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Supplementary Information for

Rhodopsin-based voltage imaging tools for use in muscles and neurons of *Caenorhabditis elegans*

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SUPPLEMENTARY MATERIALS AND METHODS

C. elegans culture and transgenic animals

Worms were cultivated on nematode growth medium (NGM) plates, seeded with *E. coli* OP50-1 strain (1). Generation of transgenic animals was performed by microinjection of plasmid DNA, at varying concentrations (2). For transgenic strains ZX1917, ZX1918 and ZX1958, 3 ng/µL of the respective voltage sensor DNA was injected into the gonads of mother animals. For strains ZX1920, ZX1953, ZX2476, ZX2479 and ZX2483, 10 ng/µL, for ZX2560, 30 ng/µL and for ZX2477, 40 ng/µL of the respective voltage sensor plasmids were used in combination with either 1.5 ng/µL of the co-expression marker p*myo-2*::CFP or 30 ng/µL of p*elt-2*::GFP or p*myo-3*::CFP. For ZX2483 50 ng/µL of p*unc-17*::ACR2::eYFP, and for ZX2543, 100 ng/µL of p*tdc-1s*::jRCaMP1b-opti were injected.

Transgenic C. elegans *strains*

The following strains were used or generated: **DA952:** *egl-19(n582ad952),* **MT6129**: *egl-19(n2368)*, **ZX1907**: *zxEx922[pmyo2::Arch::2xMyc; pmyo-3::CFP]*, **ZX1917**: *zxEx942[pmyo-2::QuasAr::mOrange; pmyo-3::CFP]*, **ZX1918**: *zxEx943[pmyo-2::MacQ::mCitrine; pmyo-3::mCherry],* **ZX1920:** *zxIs121[pmyo-3::QuasAr::mOrange; pmyo-3::CFP]*, **ZX1951**: *egl-19(n2368); zxEx942[pmyo-2::QuasAr::mOrange; pmyo-3::CFP]*, **ZX1952**: *egl-19(n2368); zxEx942[pmyo-2::MacQ-mCitrine; pmyo-3::mCherry]*, **ZX1953:** *zxIs120[pmyo-3::ArchD95N::2xmyc-tag; pmyo-3::CFP]*, **ZX1954:** *zxIs6; zxIs120[pmyo-3::ArchD95N::2xmyc-tag; pmyo-3::CFP]*, **ZX1955**: *egl-19(n582ad952); zxEx942[pmyo-2::QuasAr::mOrange; pmyo-3::CFP]*, **ZX1956**: *egl-19(n582ad952); zxEx942[pmyo-2::MacQ-mCitrine; pmyo-3::mCherry]*, **ZX1958**: *zxEx944[pmyo-2::ArchD95N::2xmyc-tag; pmyo3::CFP]*, **ZX1959**: *egl-19(n582ad952); zxEx944[pmyo-2::ArchD95N; pmyo-3::CFP]*, **ZX1960**: *zxIs5[punc-17::ChR2(H134R); lin-15+]; zxIs121[pmyo-3::QuasAr::mOrange; pmyo-3::CFP]*, **ZX2476**: *zxEx1139[pmyo-3::QuasAr; pmyo-2::CFP]*, **ZX2477**: *zxEx1140[punc-17::QuasAr; pmyo-3::CFP]*, **ZX2479**: *zxIs5[punc-17::ChR2(H134R); lin-15+]; zxEx1142[pmyo-3::Archon::GFP; pmyo-2::CFP]*, **ZX2482**: *zxIs5[punc-17::ChR2(H134R); lin-15+]; zxEx1145[pmyo-3::QuasAr; pmyo-2::CFP]*, **ZX2483**: *zxEx1146[punc-17::ACR2::eYFP; pmyo-3::QuasAr; pelt-2::GFP]*, **ZX2534**: *lite-1(ce314); zxEx1169[ptdc-1s::jRCaMP1b-opti; pggr-2::loxP::ChR2::mCherry::bc::GFP; pggr-1::Cre]*, **ZX2560**: *zxEx1203[ptdc-1s::QuasAr::GFP; pelt-2::GFP].*

Molecular biology

Plasmid **pAB4** (*punc-17::ACR2::eYFP*) was described earlier (3). **pAB16** (p*myo-3*::QuasAr) was generated via Gibson Assembly based on p*myo-3*::QuasAr::mOrange and pPD96.52 (p*myo-3*, Addgene plasmid #1608), using restriction enzymes *Bam*HI and *Xba*I and primers QUASAR_fwd (5'-cccacgaccactagatccatATGGTAAGTATCGCTCTGCAG-3') and QUASAR_rev (5'-gtcctttggccaatcccgggCTCGGTGTCGCCCAGAATAG-3'). **pAB19** (p*myo-3*::Archon::GFP) was generated via subcloning from *rig-3*::wArchon1::GFP (a gift from Steven Flavell) into pPD96.52, using restriction enzymes *Kpn*I und *Sac*I. **pNH13** (p*myo-2*::QuasAr::mOrange) and **pNH12** (p*myo-2*::MacQ::mCitrine) were generated by subcloning of plasmids #59173 and #48762 (Addgene) into pPD132.102 (*pmyo-2*, #1662, Addgene) via restriction with *Bam*HI and *Eco*RI. Site-directed mutagenesis of plasmid pNH10 (p*myo-2*::Arch::2xMycTag) resulted in plasmid **pNH11** (p*myo-2*::Arch(D95N)::2xMycTag), using primers oNH7 (5´TATGCCAGGTACGCC

