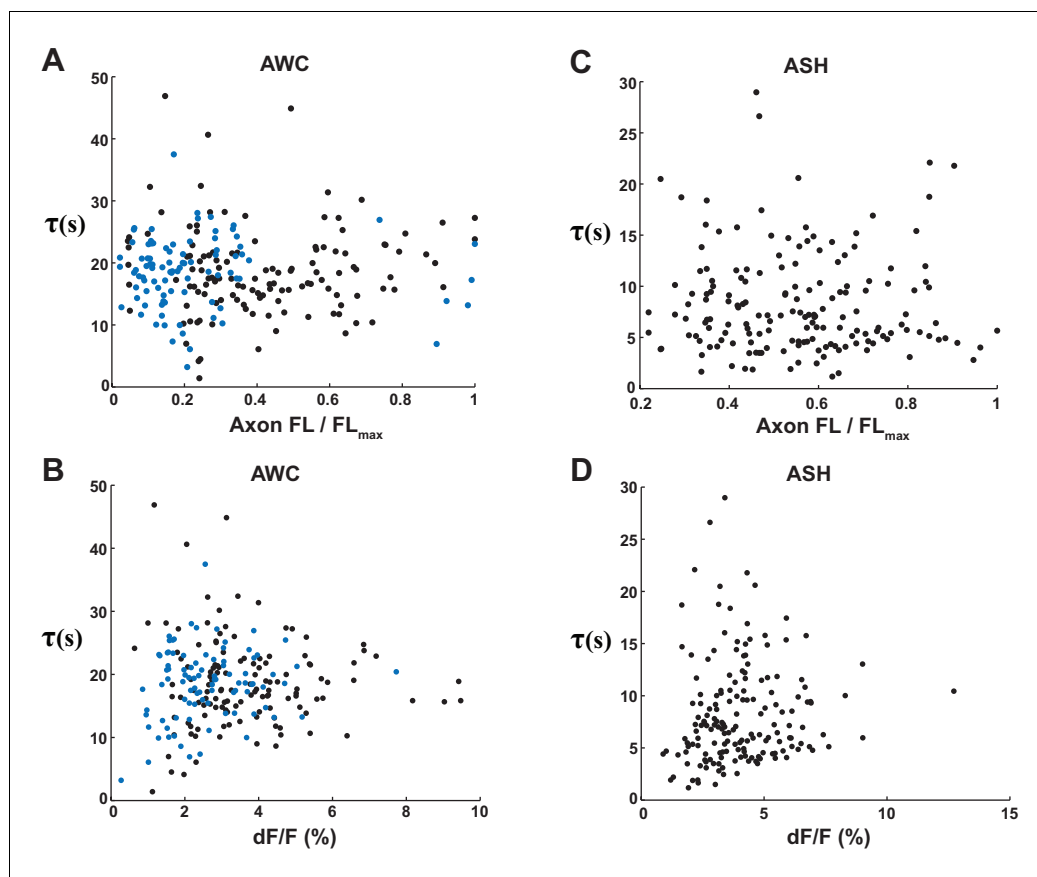
**Figure 6.** Synaptic vesicle retrieval is accelerated by AP180/CALM. (A,B) Representative single exponential fits (red) to single trials of (A) *AWC<sup>ON</sup>* and (B) *ASH* VGLUT-pH decays upon stimulus addition or removal, respectively. For each neuron, some responses were consistent with double exponential decay models (Figure 6—figure supplement 1). (C) Average time constant of *AWC<sup>ON</sup>* and *ASH* decays from single exponential fits. *AWC<sup>ON</sup>*  $n = 218$  (76 animals, 2–3 trials each). *ASH*  $n = 168$  (56 animals, 2–3 trials each). \*\*\* $p < 0.0001$ , unpaired t-test. (D) Empirical cumulative distribution plot of data in (C). Distributions differ by Kolmogorov-Smirnov test, \*\*\* $p < 0.0001$ . (E,F) Average VGLUT-pH responses in *unc-11(e47)* mutants. (E) *AWC<sup>ON</sup>* responses in *unc-11(e47)*  $n = 27$  (10 animals, 2–3 trials each), wt  $n = 15$  (5 animals, three trials each). One *unc-11(e47)* animal did not respond and was removed from the analysis. (F) *ASH* responses in *unc-11(e47)* mutants. wt  $n = 21$  (7 animals, three trials each). *unc-11(e47)*  $n = 23$  (8 animals, 2–3 trials each). Red trace: mean of 9 trials (five animals, 1–2 trials each) with clear responses to odor addition. Magnitude of response does not differ from wt ( $p = 0.72$ , peak odor response); Gray trace: mean of 14 trials that produced weak or non-detectable responses to odor addition, significantly different from wt ( $p < 0.0001$ , peak odor response). One-way ANOVA, Tukey's correction. (G,H) Average time constants from single exponential fits (initial 20 s of decay) of data in (E, F). For *ASH* *unc-11(e47)* mutants, only data from the red trace was used. Unpaired t-test,  $p < 0.0001$ . *AWC<sup>ON</sup>* *unc-11(e47)*  $n = 25$  (10 animals, 2–3 trials each); wt  $n = 15$  (5 animals, three trials each). *ASH*  $n$  as in (F). (I,J) Average axon fluorescence (first 5 frames of the recording). (I) *AWC<sup>ON</sup>* wt  $n = 12$  animals, *unc-11(e47)*  $n = 19$  animals. (J) *ASH* wt  $n = 7$  animals, *unc-11(e47)*  $n = 8$  animals. Unpaired t-test,  $p < 0.0001$ . Gray areas mark stimulus periods. Shading and error bars indicate S.E.M.

