



## SI Appendix for

### A fluorescent nanosensor paint detects dopamine release at axonal varicosities with high spatiotemporal resolution

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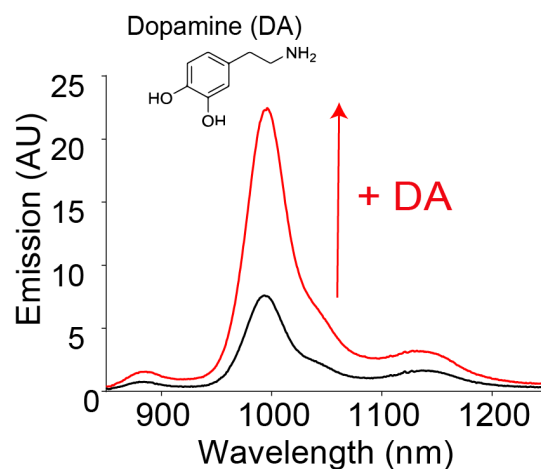
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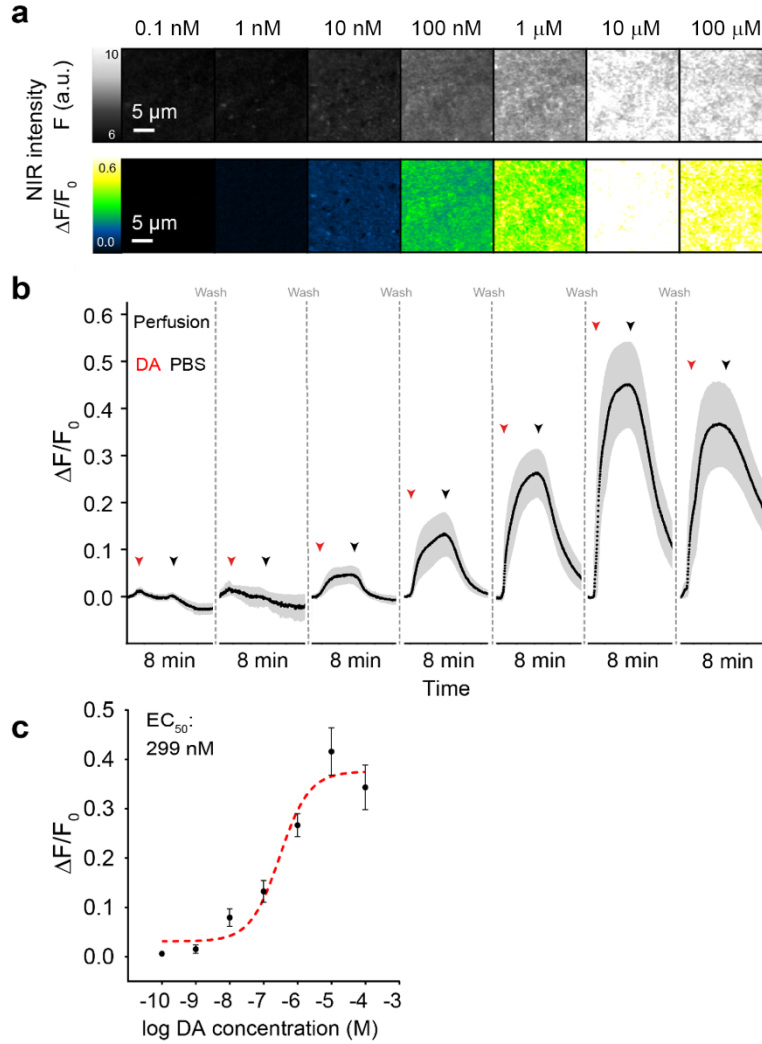
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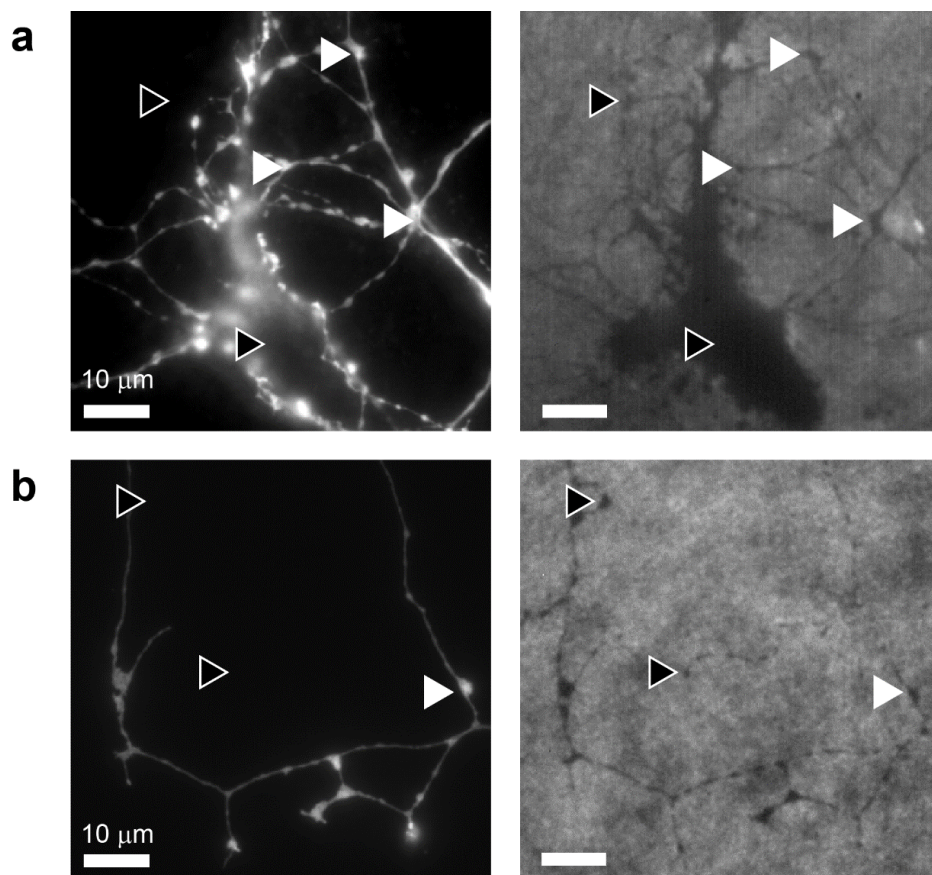
**Fig. S1: SWCNT-(GT)<sub>10</sub> increase their NIR fluorescence in response to dopamine binding**

SWCNT-(GT)<sub>10</sub> nanosensors report dopamine (DA) through fluorescence increase in the near infrared (NIR) (peak at 990 nm). The fluorescence emission spectra of nanosensors are shown before (black trace) and after addition of 100  $\mu$ M DA (red trace). Spectra were obtained with nanosensors dispersed in PBS (not immobilized on glass).



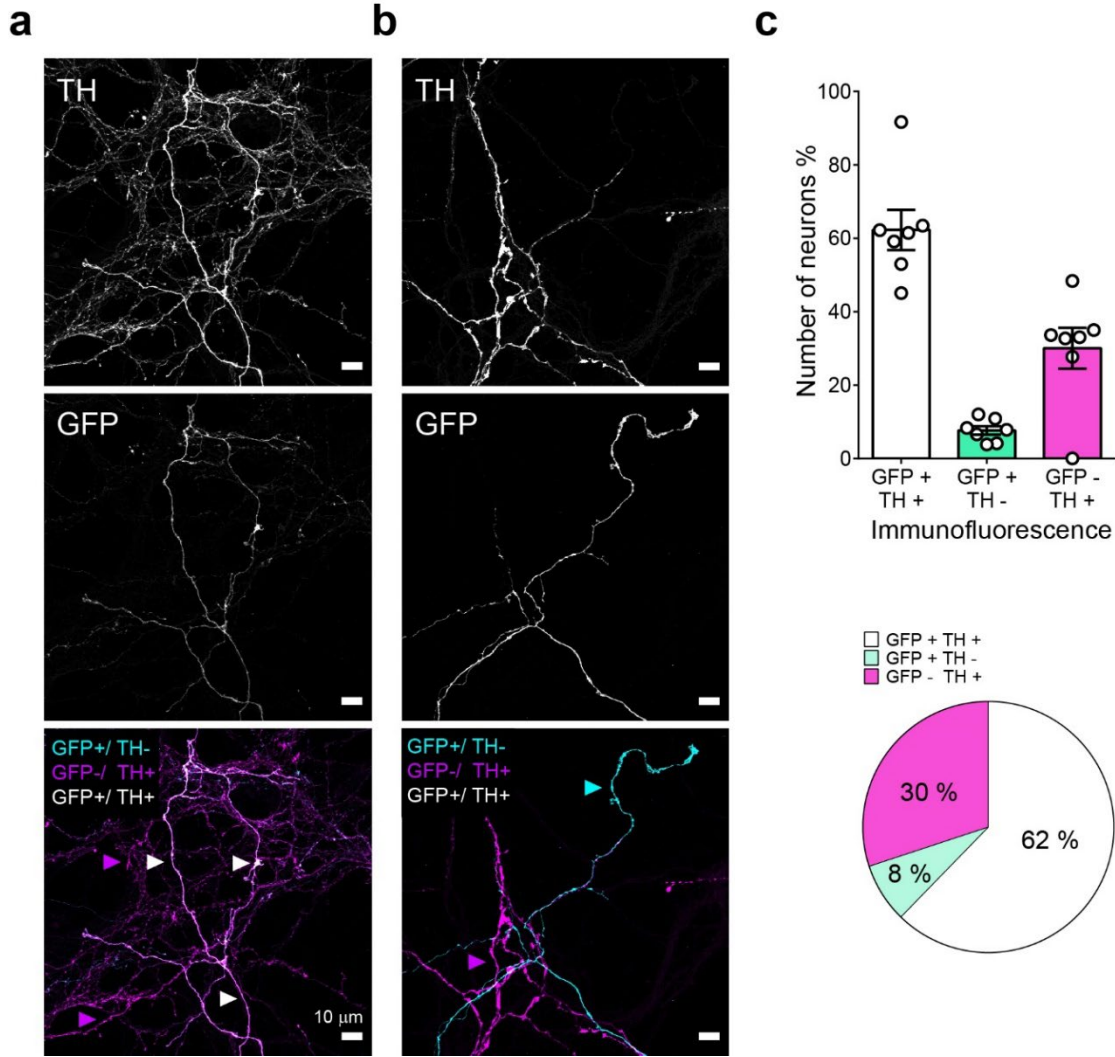
**Fig. S2: Dose-dependency and reversibility of AndromeDA activation by DA**

**a**, Raw (upper) and normalized (lower) images of AndromeDA prepared on glass coverslips during perfusion with increasing DA concentrations (0.1 nM, 1 nM, 10 nM, 100 nM, 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M). **b**, Plots showing normalized average NIR AndromeDA fluorescence over time in response to perfusion with increasing DA concentrations on a single coverslip. For each concentration of DA, DA (in PBS) was applied at the time point shown (red arrowheads), followed by a PBS wash to remove the DA (black arrowheads). Imaging was carried out during DA application and washout for 8 minutes. Imaging was then temporarily stopped (indicated by breaks in the x-axis) and washing continued while the next DA sample was prepared for application. The solid black line represents the mean values of the data over time, while the shaded areas represent the range of the SEM around the mean value.  $n = 3$  independent experiments (nanosensors batches). **c**, Dose-response curve calculated from the mean maximum peak responses of a DA nanosensor coated glass surface to perfusion with DA, demonstrating the dynamic range (10 nM DA – 10  $\mu$ M DA) and the half maximal effective DA concentration ( $EC_{50} = 299$  nM). Individual values represent mean  $\pm$  SEM.