AACTGGCTGTTTACCACC3´) and oNH8 (5´-GGTGGTAAACAGCCAGTCGGCGTACCTG GCATA-3´). **pAB17** (p*unc-17*::QuasAr) was generated via Gibson assembly based on p*myo-3*::QuasAr (pAB16) and RM#348p (p*unc-17*, a gift from Jim Rand), using NheI restriction and primers punc17_QuasAr_fwd (5'-attttcaggaggacccttggATGGTAAGTATCGCTCTGCAG-3') and punc17_QuasAr_rev (5'-ataccatggtaccgtcgacgCTCGGTGTCGCCCAGAATAG-3'). **pAB23** (p*tdc-1s*::QuasAr::GFP) was generated via Gibson assembly based on p*tdc-1s*::GFP and p*myo-3*::QuasAr (pAB16), using AgeI restriction and primers p*tdc-1s*_QuasAr_GFP_fwd (5'-cattttttcaggaccgcccaaatggtaagtatcgctctgcagg-3') and ptdc-1s_QuasAr_GFP_rev (5'-tcctttactca ttttttctagctcggtgtcgcccagaa-3'). **pXY10** (p*tdc-1s*::jRCaMP1b-opti) was generated by subcloning based on pXY09 (p*unc-17*::jRCaMPb-opti), cut with SphI & NheI (blunted) and pXY07 cut with SphI & AgeI (blunted). **pXY09** was generated from RM#348p (p*unc-17*; a gift from J. Rand), cut with KpnI & EcoRV, and insertion of a synthesized, codon-optimized version of jRCaMP1b (4). **pXY07** (ptdc-1s::GFP): The ptdc-1 promoter was amplified using primers oM010 5`-TCATgcatgcATTTCTGTATGAGCCGCCCG-3' & oM015 5`-TTGGACCGGTTGGGCGGTC CTGAAAAATGC-3' and subcloned with SphI & AgeI cuts.

Voltage and Ca^{2+} imaging of immobilized animals

One day prior to the experiments, transgenic animals at L4 stage were transferred onto NGM plates seeded with OP50-1 from bacterial suspensions supplemented with ATR (Sigma-Aldrich, USA) or the respective ATR-analogues DMAR or VI (final ATR concentration: 0.01 mM for experiments with ChR2 or ACR2 blue-light stimulation, 1 mM for imaging experiments or Arch(D95N) in the pharynx, and 0.1 mM for imaging eFRET sensors); note that for analog VI we used Arch wt, and an HBO lamp (30 mW/mm² excitation at 620 nm). DMAR could be visualized well with HBO illumination, however, APs were observed clearly only with 637 nm laser excitation, 180 mW/mm². For imaging, worms were placed onto 10 % agarose pads (M9 buffer) on microscope slides and immobilized with polystyrene beads (0.1 µm diameter, at 2.5% w/v, Sigma-Aldrich). Imaging was performed on an inverted microscope (Zeiss Axio Observer Z1), equipped with 40x oil immersion objective (Zeiss EC Plan-NEOFLUAR 40x/ N.A. 1.3, Oil DIC ∞ / 0.17), a 470 nm LED light source (KSL 70, Rapp OptoElectronic, Hamburg, Germany) or monochromator (PolychromeV, Till Photonics) for photostimulation, a 637 nm laser (OBIS FP 637LX, Coherent) for excitation of voltage sensors (1.8 W/mm^2 for imaging of QuasAr and Archon in BWMs and 180 mW/mm² for PM measurements or for imaging Arch(D95N)-DMAR in BWMs), a galilean beam expander (BE02-05-A, Thorlabs), and an EMCCD Camera (Evolve 512 Delta, Photometrics). Voltage sensor fluorescence was imaged at 700 or 747 nm emission (700/75 ET Bandpass filter, integrated in Cy5 filter cube, AHF Analysentechnik) for ATR, and at 780 nm (780 LP ET Longpass Filter) for DMAR. eFRET sensors were imaged with 545/30 or 472/30 nm excitation (HBO100 100 W mercury lamp, Zeiss, at 30 mW/mm² for 545/30 nm and 10 mW/mm² for 472/30 nm) and 610/75 or 520/35 nm emission (472/30 ET Bandpass, 610/75 ET Bandpass and 520/35 ET Bandpass Filters, AHF Analysentechnik), respectively. Stimulation of optogenetic actuators (ChR2 or ACR2) was performed with 300 µW/mm^2 at 470 nm. Acquisition times, frame rates and gain used for imaging rhodopsin voltage sensors were 1 or 2 ms, 158 or 190 fps and gain 1 (for DMAR) or gain 10 or 50 (for ATR). For eFRET sensors, it was 1 ms exposure, 190 fps and gain 10, or as stated.

