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Supplemental Information

cAMP Signals in *Drosophila* Motor Neurons Are Confined to Single Synaptic Boutons

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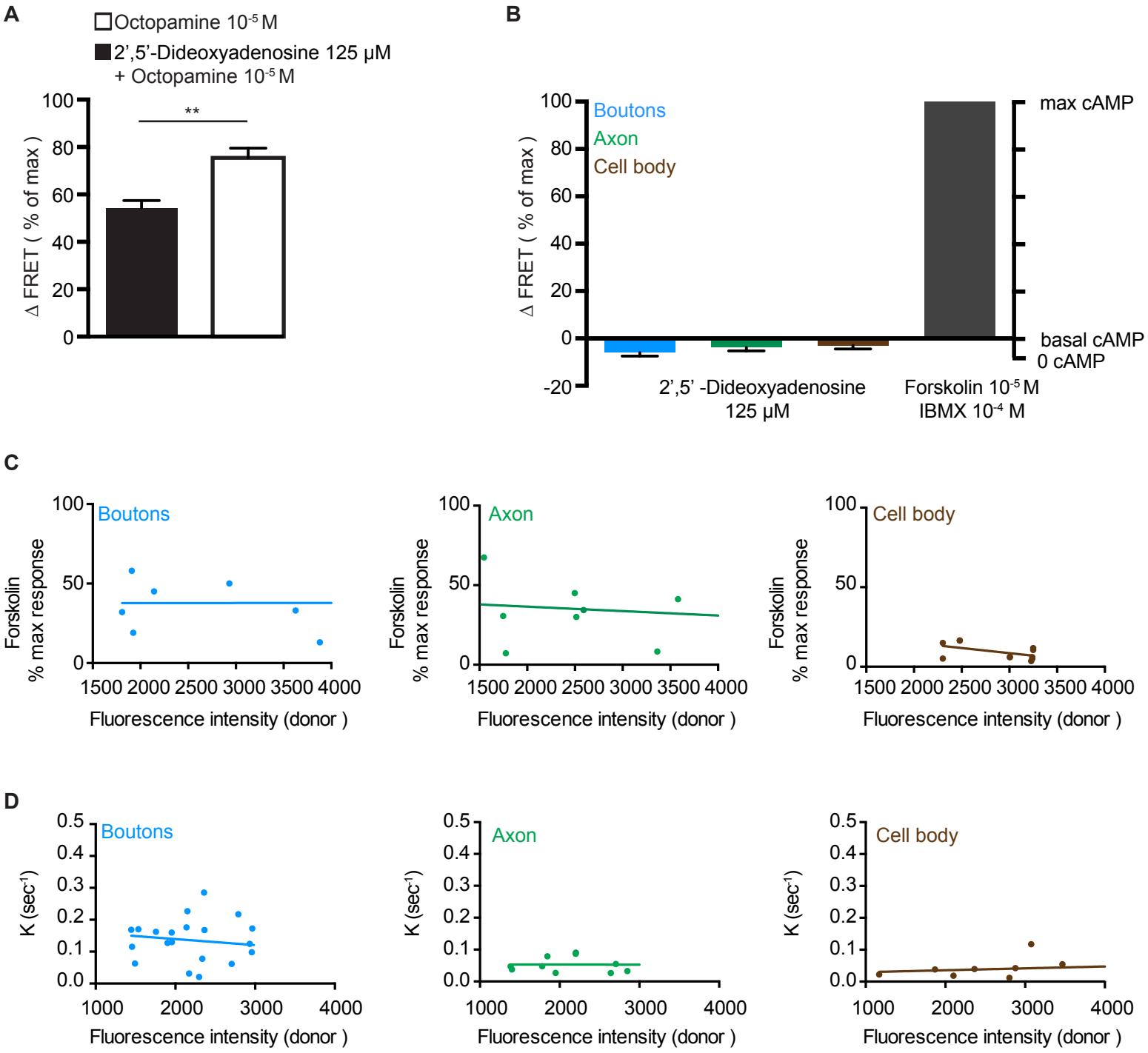