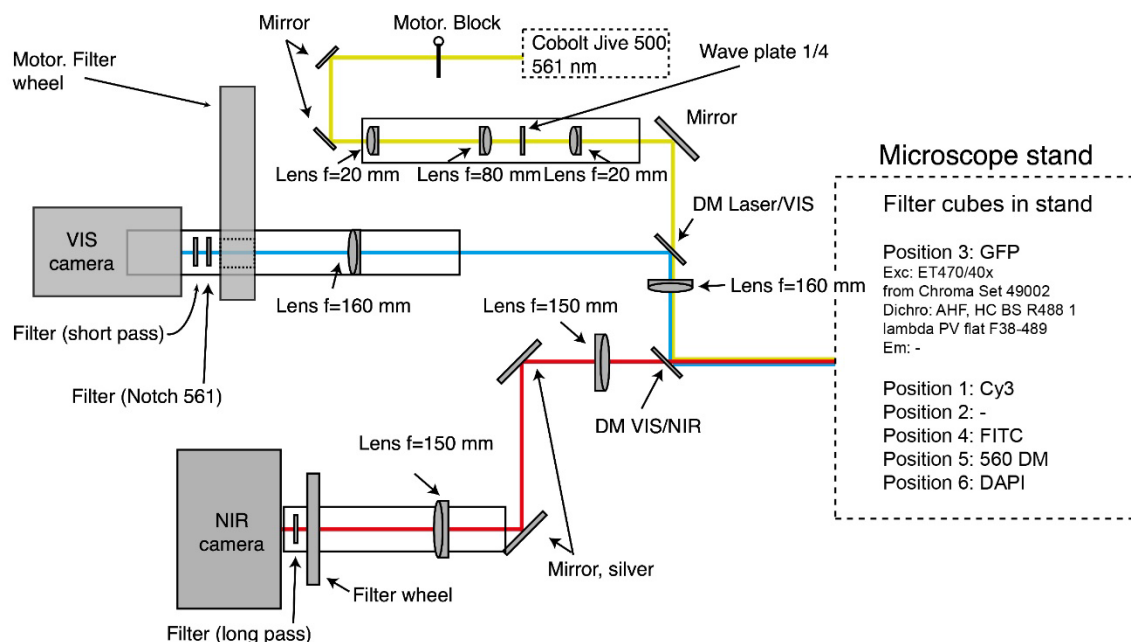
**Fig. S3 – AndromeDA is a painted monolayer of nanosensors that surrounds neuronal structures**

**a** and **b** show representative images of ventral midbrain neuron cultures from TH-EGFP mice on which nanosensors have been painted, creating an AndromeDA surface. Left panels show fluorescence microscope images of EGFP-positive axons, while right panels show corresponding NIR fluorescence microscope images of AndromeDA. AndromeDA appears as regions of NIR fluorescence (grey to white), while darker areas indicate a lack of nanosensors. Dopaminergic neurons form abundant varicosities in culture (examples indicated by white arrowheads), which are also evident in NIR images as dark regions (white arrowheads), indicating that AndromeDA is absent from cellular structures. EGFP-negative cellular structures, presumably formed by non-dopaminergic neurons, are also observed in NIR images (black arrowheads). Overall, these observations indicate that AndromeDA is present on glass surrounding the neurons but is apparently absent from cellular structures, either on the cell surface or within the cell cytosol. Scale bars represent 10 μm.



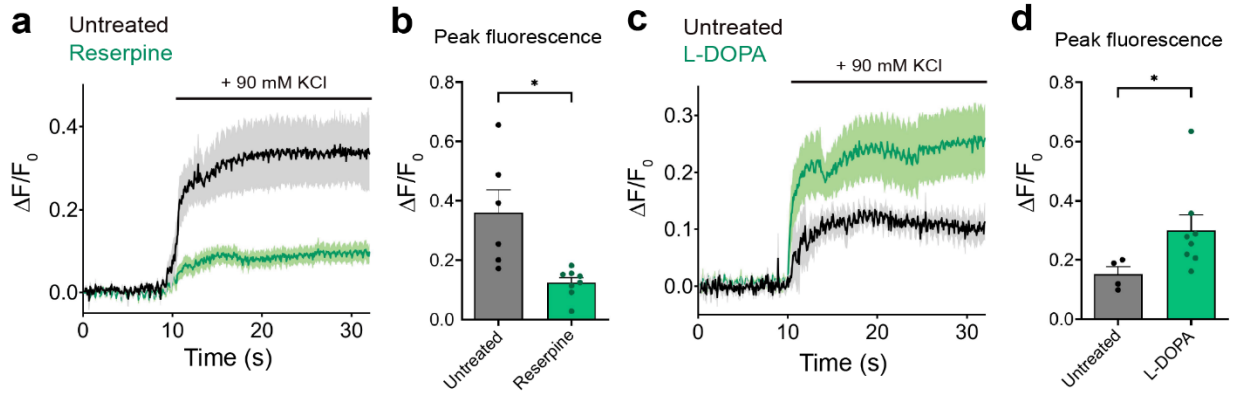
**Fig. S4: Expression of EGFP in dopaminergic neurons from TH-EGFP mice**

Immunolabeling of TH-EGFP ventral midbrain neurons against GFP and TH. Lower panels show the overlay of both channels (TH in magenta, GFP in cyan), neurons expressing both markers appear in white. Fluorescence images show axonal regions. **a**, Neuronal processes that are positive for both anti-TH and anti-GFP labeling are indicated with white arrows, neuronal processes that are positive for anti-TH and negative for anti-GFP labeling are indicated with magenta arrows. **b**, Neuronal processes that are negative for anti-TH and positive for anti-GFP labeling are indicated with cyan arrows, neuronal processes that are positive for anti-TH and negative for anti-GFP labeling are indicated with magenta arrows. **c**, Percentage of neurons expressing both or only one marker, respectively. Neuronal cell bodies were counted. In column charts, white circles represent experiments across three independent neuron cultures, bars show mean  $\pm$  SEM. The pie chart shows the mean contribution as a percentage made by each neuronal population to the total of all neurons. Scale bar = 10  $\mu$ m.  $n = 7$  coverslips from 3 independent neuron cultures.



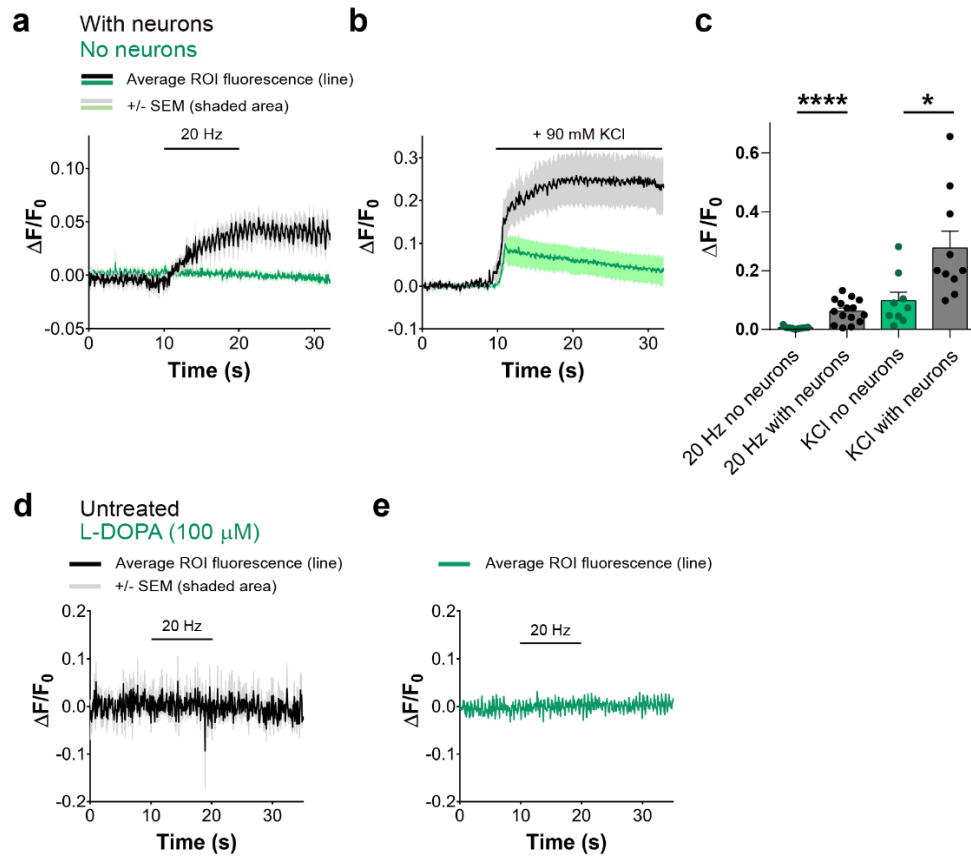
**Fig. S5: Imaging setup schematic**

This schematic shows the components and light paths of the custom-built dual-camera VIS/NIR imaging system utilized in this study. The system permits simultaneous imaging of both EGFP and NIR fluorescence. EGFP fluorescence is excited by a conventional fluorescence lamp (xCite 120Q) and imaged using a CMOS camera. NIR fluorescence is excited by a 561 nm laser and imaged using an InGaAs camera. To allow simultaneous imaging of these signals, light from the xCite 120Q lamp is passed to the microscope stand through an EGFP excitation filter, while the 561 nm laser for the SWCNT excitation enters the microscope from the bottom (yellow line), passing through a dichroic mirror (DM Laser/VIS). The microscope stand also contains other excitation filters, as indicated. Subsequent to excitation, emitted EGFP (blue line) and NIR fluorescence (red line) originating from the sample are split by another dichroic mirror (DM VIS/NIR) after leaving the microscope stand. NIR fluorescence is guided to the InGaAs NIR camera by two lenses and two mirrors as well as a long-pass filter directly in front of the camera for background reduction, with the opportunity for selecting wavelength regions by a filter wheel. Visible light fluorescence is guided to the CMOS camera using two lenses and the Laser/VIS dichroic mirror, which reflects <550 nm and thus enables the passage of the 561 nm laser light for excitation (see blue and yellow lines in the diagram). In order to select only the visible fluorescence of choice (e.g. EGFP) and omit unwanted wavelengths (e.g. cellular auto-fluorescence or residual laser excitation light), the light is additionally passed through a 650 nm short-pass filter, a 561 nm notch filter and the fluorescence emission filter of choice (e.g. 525/50 nm bandpass filter for EGFP-detection).