To induce pumping under imaging conditions, animals were incubated in 3 µl serotonin (20 mM, in M9 buffer) for 3 min prior to the experiments. For drug assays, animals were exposed to 30 nM nemadipine A in 0.2 % DMSO in M9, or to the vehicle control.
For imaging neurons, animals were cultivated in the presence of 0.1 mM ATR, immobilized in polystyrene beads, and excited by 1.8 W/mm² 637 nm laser light. Exposure time was 5 ms, binning was 4x4, a 700/75 ET bandpass filter, integrated in Cy5 filter cube, was used to acquire images on an EMCCD camera (Evolve 512 Delta).
For imaging Ca^{2+} via jRCaM1b, a 590 nm LED (KSL 70, Rapp OptoElectronic, Hamburg, Germany; 0.6 mW/mm^2) and an EMCCD Camera (Evolve 512 Delta; EM-gain: 50) were used, as well as these filters: GFP/mCherry Dualband ET Filter was combined with a 647/57 nm emission filter and a 605 nm longpass beamsplitter (F56-019, F37-647 and F38-605, respectively, all AHF Analysentechnik, Germany). RCaMP movies were acquired at 70 ms exposure, ca. 14 fps.

Processing and automated data analysis of pharyngeal voltage and pump events
Movie acquisition (with frame rates of 160 or ca. 190 fps, at 1 ms exposure time) was performed by using the *Photometrics PVCAM Device Adapter for µManager* (5). For BWM experiments, raw image sequences were analyzed via ROI selection of signal and background and *multi-measure* function in ImageJ (National Institutes of Health, USA; https://imagej.nih.gov/ij/index.html). Changes in fluorescence were calculated as $\Delta F/F_0$, or $\Delta F/F_{mean}$, where F_{mean} (or F, for simplicity) is the mean fluorescence (F_{mean}) of the ROI, calculated for the entire imaging period. Contrast was calculated as the ratio between mean fluorescence of a ROI across the pharyngeal or BWM tissue expressing the voltage sensor, and the mean fluorescence of a ROI within the animal, but chosen in tissue not expressing sensors and showing no autofluorescence. Signal to noise ratio (SNR) was calculated as the ratio of the mean fluorescence of all peaks (= APs) observed during a pump train, and the standard deviation of the fluorescence in the ROI during resting phases.
To register voltage signals in the PMs, a ROI was defined around the whole pharynx, whereas a smaller circular ROI was positioned over the terminal bulb lumen with the grinder, to track contractions via the accompanying lumen opening (lowering of the fluorescence in this ROI, as fluorescing tissue retracts radially). For background correction, another ROI was defined in an arbitrary dark region outside the worm, or, for contrast measurements, 'inside' the worm, but avoiding the gut or tissue expressing the respective voltage sensor. ΔF/F was calculated in Excel (Microsoft). A custom workflow in KNIME 3.6.2 (KNIME AG, Zurich, Switzerland (6)) was used to synchronize pump and voltage events across animals by calling an R (R 3.5.1, The R Foundation for Statistical Computing) script to process each of the Excel tables (the respective script will be provided on a publicly available server; see main manuscript). The R script (included in the KNIME workflow) proceeds to fit a spline curve to the input data with as many anchor points as data points present. The fit curve is sampled at constant 200 data points per second to account for the variable camera timing. Pumps are found as local minima with a centered time window of 625 ms. A manual input of indices can be used to correct this peak registration. The signals that passed manual control are synchronized first to the pump minimum. The voltage signal is then analyzed for the peak onset by searching for the minimum of the scaled difference with a lag of 50 ms of a centered moving average with window size of 105 ms. The events are subsequently synchronized to the onset of the voltage peak and grouped per animal, or

analyzed individually, where appropriate. The signals are further analyzed to extract start and end of peak by searching for the first and last data point to cross a line at 10 % of the distance from the baseline to the peak minimum, respectively. Analogously, the full width at half maximum (FWHM) is calculated by searching for the two data points that cross the 50 % line between baseline and peak minimum. Duration is defined as the time difference between start and end of a peak. The kinetic parameters τ_{ON} and τ_{OFF} are modelled each with a mono-exponential curve fit accounting only for the time frames from (-50 ms, $FWHM_{start}$) and ($FWHM_{end}$, 305 ms), respectively. The area (voltage integral) is calculated as the integral from start to end of the peak from the baseline. The amplitude is calculated as the peak minimum to the maximum value at negative time after synchronization in the depicted time frame. The delay is reported as the time difference between the $FWHM_{start}$ of the voltage and $FWHM_{start}$ of the pump peaks.