DOI: <https://doi.org/10.7554/eLife.31234.015>



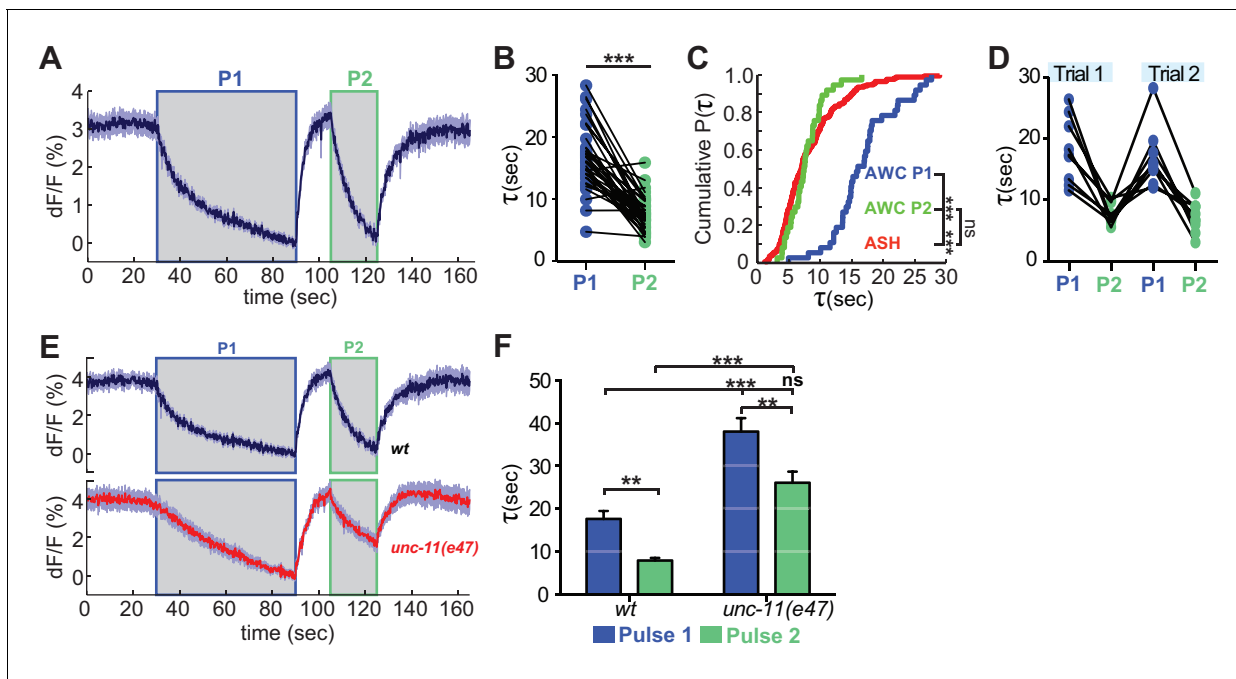
**Figure 6—figure supplement 1.** A proportion of VGLUT-pH recordings are consistent with multiple retrieval time constants. (A,B) Three individual examples of AWC<sup>ON</sup> (A) or ASH (B) VGLUT-pH decays that fit a double exponential decay model with greater than 95% likelihood based on AICp (see Materials and methods). Gray areas mark stimulus periods. (C,D) Percent of VGLUT-pH data that fits a double or triple exponential model with greater than 50% likelihood (AICp >50%). Box and whisker plot created by 10,000 sampled bootstrap sampling. (E) Frequency histogram of AICp-based likelihood values for double exponential fits to VGLUT-pH data. An AICp value of >50 supports a double exponential fit with increasing likelihood; an AICp of less than 50 supports a single exponential with increasing likelihood. (C–E) AWC<sup>ON</sup> data: n = 218 (76 animals, 2–3 trials each). ASH data: n = 168 (56 animals, 2–3 trials each). (F,G) Average decay constants from double exponential fits. Note similarity of two AWC<sup>ON</sup> timescales to effects of plasticity in **Figure 7F**. Fits that minimized tau1 or tau2 to ~zero were excluded from the average. AWC<sup>ON</sup> data: n = 211 (76 animals, 2–3 trials each). ASH data: n = 125 (56 animals, 2–3 trials each). Error bars = standard deviation. t-test p<0.0001. All ASH and AWC<sup>ON</sup> data pooled from wild-type data used in this study. AWC<sup>ON</sup> stimulus = 11.2  $\mu$ M butanone (60 s). ASH stimulus = 500 mM NaCl (10 s).