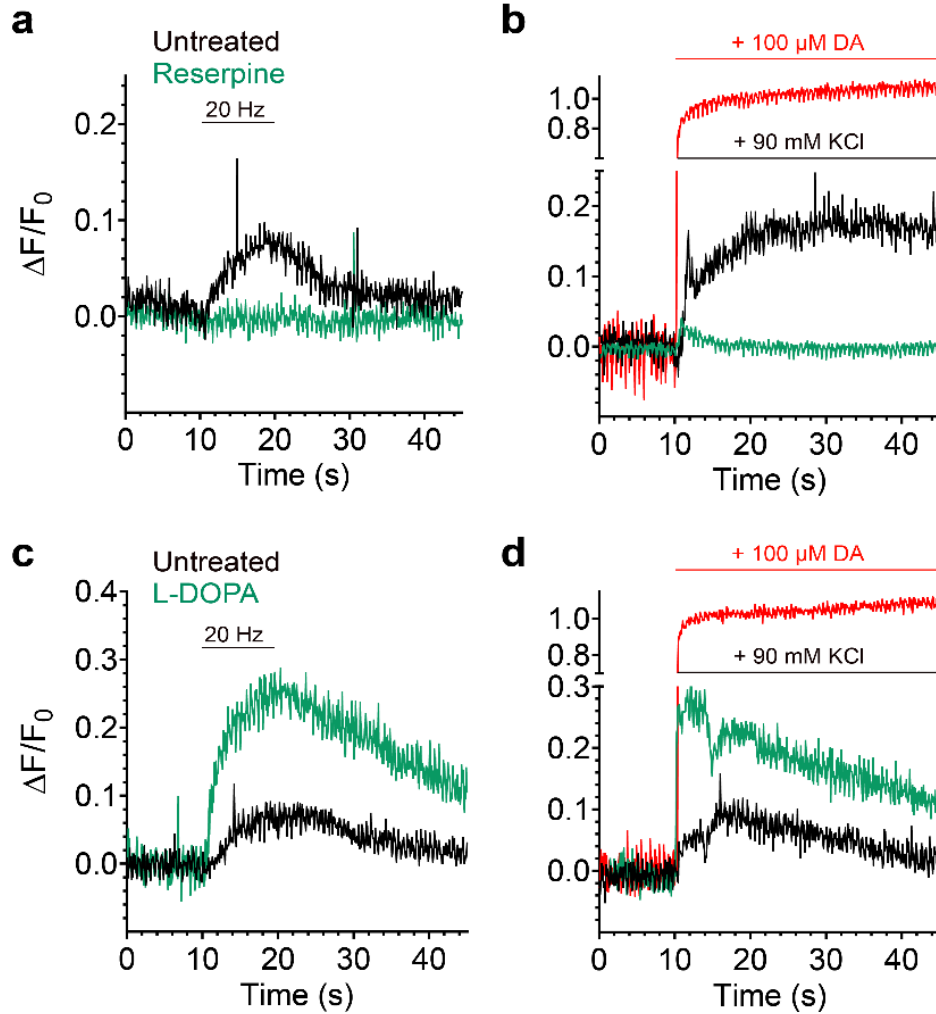
**Fig. S6: Detection of DA release and diffusion using AndromeDA in neurons stimulated with KCl**

AndromeDA fluorescence within axonal ROIs was quantified as described in Figure 2 in neurons stimulated using 90 mM KCl. **a**, KCl stimulation caused a sustained increase in AndromeDA fluorescence over time, consistent with evoked DA release into the extracellular space. Treatment of neurons with 1  $\mu$ M reserpine (90 min) decreased this KCl-mediated activation of AndromeDA compared to untreated neurons. **b**, Peak fluorescence (mean  $\pm$  SEM) for each stimulus method, showing a significant reduction in neurons treated with reserpine.  $n = 6$  experiments (untreated) and 8 (reserpine). **c**, Neurons were pre-treated with L-DOPA (100  $\mu$ M, 45 min) and stimulated with KCl. Average AndromeDA fluorescence is shown over time for neurons stimulated with KCl, either untreated or with L-DOPA pre-treatment. **d**, Peak fluorescence (mean  $\pm$  SEM) during KCl stimulation shows that L-DOPA increases the evoked AndromeDA fluorescence compared to untreated neurons.  $n = 4$  (untreated) and 8 (L-DOPA) experiments. Scale bars = 10  $\mu$ m. In line graphs, the solid lines represent mean values, shaded areas represent SEM. All statistical comparisons used a two-tailed Welch's t-test. \* denotes  $p < 0.05$ .



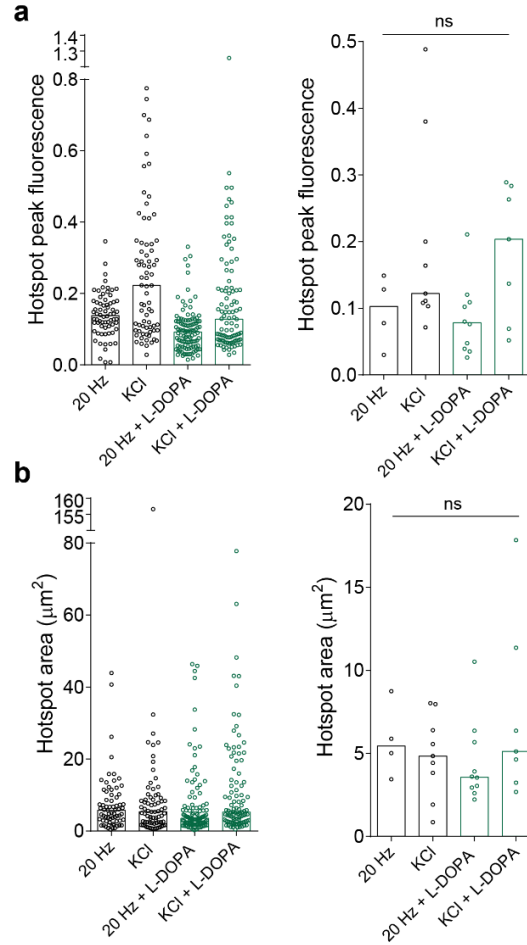
**Fig. S7: AndromeDA NIR fluorescence responses in the absence of dopaminergic neurons**

To verify that AndromeDA fluorescence was specifically caused by evoked DA release, AndromeDA fluorescence over time during electrical or KCl stimulation in the absence of neurons was measured and then compared to responses when dopaminergic neurons were present. **a**, This graph shows the average normalized NIR AndromeDA fluorescence over time of experiments using electrical stimulation at 20 Hz, either with neurons (black) or without neurons (green) on the coverslip. **b**, This graph shows the average normalized NIR AndromeDA fluorescence over time of experiments using treatment with 90 mM KCl, either with neurons (black) or without neurons (green) on the coverslip. **c**, Quantification of the peak fluorescence for each condition presented in **a** and **b**. \* indicates  $p < 0.05$ , \*\*\*\* indicates  $p < 0.0001$ . 20 Hz data were compared using the Mann-Whitney test and KCl data were compared using a two-tailed Welch's t-test. Data for stimulated neuron samples was derived from the untreated samples presented in Figure 2.  $n$  values for each sample were as follows: 20 Hz with neurons = 15, 20 Hz no neurons = 9, KCl with neurons = 10, KCl no neurons = 9. AndromeDA fluorescence was then examined in electrically stimulated hippocampal neuron cultures, in the absence of dopaminergic neurons. **d**, This graph shows the average normalized NIR AndromeDA fluorescence over time of untreated hippocampal neurons electrically stimulated at 20 Hz ( $n = 4$ ). **e**, Average normalized NIR AndromeDA fluorescence over time of L-DOPA (100  $\mu$ M) treated hippocampal neurons electrically stimulated at 20 Hz ( $n = 2$ ). For all data,  $n$  values represent the number of individual experiments analyzed. In line graphs, the solid lines represent the mean values of the data over time, while the shaded areas around each line represent the range of the SEM around the mean value. In column graphs, columns represent mean values and error bars represent +/- SEM.