Electrophysiology

Recordings were conducted from dissected BWM cells on the ventral side of the body anterior to the vulva as described earlier (1). Animals were immobilized with Histoacryl glue (B. Braun Surgical, Spain) and a lateral incision was made to access neuromuscular junctions along the ventral nerve cord. The basement membrane overlying muscles was removed by incubation in 0.5 mg/ml collagenase for 10 s (C5138, Sigma-Aldrich, Germany). BWMs were patch-clamped in whole-cell mode at 22˚C using an EPC10 amplifier with head stage connected to a standard HEKA pipette holder for fire-polished borosilicate pipettes (1B100F-4, WPI, USA) of 4-7 MΩ resistance. The extracellular (bath) solution contained: NaCl 150 mM; KCl 5 mM; $CaCl_2$ 5 mM; $MgCl_2$ 1 mM; glucose 10 mM; sucrose 5 mM; HEPES 15 mM, pH7.3 with NaOH, ≈330 mOsm. The pipette solution contained: K-gluconate 115 mM; KCl 25 mM; $CaCl_2$ 0.1 mM; $MgCl_2$ 5 mM; BAPTA 1 mM; Hepes 10 mM; Na_2ATP 5 mM; Na_2GTP 0.5 mM; cAMP 0.5 mM; cGMP 0.5 mM, pH7.2 with KOH, ≈320mOsm. Current clamp recordings were conducted using Patchmaster software (HEKA, Germany) (3). Light activation was performed using an LED (KSL-70, Rapp OptoElectronic, Hamburg, Germany; 470 nm, 1 mW/mm²) and controlled by the EPC10 amplifier. Data were analyzed by Patchmaster software.

For electropharyngeogram (EPG) recordings, L4 animals were placed for ca. 24 h on NGM plates seeded with OP50 and supplemented either with or without ATR; for PM intracellular recordings, this time was extended to 2-3 days. Animals were transferred into a recording chamber containing a Sylgard-coated coverslip and filled with 1.5 mL of EmD50 buffer (NaCl, 140 mM; KCl, 3 mM; $CaCl_2$, 3 mM; $MgCl_2$, 1 mM; Hepes, 10 mM; D-Mannitol, 50 mM; pH 7.3 adjusted with NaOH) containing 2-20 µM 5 Hydroxytryptamin to stimulate pumping. Cut-head preparation and EPG recording of strain ZX1918 were performed as described previously (7). Intracellular recordings were performed following a previously described protocol (8), also for the manufacturing of intracellular electrodes and the positioning of the holding and the intracellular electrodes. For combined voltage imaging an EMCCD Camera (Evolve 512 Delta) was used. Data were analyzed by OriginPro.

Software and statistics

Knime 3.6.2 (KNIME AG, Zurich, CH), R 3.5.1 (The R Foundation for Statistical Computing).
Statistics used were ANOVA with Bonferroni correction, after verification of normal distribution of the data

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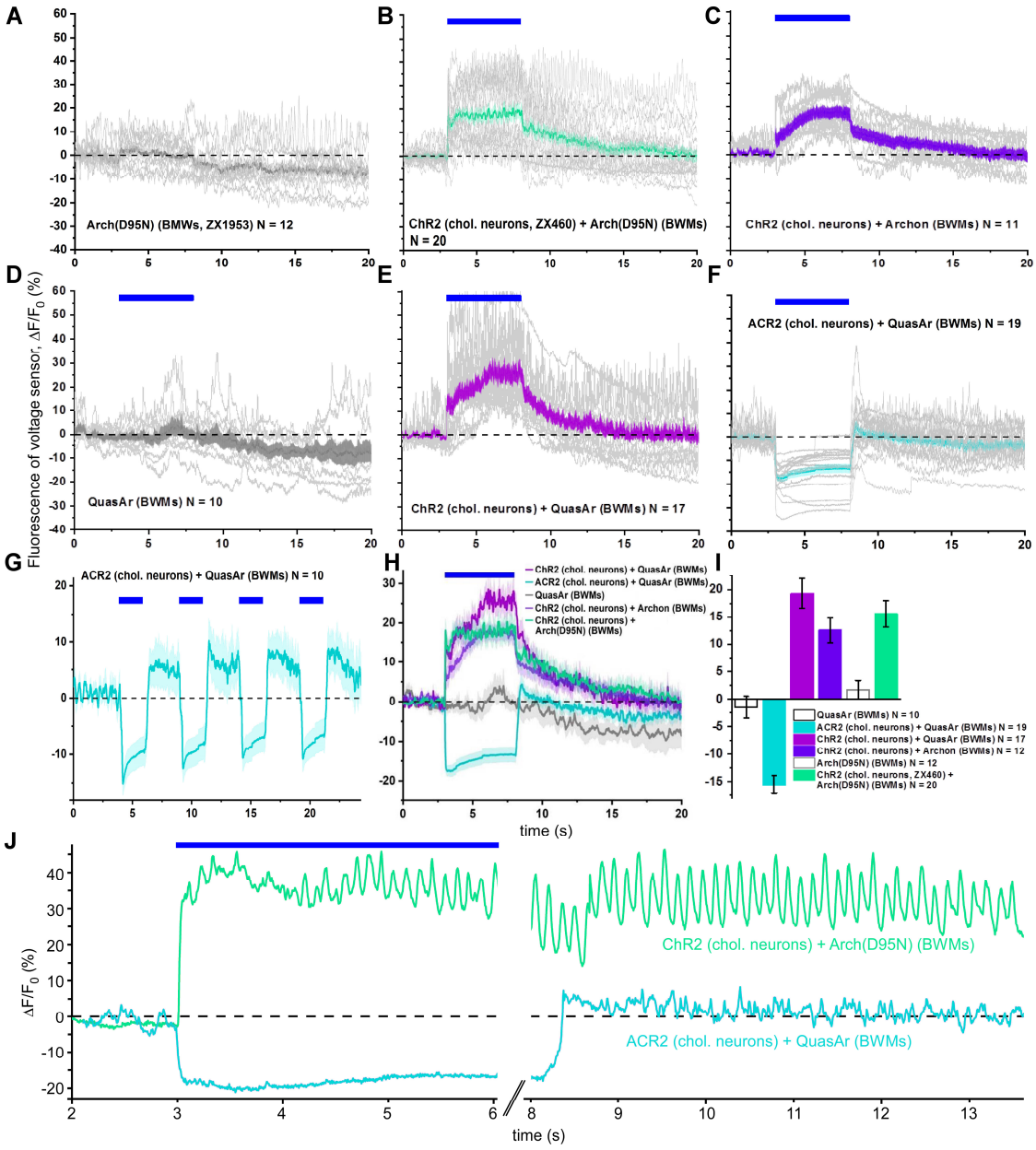