DOI: <https://doi.org/10.7554/eLife.31234.016>



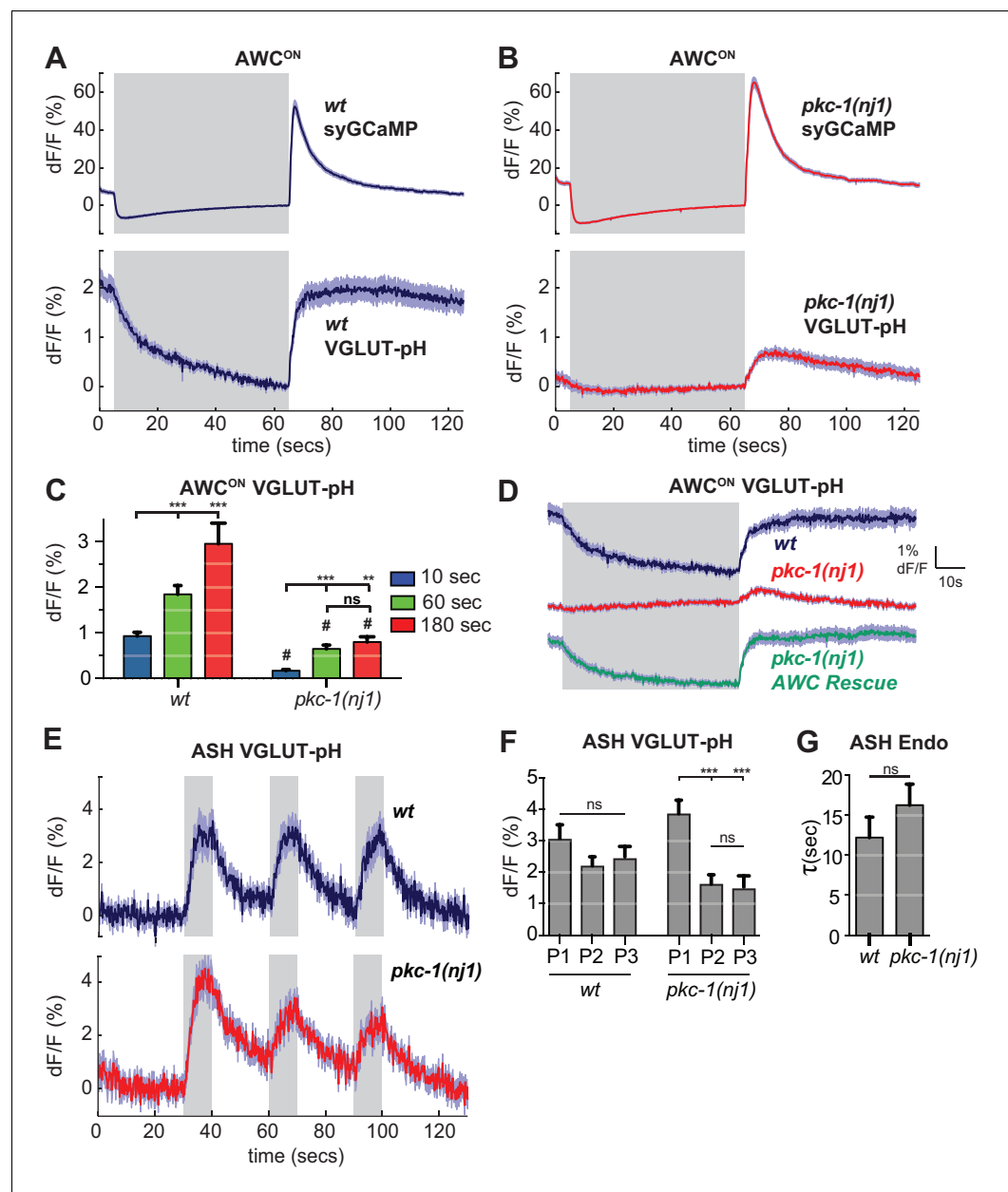
**Figure 6—figure supplement 2.** VGLUT-pH decay time constants are not correlated with expression level or response magnitude. (A) Scatter plot of AWC<sup>ON</sup> VGLUT-pH decay time constants versus baseline axon fluorescence intensity. Two different AWC<sup>ON</sup> VGLUT-pH transgenic lines with different expression levels are represented by the black and blue points. Axon intensities were normalized to the brightest axon detected in the line (FL<sub>MAX</sub>). Time constants are from single exponential fits, as in **Figure 6**. Correlation coefficient = 0.01,  $p=0.89$ , not significant. (B) AWC<sup>ON</sup> decay time constants plotted against the magnitude of basal release, dF/F<sub>0</sub> prior to stimulus onset; F<sub>0</sub> is fluorescence level prior to stimulus removal. Correlation coefficient = 0.024,  $p=0.73$ , not significant. (C) Scatter plots of ASH VGLUT-pH decay time constants versus baseline axon fluorescence intensity. Correlation coefficient = -0.029,  $p=0.72$ , not significant. (D) ASH decay time constants after stimulus removal, compared to peak dF/F during stimulus. Correlation coefficient = 0.058,  $p=0.46$ , not significant. AWC<sup>ON</sup>  $n = 218$  (76 animals, 2–3 trials each). ASH  $n = 168$  (56 animals, 2–3 trials each).