Figure S1 Related to Figure 1

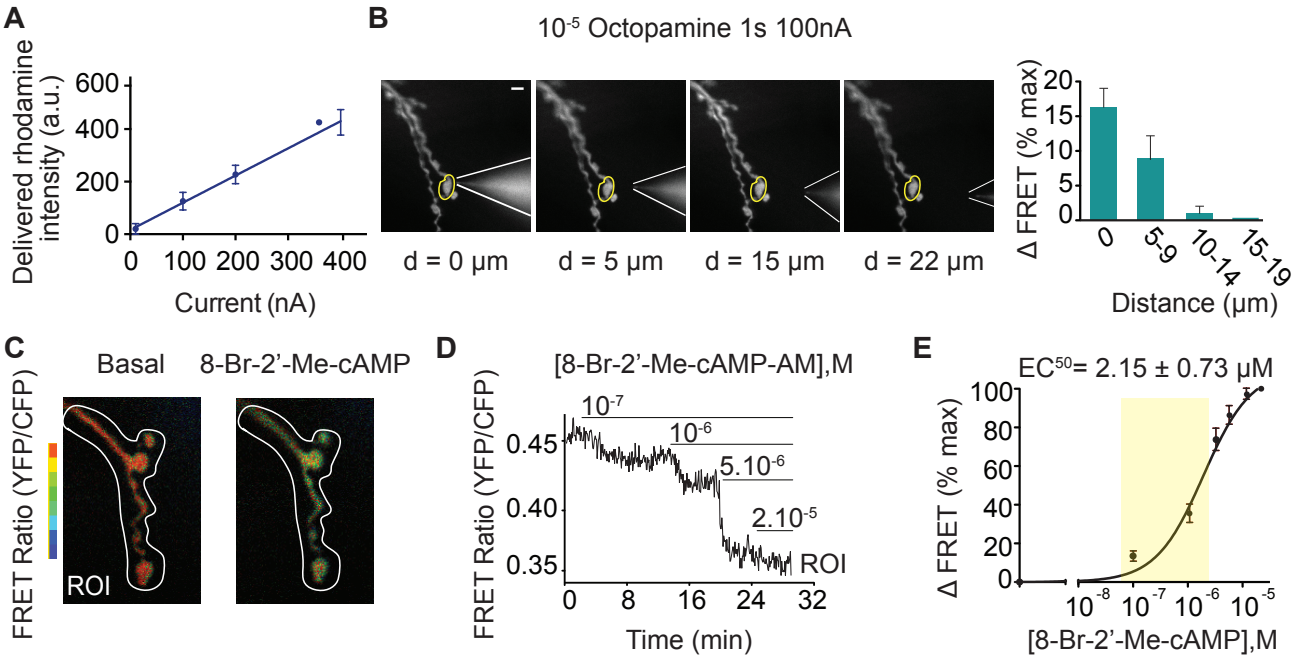

Figure S2 Related to Figure 2

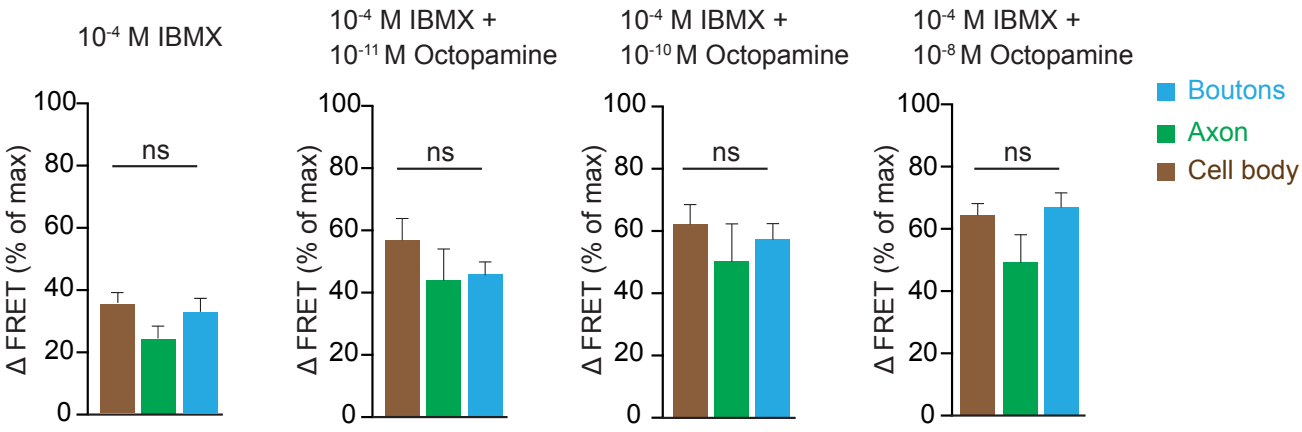

Figure S3 Related to Figure 3

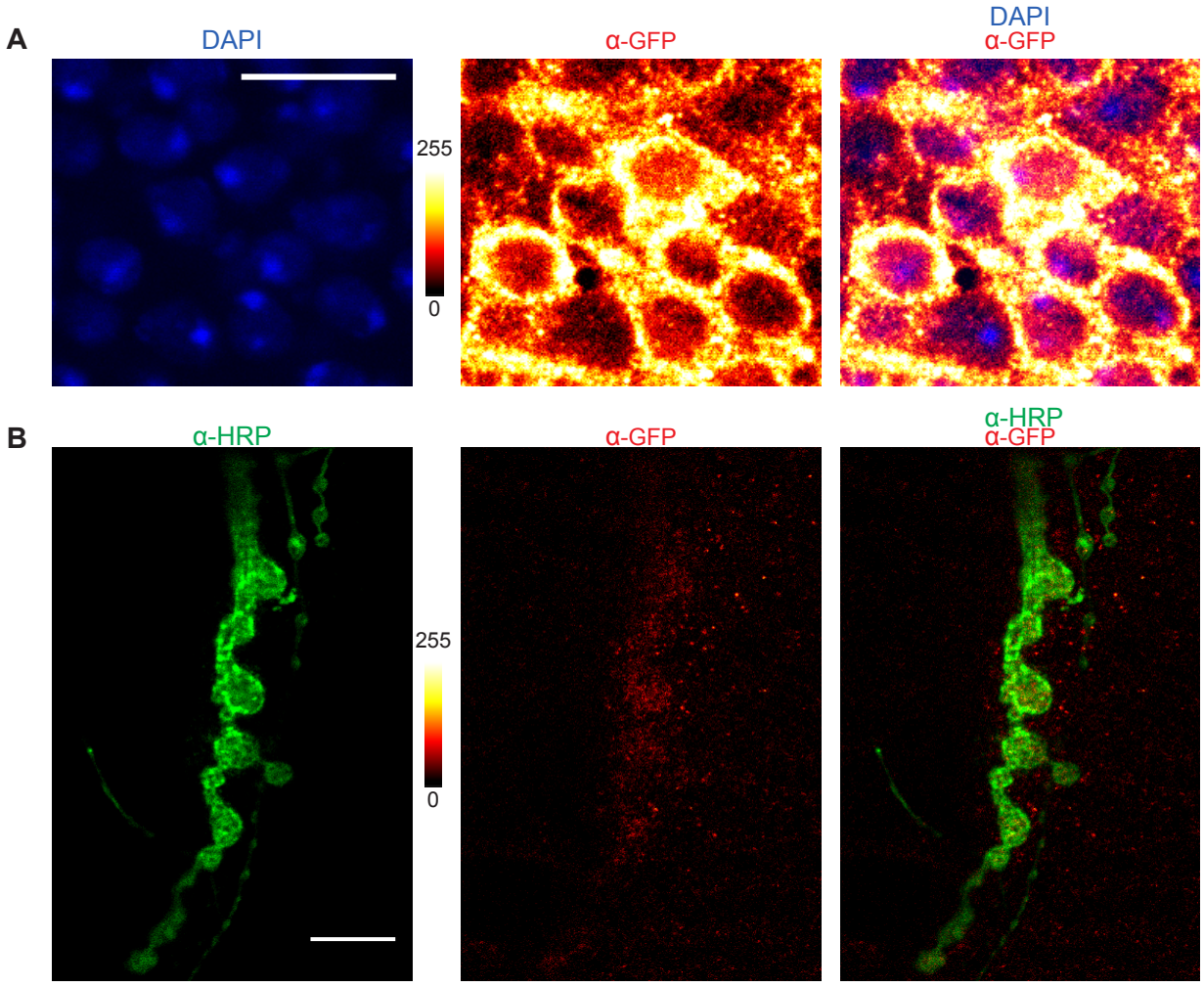

Figure S4 Related to Figure 3

Supplemental Information

Figure S1 Characterization of the transgenically expressed Epac1-camps sensor in *Drosophila* motor neurons, Related to Figure 1

(A) Efficacy of the adenylyl cyclase inhibitor 2',5'-dideoxyadenosine in *Drosophila* larvae. Motor neurons expressing the Epac1-camps sensors were preincubated with 2',5'-dideoxyadenosine and stimulated with octopamine. ΔFRET values were calculated as described in **Figure 1C**. Data are shown as mean ± s.e.m. **, P<0.01 by Student´s t-test. (n = 8).