Fig. S1. Summary of individual and mean voltage fluorescence traces for various rhodopsin voltage sensors expressed in BWMs. Related to Figs. 1 and 3. A) Arch(D95N)-ATR, spontaneous activity. **B)** Arch(D95N)-ATR with ChR2 expressed in cholinergic neurons. **C)** Archon with ChR2 expressed in cholinergic neurons. **D)** QuasAr, spontaneous activity. **E)** QuasAr with ChR2 expressed in cholinergic neurons. **F, G)** QuasAr with ACR2 expressed in cholinergic neurons. **H)** Summary of mean fluorescence changes in (B, C, E, F). Blue bars in (A-H) indicate photo-stimulation periods. **I)** Statistical analysis of the mean±SEM fluorescence changes of data shown in H, averaged across the 5 s stimulation periods. **J)** Single traces of Arch(D95N) and QuasAr expressed in BWMs, before during (blue bar) and after photoactivation of ChR2 or ACR2 in cholinergic neurons, as indicated.

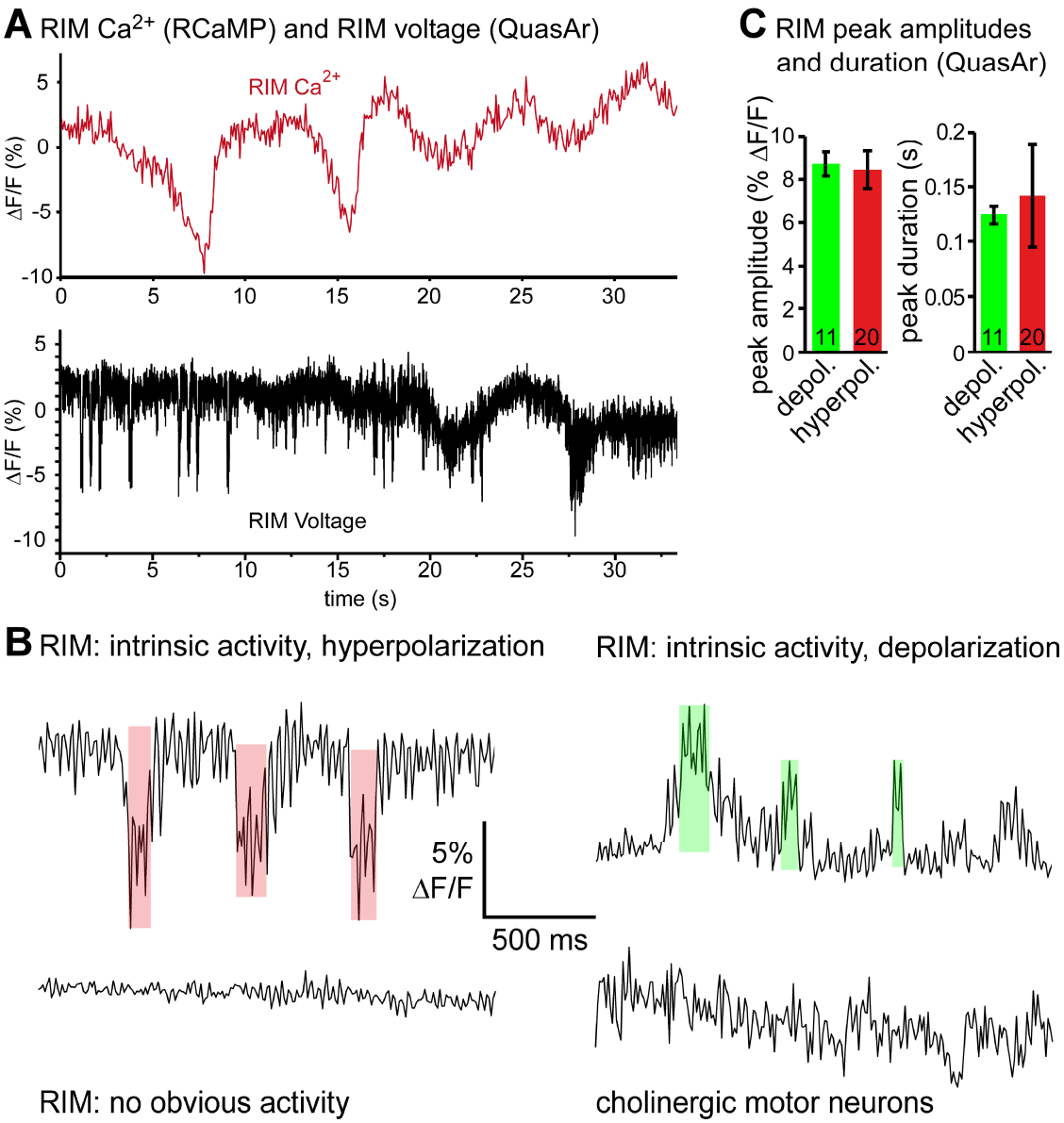

Fig. S2. Neuronal activity can be observed by Ca^{2+}- and QuasAr voltage imaging: A) Comparison of activity in RIM, measured by jRCaMP1b Ca^{2+} imaging (red, upper panel) as well as by QuasAr (black, lower panel). Similar slow bouts of hyperpolarizing activity can be