DOI: <https://doi.org/10.7554/eLife.31234.017>



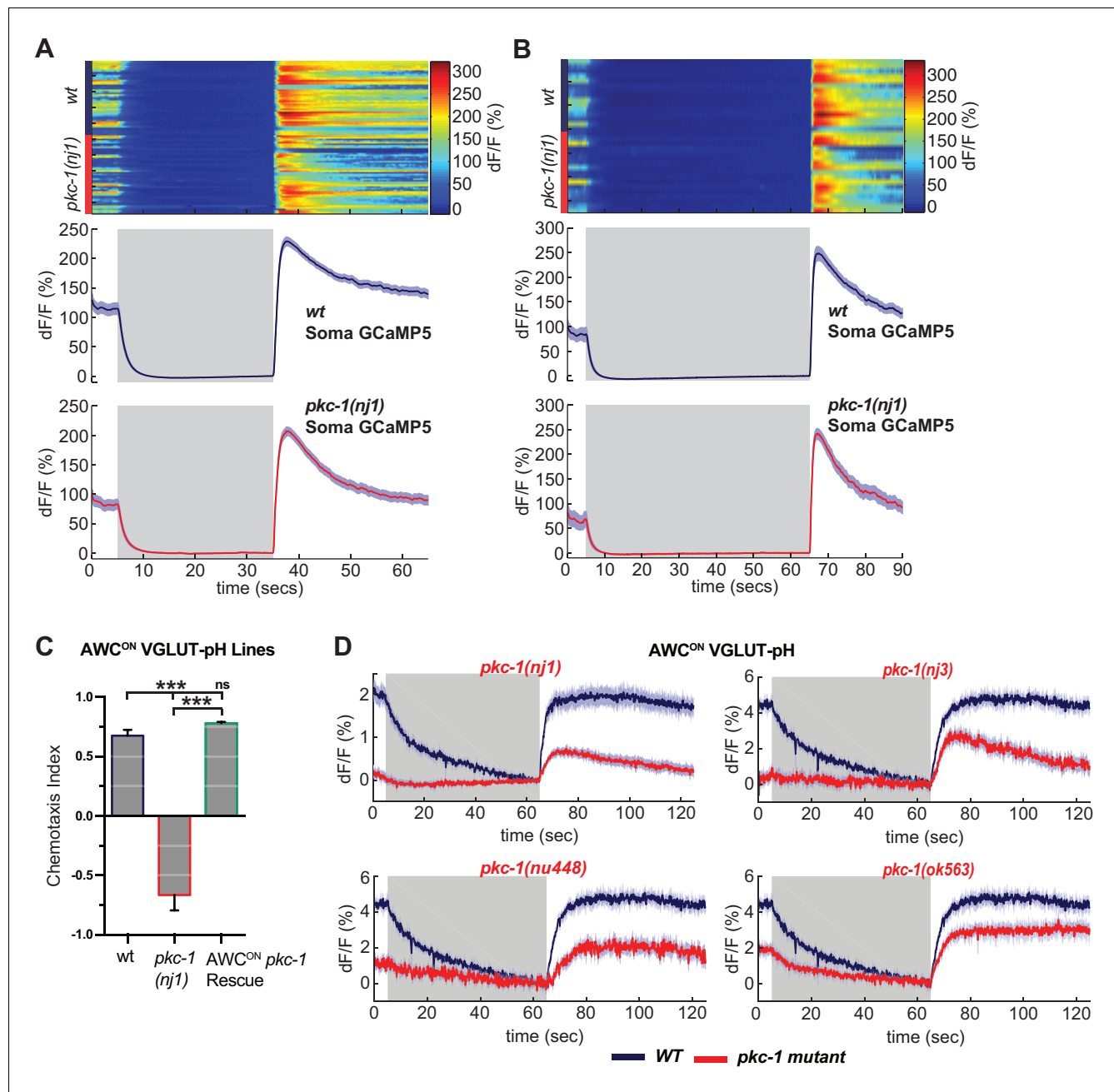
**Figure 7.** Recent neural activity modulates AWC<sup>ON</sup> VGLUT-pH retrieval. (A) Average AWC<sup>ON</sup> VGLUT-pH response to two successive odor stimuli, applied for 60 s (P1) and 20 s (P2).  $n = 39$  (13 animals, three trials each). (B) Time constants from single-term exponential fits to P1 and P2 from (A) performed on the initial 20 s of the decay for each pulse.  $n = 37$  (13 animals, 2–3 trials each). Paired t-test, \*\*\* $p < 0.0001$ . (C) Cumulative distribution plot of time constants for AWC P1, AWC P2, and ASH VGLUT-pH decays. AWC P1 and AWC P2 data from (B) and ASH data from Figure 6D. Kruskal-Wallis and Dunn's test for multiple comparisons, \*\*\* $p < 0.0001$ , ns  $p = 0.1$ . (D) Time constants for P1 and P2 from two consecutive trials of the stimulation protocol in (A) ( $n = 8$  animals, 70 s between trials). (E) Average AWC VGLUT-pH signals in wt and *unc-11(e47)* mutants. wt  $n = 21$  (7 animals, three trials each). *unc-11(e47)*  $n = 25$  (9 animals, three trials each) (two non-responding trials removed). (F) Average time constants from single exponential fits (initial 20 s of decay) of data in (E). wt  $n = 21$  (7 animals, three trials each). *unc-11(e47)*  $n = 22$  (8 animals, 2–3 trials each). Two-way ANOVA, \*\*\* $p < 0.0001$ , \*\* $p < 0.008$ , ns ( $p = 0.07$ ). Gray areas marks stimulus periods. Shading and error bars indicate S.E.M. ns = not significant.