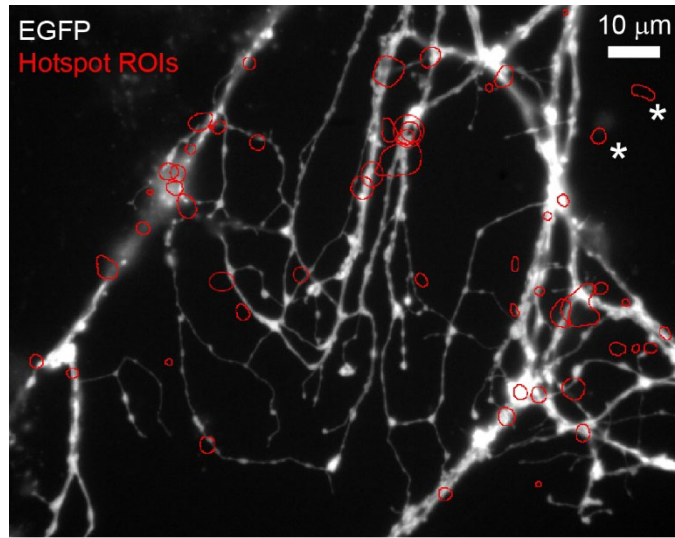
**Fig. S8: Representative traces of AndromeDA responses in ventral midbrain neurons following reserpine or L-DOPA treatment**

**a**, Representative traces showing AndromeDA fluorescence during 20 Hz electrical field stimulation from a single imaging experiment in untreated neurons (black) and a single experiment in neurons treated with reserpine (1  $\mu$ M, 90 min, green). **b**, Representative traces showing AndromeDA fluorescence during 90 mM KCl stimulation from a single imaging experiment in untreated neurons (black) and a single experiment in neurons treated with reserpine (green). **c**, Representative traces showing AndromeDA fluorescence during 20 Hz electrical field stimulation from a single imaging experiment in untreated neurons (black) and a single experiment in neurons treated with L-DOPA (100  $\mu$ M, 45 min, green). **d**, Representative traces showing AndromeDA fluorescence during 90 mM KCl stimulation from a single imaging experiment in untreated neurons (black) and a single experiment in neurons treated with L-DOPA (green). All data sets were normalized to the corresponding AndromeDA response to 100  $\mu$ M DA (red traces in b and d).



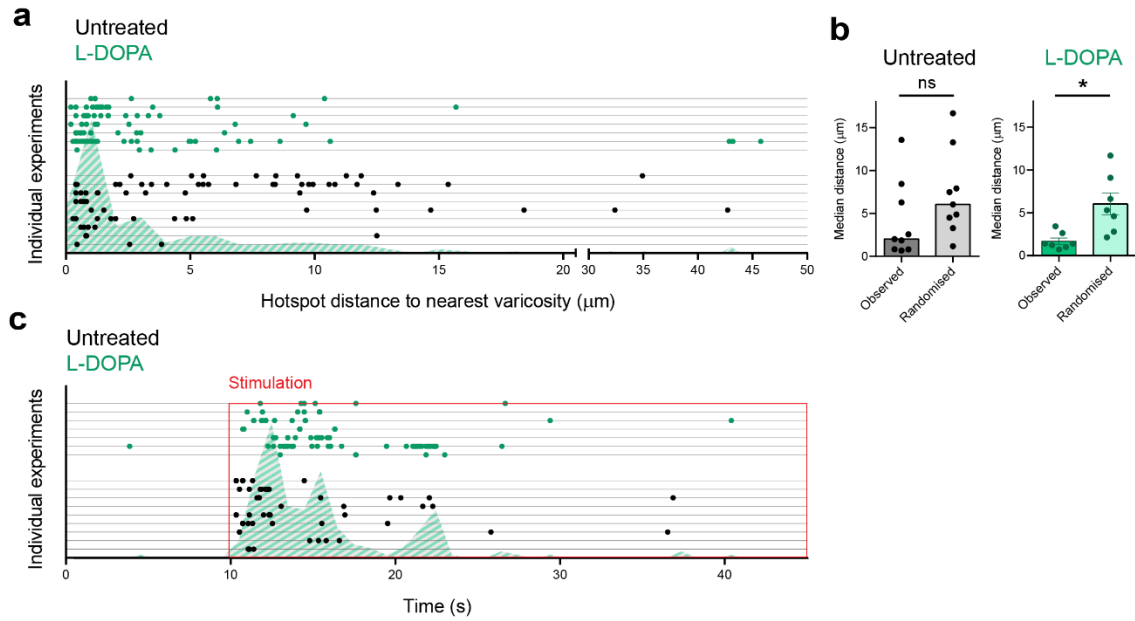
**Fig. S9: Hotspot area and peak fluorescence**

Hotspots of Andromeda activation were detected and analyzed in images acquired from neurons stimulated with 20 Hz electrical or KCl simulation, as indicated. Neurons were either untreated (black) or pre-treated with L-DOPA (100  $\mu\text{M}$ , 45 min, green). **a**, The left bar graph shows the peak fluorescence intensity at hotspots, with data from each hotspot represented as a single dot and bar height representing the median of all hotspots for each condition. The right bar graph shows the median values for each experiment as individual dots and bar height representing the average of the experimental median values. **b**, The left bar graph shows the area occupied by individual hotspots, with data from each hotspot represented as a single dot and bar height representing the median of all hotspots for each condition. The right bar graph shows the median values for each experiment as individual dots and bar height representing the average of the experimental median values. Hotspot fluorescence was calculated by subtracting the mean signal of the extracellular space ( $\Delta F/F_0$ ) from the mean signal within the hotspot ROIs ( $\Delta F/F_0$ ) to highlight local DA release against global DA diffusion. Hotspot peak fluorescence and area were not statistically different between any of the conditions examined. For 20 Hz, 68 hotspots are represented from  $n = 4$  experiments. For 20 Hz + L-DOPA, 110 hotspots are represented from  $n = 10$  experiments. For KCl, 73 hotspots are represented from  $n = 9$  experiments. For KCl + L-DOPA, 100 hotspots are represented from  $n = 7$  experiments. ns = not significant. Non-parametric distribution of data was confirmed using the Shapiro-Wilk normality test and samples compared using Kruskal-Wallis test with multiple comparisons ( $p < 0.05$ ).



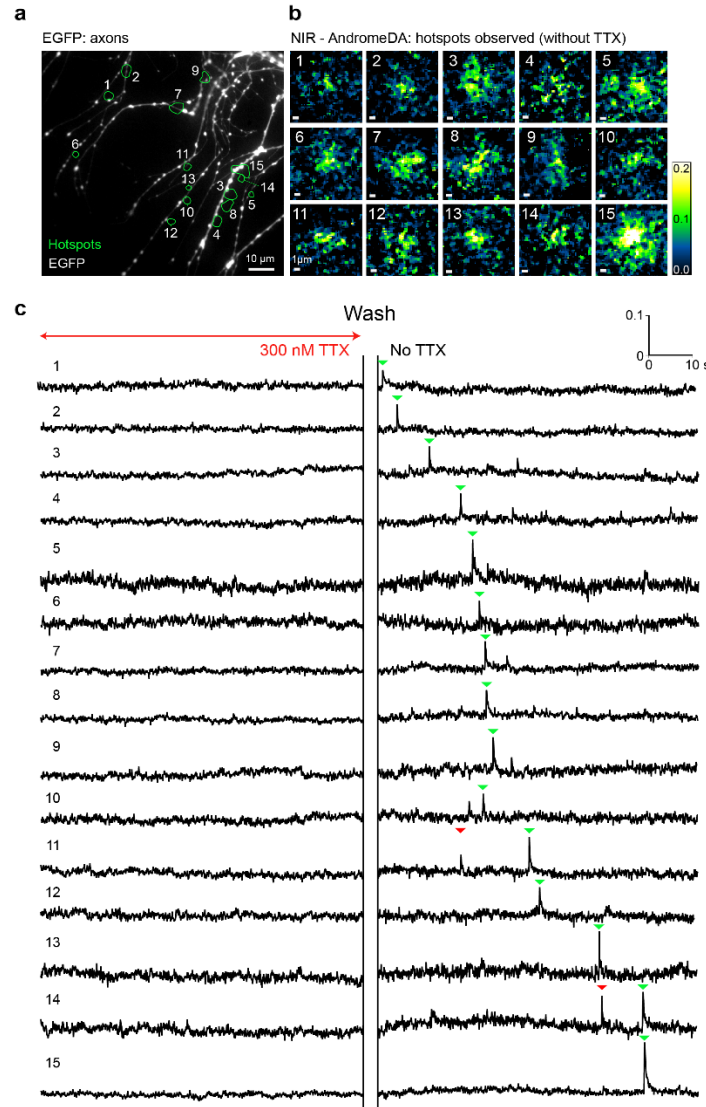
**Fig. S10: Spatial relationship between axons and hotspots**

An image is shown overlaying the EGFP fluorescence signal (white) from a dopaminergic axon and the hotspot ROIs identified by DART during AndromeDA imaging of this neuron (red outlines). Most ROIs either overlap with, or are located very close to, the axons. Two ROIs (\*) are indicated that are relatively distant from the axons, most likely resulting from DA release by a dopaminergic axon that is not expressing EGFP, and is thus not visible.



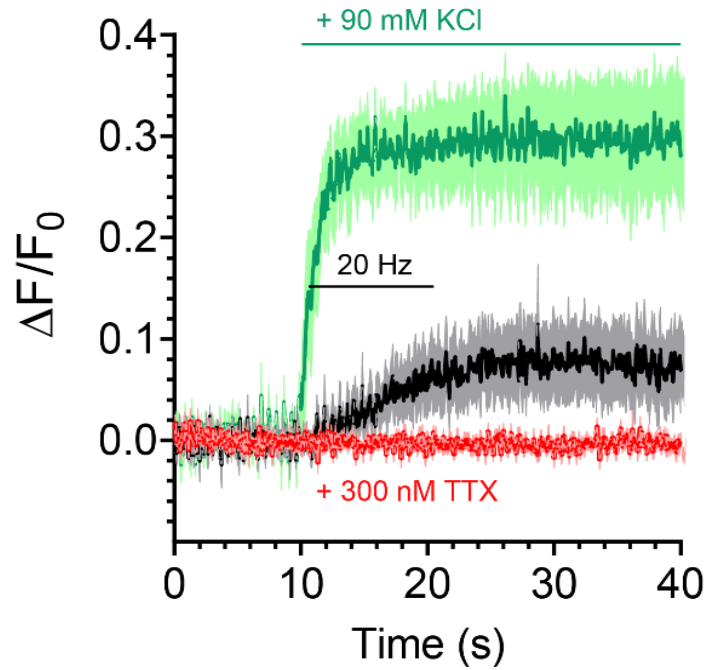
**Fig. S11: Spatiotemporal analysis of KCl-evoked hotspots**

Hotspots from both untreated (black dots) and L-DOPA treated (100 μM, 45 min green dots) neurons stimulated with KCl shown in **a** and **c** (arranged in horizontal lines along the y-axis according to the individual experiments in which the hotspots were observed). Hatched areas show the frequency distribution of all a total of 173 observed hotspots. Hotspot fluorescence was calculated by subtracting the mean signal of the extracellular space ( $\Delta F/F_0$ ) from the mean signal within the hotspot ROIs  $\Delta F/F_0$  to highlight local DA release against global DA diffusion. **a**, The distance between the center of individual hotspots and the center of the nearest varicosity was measured for all hotspots. **b**, Measured distance data was compared against 'randomized' data generated as described in the main text. Varicosity to hotspot distance is larger when non-corresponding EGFP images are used (randomized). **c**, Time to local peak maximum was measured for all hotspots. In column graphs, circles represent medians per experiment and columns represent median (b, untreated) or mean values (b, L-DOPA). For untreated neurons  $n = 9$  experiments, for L-DOPA treated neurons  $n = 7$  experiments. \* denotes  $p < 0.05$ ; ns = not significant. Data were compared using the two-tailed Mann-Whitney test (b, untreated) or two-tailed Welch's t-test (b, L-DOPA).