observed. **B)** RIM::QuasAr voltage traces show brief de- (green) and hyperpolarizing (red) spikes. **C)** Statistical analysis of representative de- and hyperpolarizing peaks in RIM::QuasAr recordings, as demarcated in B). Peak amplitude and peak durations, means±SEM, n, number of animals, is indicated.Type or paste caption here. Paste figure above the caption.

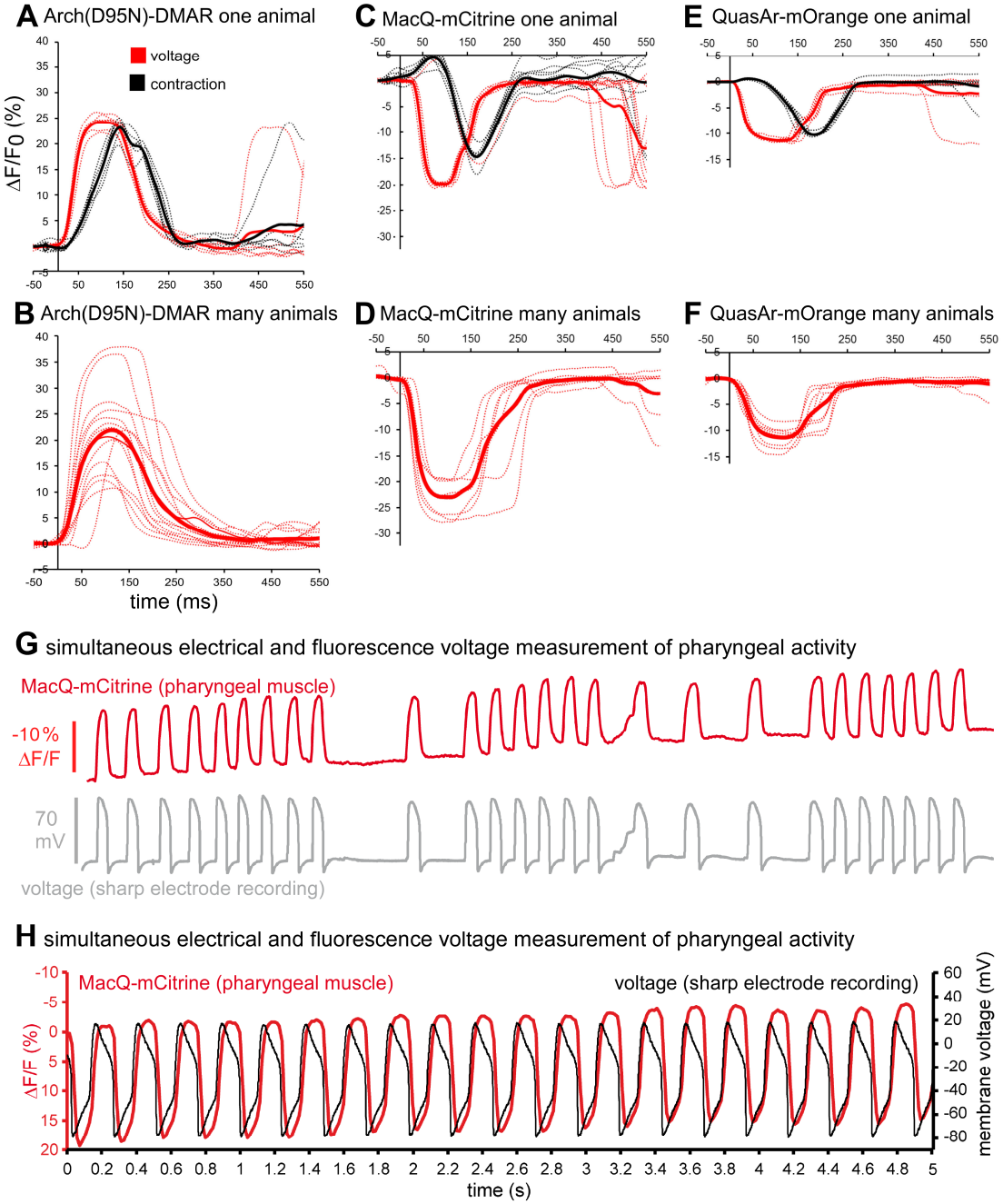

Fig. S3. Comparison of voltage traces recorded using rhodopsin and eFRET voltage sensors, as well as by sharp electrodes in the pharynx during pump trains in single animals, and across animals. Related to Fig. 4. A) Arch(D95N)-DMAR, voltage and pump fluorescence signals of a single animal, aligned and averaged. Red: voltage trace; black: pump trace. **B)** As in (A), but mean pump events of 11 animals. **C, D)** Single animal events measured with MacQ-mCitrine in the pharynx (C) and mean events observed in 7 animals (D). **E, F)** As in (C, D), but using QuasAr-mOrange (n=7 animals in F). **G, H)** Dissected pharynx, impaled by sharp electrode, allows for membrane potential recording (grey and black traces, in G, H, respectively) and simultaneous optical recording using MacQ-mCitrine (red traces in G, H).