DOI: <https://doi.org/10.7554/eLife.31234.019>



**Figure 8.** *pkc-1* regulates AWC<sup>ON</sup> glutamate release downstream of calcium influx. (A,B) AWC<sup>ON</sup> synaptic calcium (top) and VGLUT-pH (bottom) responses in (A) wild-type and (B) *pkc-1(nj1)* mutant animals. *wt* SyGCaMP *n* = 21 (7 animals, three trials each), *pkc-1* SyGCaMP *n* = 25 (9 animals, 1–3 trials each). *wt* VGLUT-pH *n* = 29 (10 animals, 2–3 trials each), *pkc-1(nj1)* VGLUT-pH *n* = 48 (17 animals, 2–3 trials each). (C) Average AWC<sup>ON</sup> VGLUT-pH peak response magnitude after odor removal for indicated stimulation durations. #, different from wild-type *p* < 0.0001. \*\**p* = 0.0026, \*\*\**p* < 0.0001. Two-way ANOVA Tukey's correction. 10 s pulses: *wt* *n* = 60 (10 animals, six trials each). *pkc-1(nj1)* *n* = 102 (17 animals, 4–6 trials each). 60 s pulses: as in (A,B). 3 min pulses: *wt* *n* = 8 (8 animals, one trial each). *pkc-1(nj1)* *n* = 15 (15 animals, one trial each). (D) Expression of *pkc-1* cDNA in AWC<sup>ON</sup> rescues *pkc-1(nj1)* VGLUT-pH responses. *wt* *n* = 18 (6 animals, three trials each). *pkc-1(nj1)* *n* = 30 (10 animals, three trials each). *pkc-1(nj1)* AWC<sup>ON</sup> rescue *n* = 30 (10 animals, three trials each). (E) ASH VGLUT-pH responses in *pkc-1(nj1)* mutants. *wt* *n* = 15 (5 animals, three trials each). *pkc-1(nj1)* *n* = 15 (6 animals, 2–3 trials each). (F) Average ASH VGLUT-pH peak responses for each stimulus pulse (P1–3) within a trial. Data from (E). Two-way ANOVA Tukey's correction. \*\*\**p* < 0.0001. (G) Average time constants from single exponential fits of data in (E, first pulse). Supporting statistical analysis for all panels is detailed in **Supplementary file 3C** and Source Data. Gray areas mark stimulus periods. Shading and error bars indicate S.E.M. ns = not significant.

DOI: <https://doi.org/10.7554/eLife.31234.021>



**Figure 8—figure supplement 1.** *pkc-1(lf)* alters synaptic release downstream of calcium influx. (A,B) AWC<sup>ON</sup> GCaMP5 responses to 30 s (A) and 60 s (B) pulses of butanone, measured at the soma. Top panels: Heat map of individual trials. Bottom: average across trials. (A) *pkc-1(nj1)* n = 50 (17 animals, 2–3 trials each), wt n = 47 (16 animals, 2–3 trials each). (B) *pkc-1(nj1)* n = 16 (16 animals), wt n = 16 (16 animals), one trial each. (C) Butanone chemotaxis index (see Materials and methods) of AWC<sup>ON</sup> VGLUT-pH transgenics used in **Figure 8D**, showing that expression of *pkc-1* cDNA in AWC<sup>ON</sup> under the *str-2* promoter rescues *pkc-1(nj1)* chemotaxis. n = 3 population assays per genotype. One-way ANOVA, \*\*\*p<0.0001. ns = not significant. Error bars = S.E.M. (D) VGLUT-pH responses in *pkc-1* mutant alleles. *nj1* and *nu488* disrupt the kinase domain, *nj3* terminates the protein before the kinase domain, and *ok563* disrupts two of three isoforms (see **Supplementary file 2**). *pkc-1(nj1)* data are from **Figure 8B**. *pkc-1(nu488)* n = 10 (4 animals, 2–3 trials each), *pkc-1(nj3)* n = 18 (6 animals, three trials each), *pkc-1(ok563)* n = 48 (16 animals, three trials each), wt n = 36 (12 animals, three trials each). Alleles *nu488*, *nj3* and *ok563* are plotted with the mean response of pooled wild-type controls from those experiments. Gray areas mark stimulus periods, 11.2  $\mu$ M butanone. Shading = S.E.M.

DOI: <https://doi.org/10.7554/eLife.31234.022>