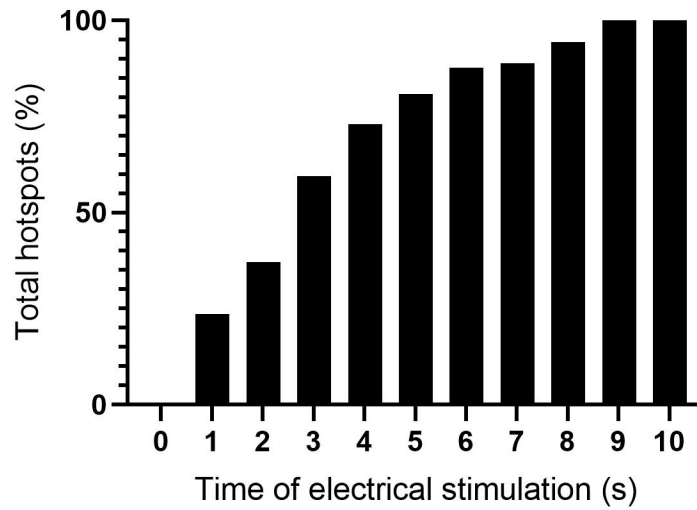
**Fig. S12: TTX abolishes hotspots of DA release**

**a**, Primary neurons from the ventral midbrain of TH-EGFP mice were pre-treated with L-DOPA (100  $\mu\text{M}$ , 45 min), painted with AndromeDA and imaged for 80 seconds in the presence of 300 nM TTX. An overview of the imaging field captured in the visible light spectrum shows EGFP-positive varicose axons of living dopaminergic neurons. During TTX application, no AndromeDA hotspots were observed. After TTX removal, neurons were imaged for another 80 seconds. After the removal of TTX, 15 hotspots were detected during imaging (green ROIs, 1-15). **b**, Magnified normalized  $\Delta F/F_0$  NIR images of AndromeDA hotspots at the time point of peak hotspot fluorescence intensity. **c**, Traces show the mean pixel intensity within each hotspot ROI over time, in the presence (left) and absence (right) of TTX. Exemplary hotspot peaks corresponding to the images in a and b are marked with green arrowheads. Traces 11 and 14 also exhibited additional peaks, indicated by red arrowheads. Scale bar in a = 10  $\mu\text{m}$ , scale bar in b = 1  $\mu\text{m}$ . In c, the y-axis represents hotspot fluorescence and x-axis represents time. Hotspot fluorescence traces were generated by subtracting the signal of the extracellular space ( $\Delta F/F_0$ ) from the mean signal within the hotspot ROIs ( $\Delta F/F_0$ ) to highlight local DA release against global DA diffusion.



**Fig. S13: Neurons show normal response to stimuli after TTX treatment**

Neurons painted with AndromeDA were imaged for 80 seconds in the presence of 300 nM TTX (red trace). After washout of TTX, neurons were depolarized first by 20 Hz electrical field stimulation (black trace), and then 90 mM KCl (green trace). Graphs show average NIR fluorescence over time, quantified within ROIs of 2  $\mu\text{m}$  radius drawn around all varicosities in the imaging field. Solid lines represent the mean values, while the shaded areas represents the SEM range around the mean value. Neurons were pre-treated with L-DOPA (100  $\mu\text{M}$ , 45 min) prior to imaging.  $n = 7$  experiments.



**Fig. S14: Most hotspots exhibit peak fluorescence early in the 20 Hz electrical stimulus train**

The time after the initiation of 20 Hz stimulation at which each hotspot reached peak fluorescence measured. These values are shown here as a cumulative frequency plot, arranged into bins. Each bin represents a timeframe of 1 s during electrical stimulation, such that bin 0 represents the time between 0 and 1 s after stimulation was commenced, bin 1 represents the time between 1 and 2 s after stimulation was commenced, and so forth. The plot shows that most hotspots have their peak fluorescence during the first 4 s of electrical stimulation.  $n = 110$  hotspots  $n = 10$  experiments in L-DOPA treated neurons.

## DA Diffusion Simulation

A simulation of the diffusion after DA release was performed by numerically solving the diffusion equation (Fick's 2<sup>nd</sup> law:  $\frac{\partial c}{\partial t} = D \Delta c$ ), using an explicit Euler scheme. It was implemented in Python 3.

The simulation area covers a 2.5-dimensional geometry, where a DA release is simulated in the center of the base plane (i.e.  $z = 0$ ). From there, diffusion is possible along the base plane (positive and negative  $x/y$ -direction) and upwards (positive  $z$ -direction), but not downwards. The size of the simulated volume is 100x100  $\mu\text{m}$  in the  $x/y$ -direction and 50  $\mu\text{m}$  in the  $z$ -direction, divided into finite volume elements.

The DA concentration is simulated for diffusion with and without DA uptake. DA uptake by DAT is added only on the base plane, above that surface no more uptake is simulated. In the  $z$  direction, DA can diffuse in free space. The DA uptake is simulated using Michaelis Menten kinetics ( $\frac{dc}{dt} = -\frac{v_{\max} C}{K_m + C}$ ). For simplification,  $K_m \gg C$  is considered. This simplification may overestimate the DA uptake in areas of high DA concentration. Simulation parameters were selected based on information obtained from references as indicated.

Diffusion constant:

| D [ $\text{cm}^2/\text{s}$ ] | for temperature [ $^{\circ}\text{C}$ ] | Reference |
|------------------------------|----------------------------------------|-----------|
| $7.63 \cdot 10^{-6}$         | 37                                     | (1)       |
| $6.05 \cdot 10^{-6}$         | 25                                     | (2)       |

DA release quantal size:

|              |     |
|--------------|-----|
| 2000 - 14000 | (1) |
| 1000         | (3) |

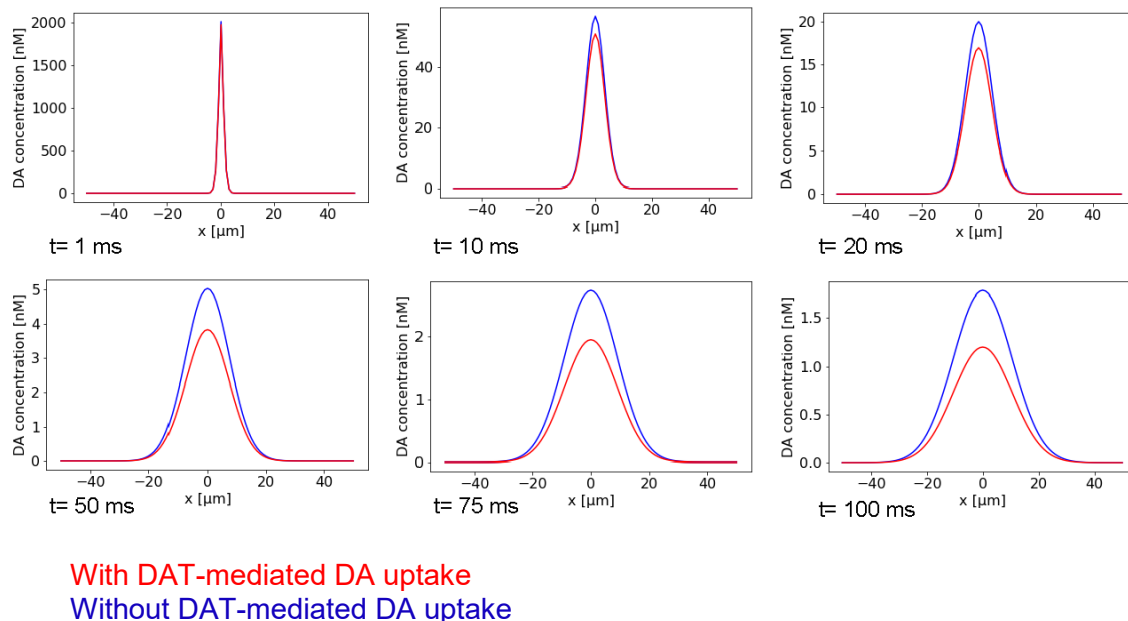
Synaptic vesicle size:

|                |     |
|----------------|-----|
| 40 nm diameter | (4) |
|----------------|-----|

DA uptake parameters:

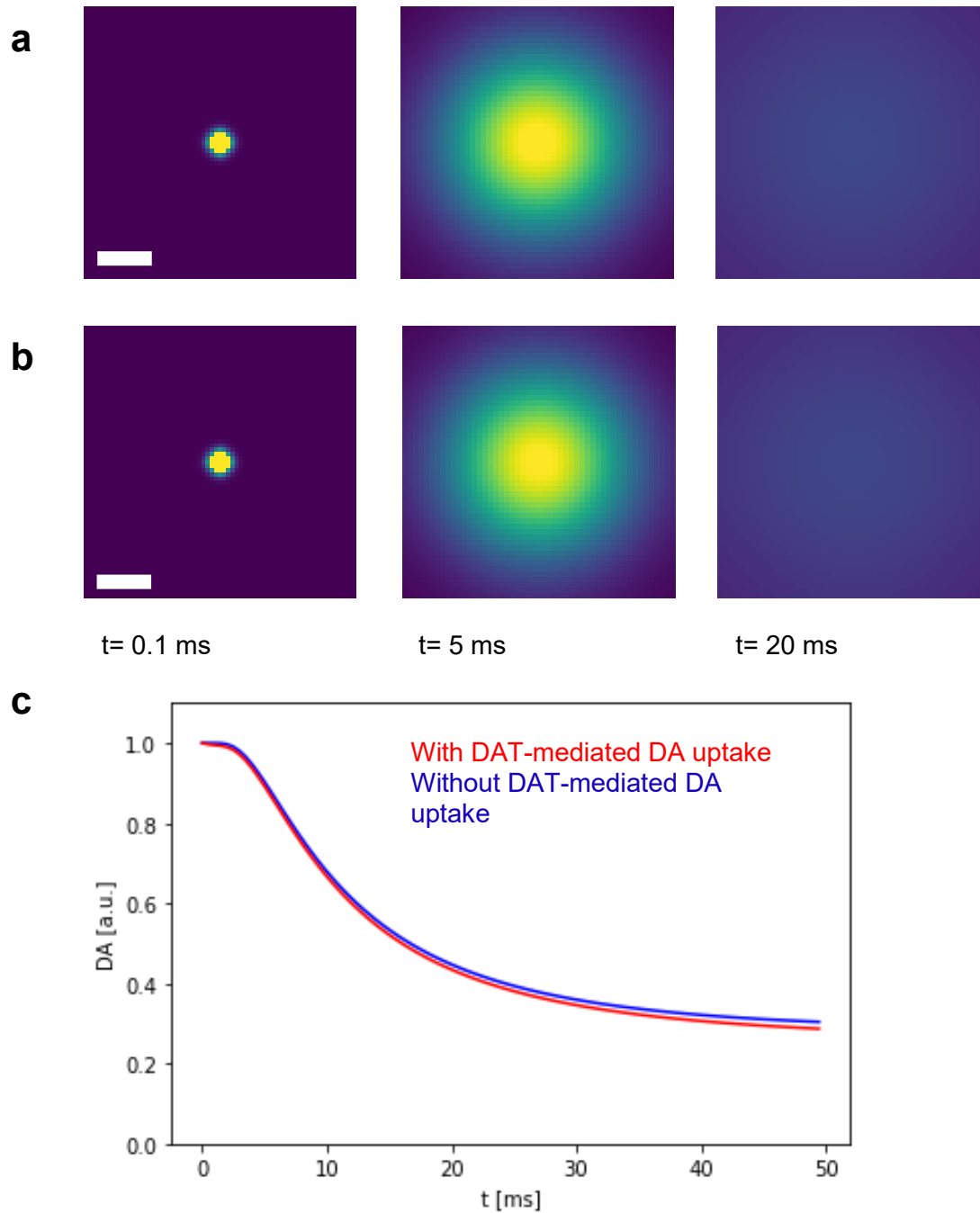
| $v_{\max}$ [ $\mu\text{M}/\text{s}$ ] | $K_m$ / $\mu\text{M}$ |     |
|---------------------------------------|-----------------------|-----|
| 4.1                                   | 0.21                  | (1) |
| 4.3                                   | 0.21                  | (5) |
| 2.4                                   | 0.21                  | (6) |

For the simulation  $D = 6.05 \cdot 10^{-6} \text{ cm}^2/\text{s}$ ,  $v_{\max} = 4.3 \mu\text{M}/\text{s}$  and  $K_m = 6.05 \mu\text{M}$  were selected, so that the DA uptake may possibly be overestimated. The simulation was run for a DA release quantal size of 10000 DA molecules. Since,  $K_m \gg C$  is considered, the DA release quantal size does not have any influence on the relative effect of DA uptake. The size of a synaptic vesicle would only need to be considered, if it were larger than the volume element (voxel) size. As the voxel size in the simulation is larger than the vesicle volume (assuming a vesicle diameter of 40 nm), vesicle size does not need to be accounted for in the simulation.



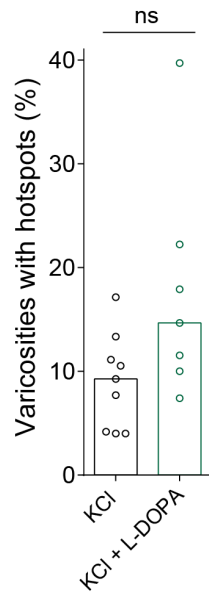
**Fig. S15: DA diffusion simulation – DA concentration on the base plane**

DA concentration on the base plane ( $z = 0$ ), along a cross-section with the spot of DA release at the center for different times after DA release. DA concentration for diffusion without (blue) and with (red) DA uptake by DAT. The origin of the  $x$ -axis denotes the site of DA release, which therefore also has the highest concentration of DA molecules ( $y$ -axis). Shortly after release, at  $t = 1$  ms, local DA concentration is high and there is essentially no difference in the DA concentration profiles whether DAT-mediated uptake is present or not. As time progresses, the local DA concentration decreases rapidly. As time progresses, the presence of DAT-mediated DA uptake has increasing impact. However, DA concentration decreases extremely rapidly with time, such that between 1 ms and 10 ms the DA concentration has decreased  $\sim 30$ -fold. As the sensor response near release sites is determined primarily by the initial high concentration of DA molecules, we conclude that DAT-mediated DA uptake does not significantly impact our data, and that loss of local nanosensor signal is attributable to DA diffusion alone.



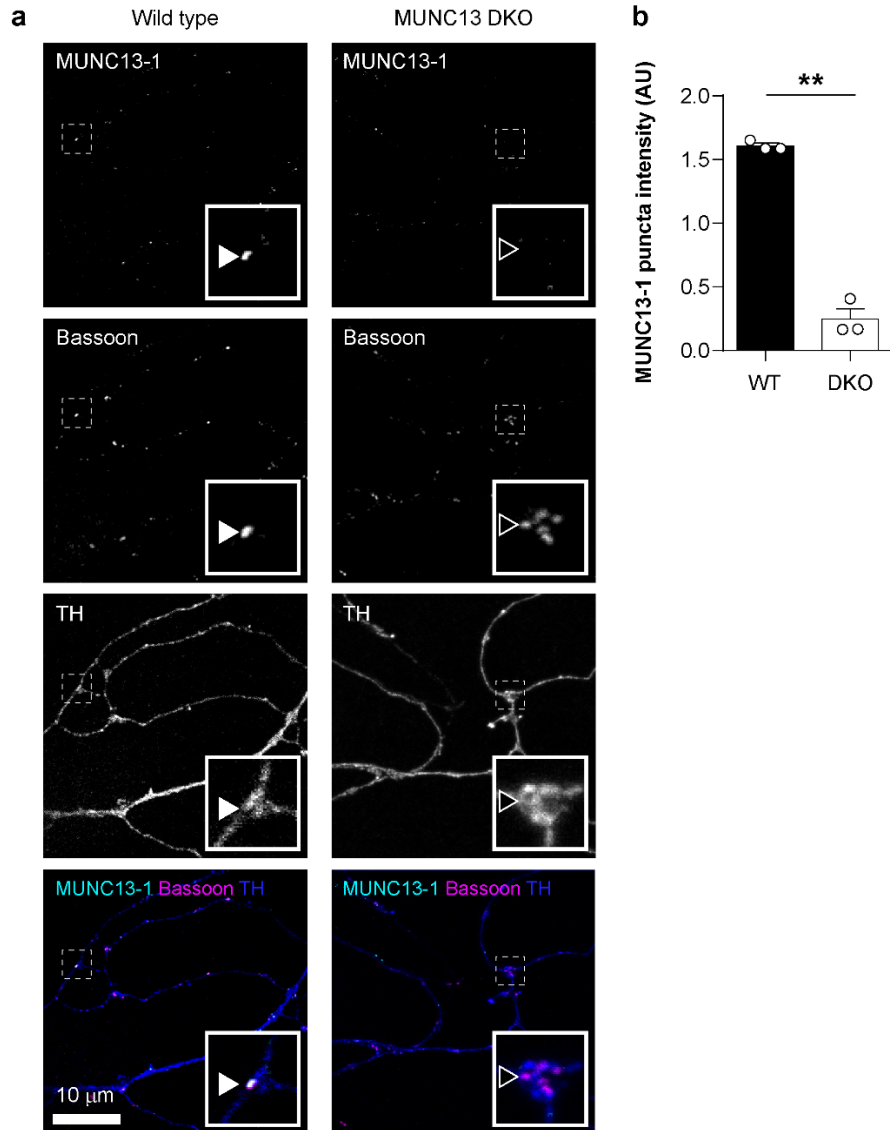
**Fig. S16: DA diffusion simulation – DA concentration in the simulation area**

**a**, Color-coded mapping of the DA concentration in the simulation area above the base surface for simulated DA diffusion without additional DA uptake by DAT. **b**, Color-coded mapping of the DA concentration in the simulation area above the base surface for simulated DA diffusion with additional DA uptake by DAT. **c**, DA concentration in the total simulation area in the time after DA release. Scale bars in **a** and **b** represent  $2 \mu\text{m}$ .



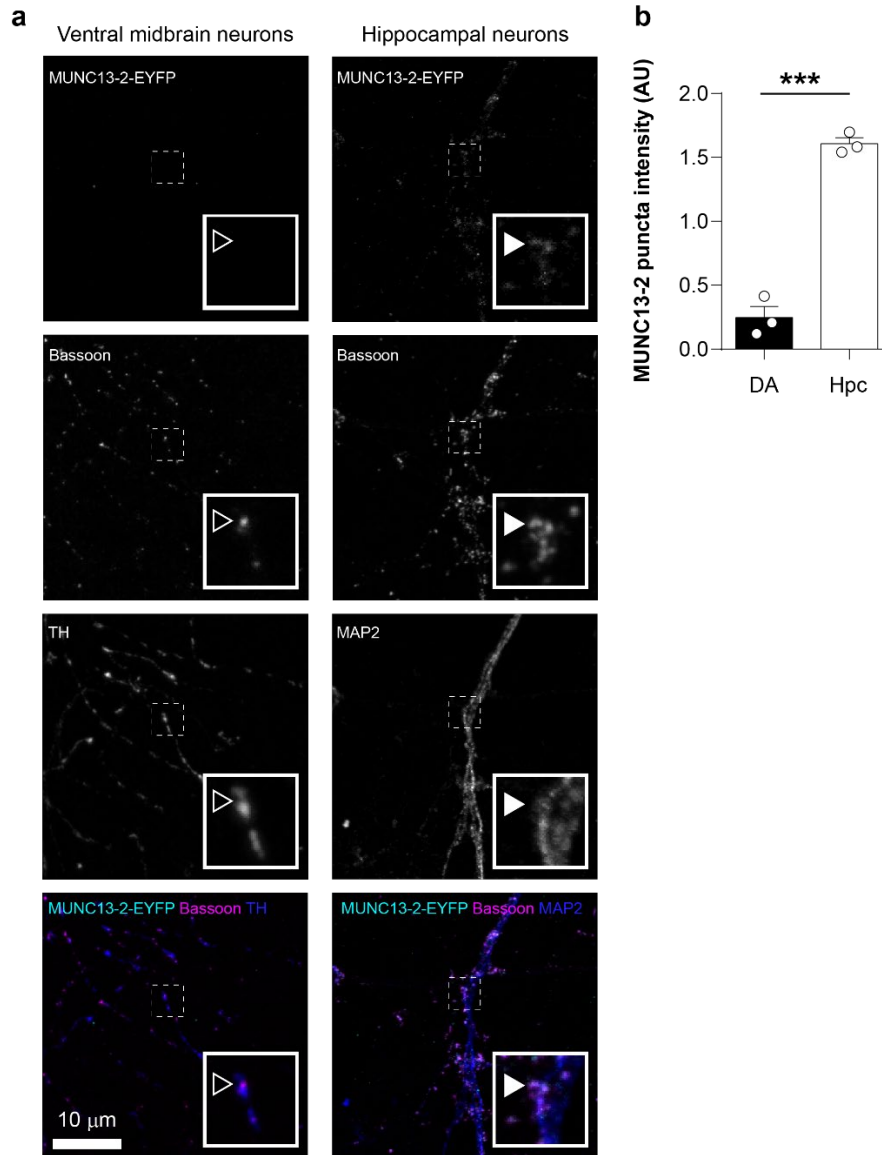
**Fig. S17: Proportion of total dopaminergic varicosities exhibiting KCl-evoked hotspots**