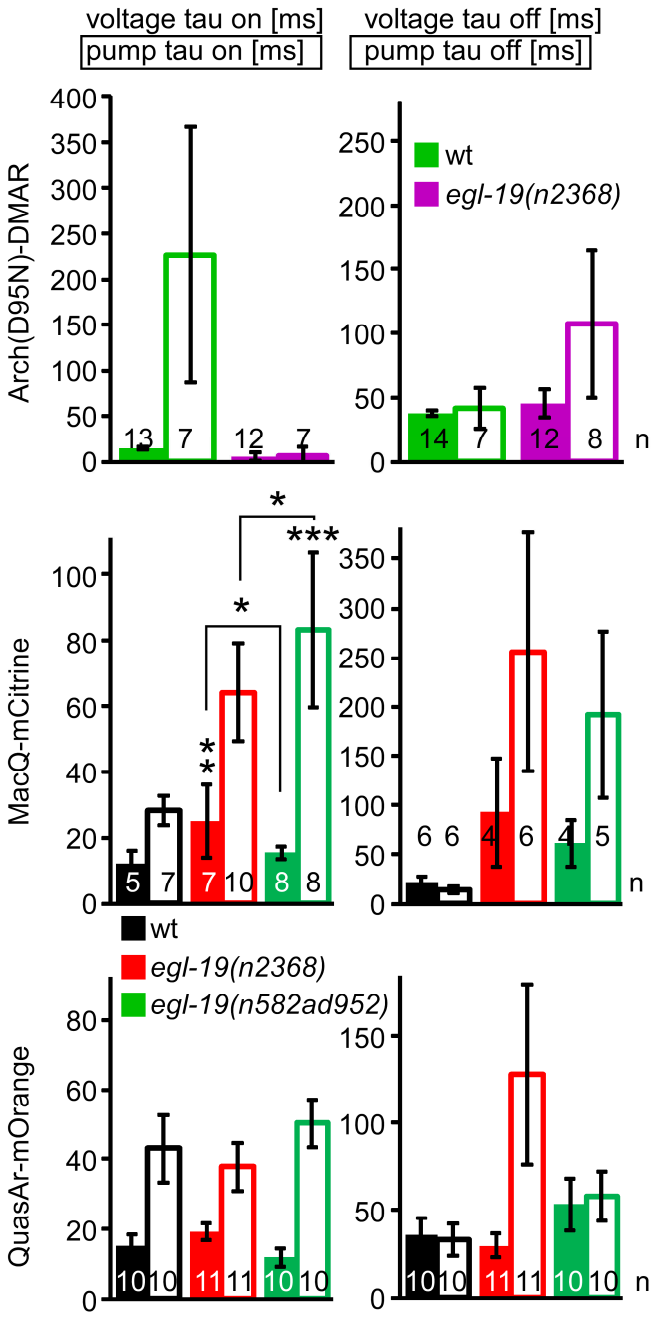

Fig. S4 Voltage and pump signal rise and decay times, automatically determined for pharyngeal APs and pump events measured with rhodopsin and eFRET sensors. Related to Fig. 5. Parameters, genotypes and number of animals as indicated, measured for animals expressing Arch(D95N), equipped with DMAR (top), MacQ-mCitrine (middle) and QuasAr-mOrange (lower panels). Means±SEM, ANOVA with Bonferroni correction, ***P≤0.001, **P≤0.01, *P≤0.05.