(B) Similar basal cAMP levels in boutons, axon and cell body. *Drosophila* motor neurons were stimulated with 2',5'-dideoxyadenosine. ΔFRET values were calculated as described in **Figure 1C**. Data were not statistically different by one-way ANOVA. (n = 8).

(C) Intracellular biosensor concentration does not affect the amplitude of the cAMP response. *Drosophila* motor neurons expressing the Epac1-camps sensors were stimulated with 10^{-6} M forskolin. The amplitude of the response to forskolin was quantified in different regions of the motor neuron (boutons, axon, cell body) as a percentage of the maximal forskolin + IBMX response and plotted against fluorescence intensity. Data are shown as mean ± s.e.m. (n= 7).

(D) Intracellular biosensor concentration does not affect the kinetics of the cAMP response. *Drosophila* motor neurons were stimulated with 10^{-5} M octopamine. The rate constant K of cAMP accumulation was calculated using a non-linear regression (monoexponential phase decay) and plotted against fluorescence intensity. Data are shown as mean ± s.e.m. (n= 10-21).

Figure S2 Iontophoretic delivery of octopamine, Related to Figure 2

(A) Linearity of the iontophoretic ejection. The glass microelectrode was filled with the fluorescent dye rhodamine (10^{-5} M). The amount of dye released upon sequential pulses of 1 s with increasing ejection current was followed by fluorescence microscopy. Data fitted to a linear model. (n = 5).

(B) Range of the delivery of octopamine. Left, YFP images showing the microelectrode gradually moved away from the target bouton. Right, dependency of the FRET changes produced by a test pulse on the distance between pipette and target bouton. ΔFRET values calculated as described in **Figure 1C**. This procedure was repeated at the end of each experiment in Figure 2. (n = 10).

(C) Pseudocolor FRET images (YFP/CFP ratios) of the NMJ on muscle 13, before (basal) and after generalized stimulation with a saturating concentration (10^{-5} M) of the cell-permeable cAMP analog 8-Br-2'-Me-cAMP-AM.

(D) FRET measurement of cAMP changes induced by direct activation of the Epac1-camps sensor with increasing concentrations of 8-Br-2'-Me-cAMP-AM. Shown is a representative trace measured in the region of interest (ROI) depicted in C.
(E) Concentration-response curves obtained from traces like those shown in D, calculated as described in **Figure 1C**. Shaded yellow area represents the cAMP concentration that can activate its downstream target. Scale bar, 5 μm. Data are shown as mean ± s.e.m. (n = 8).

Figure S3 PDE inhibition abolishes the octopamine-induced cAMP gradient, Related to Figure 3
Semi-intact larval preparations were pre-incubated with the broad PDE inhibitor IBMX (10^{-4} M) and subsequently stimulated with different concentrations of octopamine. cAMP changes were monitored in boutons, the distal axon and cell body. ΔFRET values calculated as described in **Figure 1C**. ns, statistically non-significant differences by one-way ANOVA. Data are shown as mean ± s.e.m. (n = 6-10).

Figure S4 Subcellular localization of EGFP tagged PDE dunce, Related to Figure 3
(A) Third instar *Drosophila* larva expressing *dnc-EGFP-dnc* (y^1w^*Mi{PT-GFSTF.2}dnc $^{MI03415\text{-}GFSTF.2}$). Cell bodies stained with DAPI against neuronal membranes (blue) and with anti-GFP to visualize the Dnc-EGFP-Dnc expression pattern (red hot).
(B) Specificity of the α-GFP antibody. Third instar wt (Canton-S) *Drosophila* larva. NMJ on muscle 13 stained with α-HRP against neuronal membranes (green) and with α-GFP. Scale bar, 10 μm.

Movie 1 cAMP gradient at the NMJ, Related to Figure 1
First time derivative analysis of the cAMP changes induced by generalized application of octopamine (10^{-5} M). Frame interval = 5s. Scale bar, 10 μm.

Movie2 cAMP signals confined to single synaptic boutons, Related to Figure 2
First time derivative analysis of the cAMP changes induced by local stimulation of the most distal bouton with octopamine. Frame interval = 300 ms. Scale bar, 10 μm.