Column charts showing the average percentage of varicosities exhibiting a hotspot within 3  $\mu\text{m}$ . Varicosities were manually counted based on axon morphology. Circles represent experiments and columns represent median values. ns = not significant (two-tailed Mann-Whitney test). For KCl,  $n = 9$  experiments. For KCl + L-DOPA (L-DOPA treatment at 100  $\mu\text{M}$ , for 45 min),  $n = 7$  experiments.



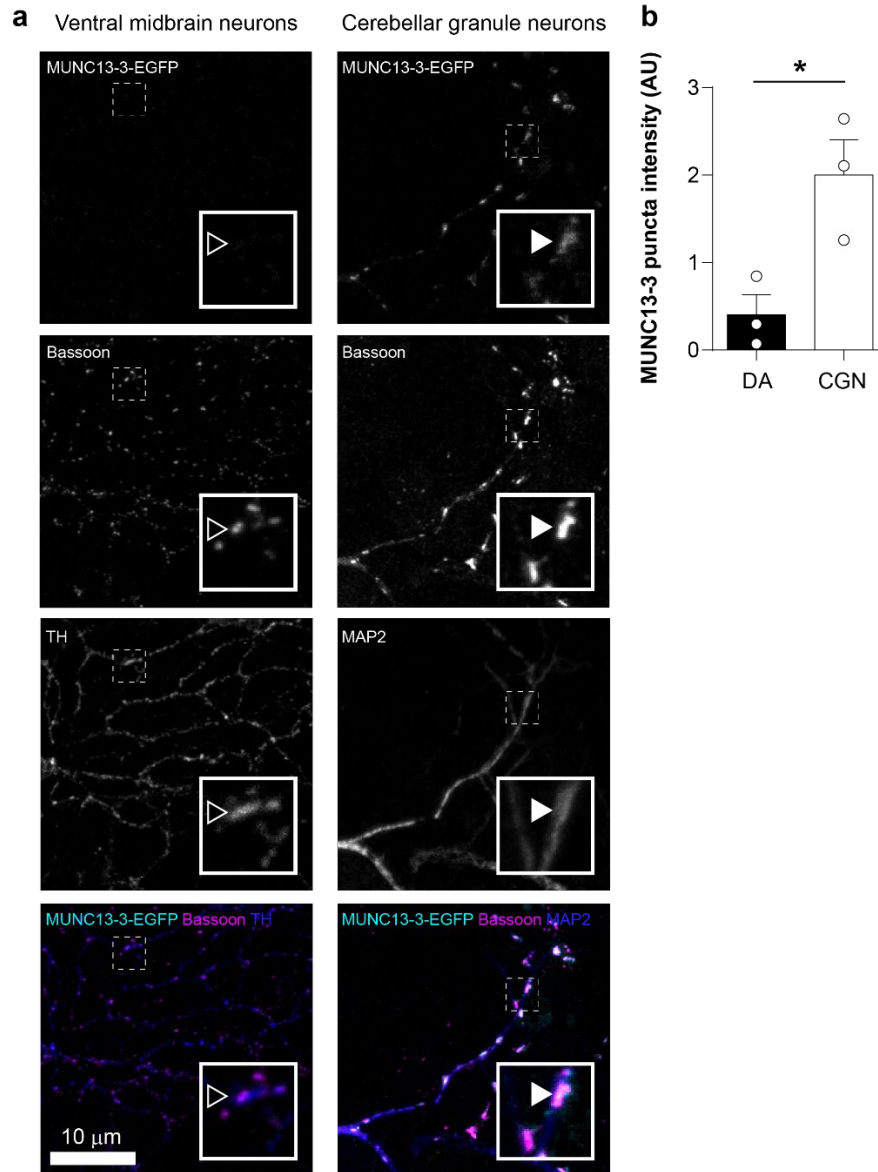
**Fig. S18: Active zones of dopaminergic neurons are positive for Munc13-1**

**a**, Ventral midbrain neuron cultures from WT (left) or Munc13-DKO (right) mice are shown with immunolabeling against Munc13-1, bassoon, and TH, as indicated. The lower panels show the three channels overlaid (Munc13-1 in cyan, bassoon in magenta, TH in blue). The region indicated by a white dashed box in each panel is shown in a higher detail inset. An example of a bassoon-positive punctum that was also positive for Munc13-1 is indicated by a white arrowhead (left panels), while an example of a bassoon-positive punctum showing no Munc13-1 immunolabeling is indicated with a black arrowhead (right panels). **b**, Quantification of the average anti-Munc13-1 immunolabeling within bassoon-positive puncta in WT (black) and Munc13-DKO (white) mice is represented as a column chart. \*\* indicates  $p < 0.01$ , data is from images acquired across 3 independent neuron cultures. Samples were compared using a two-tailed Welch's  $t$ -test.



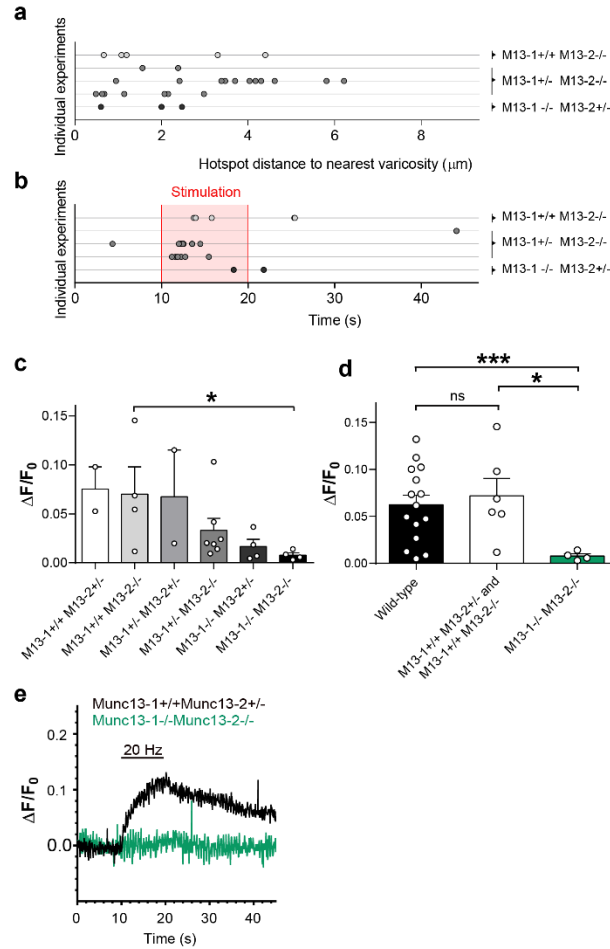
**Fig. S19: Active zones of dopaminergic neurons are negative for Munc13-2 in Munc13-2-EYFP knock-in mice**

**a**, Neurons from the ventral midbrain neuron (left) or hippocampus (right) of Munc13-2-EYFP knock-in mice are shown with immunolabeling against GFP, bassoon, and either TH to detect dopaminergic axons (left) or MAP2 to detect post-synaptic dendrites (right), as indicated. The lower panels show the three channels overlaid (GFP in cyan, bassoon in magenta, TH in blue). The region indicated by a white dashed box in each panel is shown in a higher detail inset. An example of a bassoon-positive punctum that was also positive for anti-GFP labeling is indicated by a white arrowhead (right panels), while an example of a bassoon-positive punctum showing no GFP immunolabeling is indicated with a black arrowhead (left panels). **b**, Quantification of the average anti-GFP immunolabeling within bassoon-positive puncta in dopaminergic (black) and hippocampal (white) neurons is represented as a column chart. \*\*\* indicates  $p < 0.001$ , data is from images acquired from 3 Munc13-2-EYFP knock-in mice, with the ventral midbrain and hippocampus harvested from each mouse and then cultured separately. Samples were compared using a two-tailed Welch's  $t$ -test.



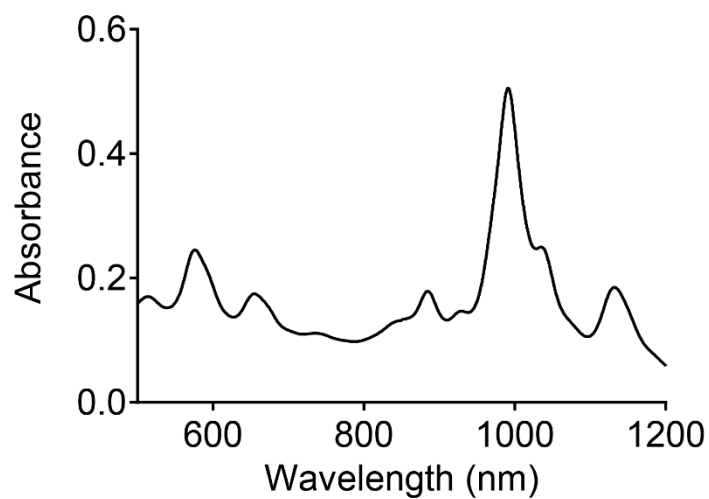
**Fig. S20: Active zones of dopaminergic neurons are negative for Munc13-3 in Munc13-3-EGFP knock-in mice**

**a**, Ventral midbrain neurons (DA, left) or cerebellar granule neurons (CGN, right) of Munc13-3-EGFP knock-in mice are shown with immunolabeling against GFP, bassoon, and either TH to detect dopaminergic axons (left) or MAP2 to detect post-synaptic dendrites (right), as indicated. The lower panels show the three channels overlaid (GFP in cyan, bassoon in magenta, TH in blue). The region indicated by a white dashed box in each panel is shown in higher detail inset. An example of a bassoon-positive punctum that was also positive for anti-GFP labeling is indicated by a white arrowhead (right panels), while an example of a bassoon-positive punctum showing no GFP immunolabeling is indicated with a black arrowhead (left panels). **b**, Quantification of the average anti-GFP immunolabeling within bassoon-positive puncta in dopaminergic (black) and CGNs (white) is represented as a column chart. \* indicates  $p < 0.05$ , data is from images acquired across 3 independent cultures. Dopaminergic and CGN neurons were harvested from the same mouse brains. Samples were compared using a two-tailed Welch's *t*-test.