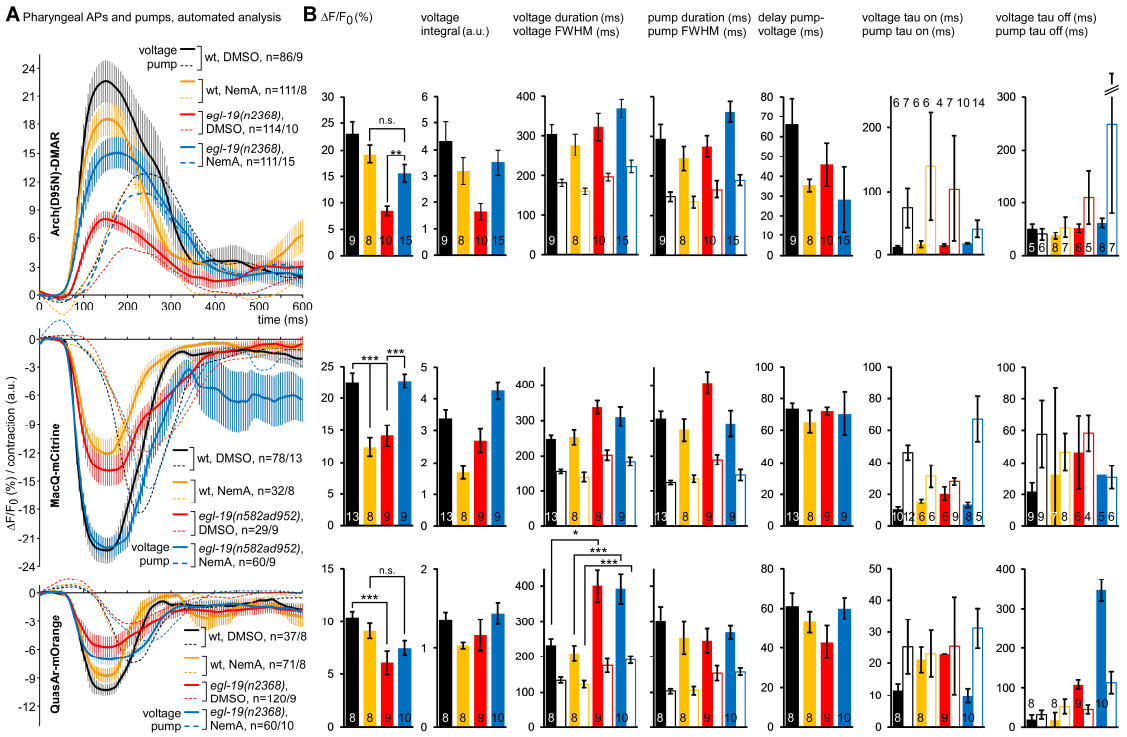

Fig. S5. Comparison of voltage and pump parameters measured in mutants and in response to nemadipine-A (NemA) in the pharynx. A) Mean±SEM values for the indicated genotypes and drug or vehicle treatments, as well as different rhodopsin voltage sensors, as indicated. Also indicated is the number of animals analyzed, as well as the number of APs and pump events. **B)** Group data of automated analysis of parameters extracted from the data shown in (A), as in Fig. 5. Frame rate in (A): 189 fps, 1 ms exposure. Means±SEM, ANOVA with Bonferroni correction, ***P≤0.001, **P≤0.01, *P≤0.05.

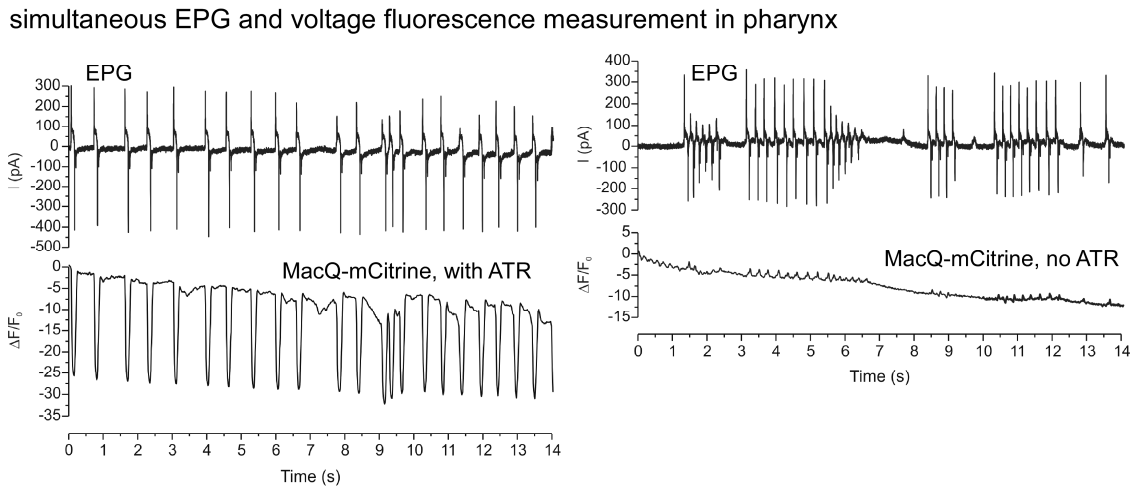

Fig. S6. Comparison of simultaneous EPG and MacQ-mCitrine fluorescence measurements in presence and absence of ATR. EPG traces (upper panels, measured as currents) and simultaneous MacQ-mCitrine voltage dependent fluorescence changes ($\Delta F/F_0$, lower panels) in the presence (left panels) or absence of ATR (right panels).

Movie S1. QuasAr-mOrange in pharynx, spontaneous pumping.

Movie S2. QuasAr in muscle arms, dorso-ventrally alternating intrinsic activity.

Movie S3. QuasAr in BWMs, intrinsic activity.

Movie S4. QuasAr in BWMs, intrinsic activity.

Movie S5. Arch(D95N)-ATR in pharynx, spontaneous pumping.

Movie S6. Arch(D95N)-DMAR in pharynx, spontaneous pumping.

Movie S7. MacQ-mCitrine in pharynx, spontaneous pumping.

Movie S8. MacQ-mCitrine in pharynx, spontaneous pumping, difference movie, 12 events aligned, averaged, 10x slowed.

Supplementary Dataset S1 (separate file)

Collected raw fluorescence traces (ROIs) and electrophysiology data of experiments underlying the data in the paper, Excel sheets, sorted by figure number and panel.