**Fig. S21: Evoked DA release in neurons from Munc13-1/2 mutant mice**

**a**, Distance between the center of each hotspot and the center of the nearest varicosity for 20 Hz electrical stimulation of the Munc13 mutants. **b** Time point of the peak NIR fluorescence of each hotspot for 20 Hz electrical stimulation of the Munc13 mutant genotypes analyzed in this study. The electrical stimulation period from 10 s to 20 s is highlighted (red). No hotspots were observed in experiments using Munc13 DKO neurons. **c**, Column chart showing the average peak Andromeda fluorescence observed during 20 Hz electrical stimulation from each of the Munc13 (M13) mutant genotypes examined. M13-1<sup>-/-</sup> M13-2<sup>-/-</sup> (Munc13 DKO) exhibits a significant reduction in peak fluorescence compared to M13-1<sup>+/+</sup> M13-2<sup>-/-</sup> mice. *n* values for each genotype were as follows: M13-1<sup>+/+</sup> M13-2<sup>+/-</sup> = 2, M13-1<sup>+/+</sup> M13-2<sup>-/-</sup> = 4, M13-1<sup>+/-</sup> M13-2<sup>+/-</sup> = 2, M13-1<sup>+/-</sup> M13-2<sup>-/-</sup> = 7, M13-1<sup>-/-</sup> M13-2<sup>+/-</sup> = 4, M13-1<sup>-/-</sup> M13-2<sup>-/-</sup> = 4. **d**, Andromeda fluorescence data from untreated wild-type neurons stimulated at 20 Hz is shown here alongside data from M13-1<sup>+/+</sup> M13-2<sup>+/-</sup> and M13-1<sup>+/+</sup> M13-2<sup>-/-</sup> mice (pooled), and from M13-1<sup>-/-</sup> M13-2<sup>-/-</sup> (Munc13 DKO) mice. **e**, Representative traces showing Andromeda fluorescence from a single experiment in a neuronal culture lacking expression of Munc13-1 and -2 (Munc13-1<sup>-/-</sup>Munc13-2<sup>-/-</sup>) and an unburdened littermate control culture (Munc13-1<sup>+/+</sup>Munc13-2<sup>+/-</sup>), during electrical stimulation. \* indicates *p* < 0.05, \*\*\* indicates *p* < 0.001. *n* values for each genotype were as follows: wild-type = 15, M13-1<sup>+/+</sup> M13-2<sup>+/-</sup> and M13-1<sup>+/+</sup> M13-2<sup>-/-</sup> = 6, M13-1<sup>-/-</sup> M13-2<sup>-/-</sup> = 4. Columns represent the average peak normalized fluorescence intensity for each sample. For all experiments, *n* represents the number of individual experiments analyzed, with each coverslip coming from a single mouse. Samples were compared using a Kruskal-Wallis multiple comparisons test. Error bars represent +/- SEM in all charts.



**Fig. S22: VIS-NIR absorbance of SWCNT-(GT)<sub>10</sub> nanosensors**

Representative VIS-NIR absorbance spectra of a 1:100 diluted stock of SWCNT-(GT)<sub>10</sub> nanosensors, which was used to determine nanosensor concentration. The stock was adjusted to 300 nM based on the (6,5)-peak at 990 nm before application in cell experiments.

## SI Video Legends

### SI Video 1: AndromeDA hotspots from neurons stimulated by electrical field stimulation

The video shows the field of view from which hotspots shown in Figure 3 were selected. The EGFP panel (on the left) shows a microscopic image of live dopaminergic axons expressing EGFP. The NIR video (on the right) shows the normalized ( $\Delta F/F_0$ ) image sequence of AndromeDA from the same imaging field during 20 Hz electrical field stimulation, with fluorescence intensity represented as a heat map. 5 exemplary varicosities (shown in the EGFP panel) with corresponding hotspots (shown in the NIR movie) are highlighted with red arrowheads. The video begins by showing 100 frames prior neural stimulation, illustrating basal levels of AndromeDA activation (noise). At 100 frames (6600.3 ms) neurons are stimulated using electrical field stimulation at 20 Hz until the end of the video. Local regions of transiently activated AndromeDA, termed 'hotspots' appear shortly after the stimulation adjacent to dopaminergic varicosities (indicated by red arrows) at different time points. AndromeDA hotspots indicate steep, local dopamine concentration gradients, showing strong fluorescence, which then rapidly decreases as dopamine diffuses from the site of initial release. Dark, unresponsive areas in the NIR movie are regions that lack nanosensors due presence of cellular structures. The image sequence was acquired at 15 frames per second and is displayed at 7 frames per second. While most hotspots occur close to EGFP-positive varicosities (Fig. 4c, d), a subpopulation are observed relatively distant from EGFP-positive structures, and are likely the result of dopaminergic neurons in the culture that lack EGFP. Scale bar = 10  $\mu\text{m}$ .

### SI Video 2: AndromeDA hotspots from neurons stimulated by 90 mM KCl application

The EGFP panel shows a microscopic image of live dopaminergic axons expressing EGFP (on the left). The NIR video shows the normalized ( $\Delta F/F_0$ ) image sequence of AndromeDA from the same imaging field during 90 mM KCl stimulation, with fluorescence intensity represented as a heat map (on the right). KCl was applied shortly before the start of the video and is present in the sample until the end of the video. In both, 10 exemplary varicosities (shown in the EGFP panel) with corresponding hotspots (shown in the NIR movie) are highlighted with red arrowheads. In the first 30 frames of the video, local regions of transiently activated AndromeDA are apparent adjacent to dopaminergic varicosities (indicated by arrowheads) at different time points. Local regions of transiently activated AndromeDA, termed 'hotspots' appear shortly after the stimulation adjacent to dopaminergic varicosities (indicated by red arrows) at different time points. AndromeDA hotspots indicate steep, local (DA) concentration gradients, showing strong fluorescence, which then rapidly decreases as dopamine diffuses from the site of initial release. Eventually (visible at frames 30 - 41), AndromeDA fluorescence increases gradually in the extracellular space due to the sustained release and diffusion of dopamine in the extracellular space. Dark, unresponsive areas in the NIR movie are regions that lack nanosensors due presence of cellular structures. Neurons have been pretreated with L-DOPA prior to experimentation. The image sequence was acquired at 15 frames per second and is displayed at 7 frames per second. Scale bar = 10  $\mu\text{m}$ .

## Extended Materials and Methods

### Animals

Hippocampal neurons were prepared from wild-type C57BL/6N postnatal day 0 (P0) mice. Ventral midbrain neurons were prepared from (B6.B6D2-Tg(TH-EGFP)21-31Koba [RBRC02095] mice at P0 (TH-EGFP) (7, 8). TH-EGFP mice were provided by RIKEN BRC through the National Bio-Resource Project of the MEXT/AMED, Japan. TH-EGFP<sup>+</sup>/<sup>-</sup> Munc13-1<sup>-</sup>/<sup>-</sup> Munc13-2<sup>-</sup>/<sup>-</sup> (THEGFP-Munc13 DKO) neonatal mice were generated by breeding the genotypes TH-EGFP<sup>+</sup>/<sup>-</sup> Munc13-1<sup>+</sup>/<sup>-</sup> Munc13-2<sup>-</sup>/<sup>-</sup> with Munc13-1<sup>-</sup>/<sup>+</sup> Munc13-2<sup>-</sup>/<sup>-</sup> mice (9, 10). Knock-in mice expressing either Munc13-2-EYFP or Munc13-3-EGFP were used to examine Munc13 expression in dopaminergic neurons (11, 12).

Mouse breeding was performed with the permission of the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES, permit numbers 33.19-42502-04-19/3254, 33.19.42502-04-15/1817 and 33.19-42502-04-18/2756). Animals were housed according to European Union Directive 63/2010/EU and ETS 123 at 21 ± 1°C, 55% relative humidity, under a 12 h/12 h light/dark cycle, and received food and tap water *ad libitum*. The sex of neonatal mice was not checked.

### Mouse genotyping

Genotyping was performed by the AGCT laboratory (Max Planck Institute for Multidisciplinary Sciences). Primers for genotyping TH-EGFP mice: 5'-TGTGGCTTTCTGAACTTGACA-3', 5'-ACCAGAGGCATACAGGGACA-3', 5'-CTACACCCTGGTCATCATCCTGC-3', and 5'-TCCAGCTCGACCAGGATG-3'. Primers for genotyping mice with the Munc13-1 deletion: 5'-CTTACCCATCTGAGAGCCGGAATTCCA-3', 5'-CTCCGAGGGGAATGCGCTTCCGTTTCCTG-3', and 5'-GAGCGCGCGCGGCGGAGTTGTTGAC-3'. Primers for genotyping mice with the Munc13-2 deletion: 5'-CTCCACTGCCCCCTTTTACTGT-3', 5'-TCAAGGGACTGTTCTAGCAATGTT-3', and 5'-GAGCGCGCGCGGCGGAGTTGTTGAC-3'. Primers for genotyping Munc13-2-EYFP mice: 5'-GATGAGACAGGCATGACCAT-3', 5'-ACAGCTAACTCTCCCTGACTGA -3' and 5'-CATGGTCCTGCTGGAGTTCGTG -3'. Primers for genotyping Munc13-3-EGFP mice: 5'-TCTCTCAGAGGACCAGCGA -3', 5'-TGGCACTTCATGGAACATTTAT -3' and 5'-CATGGTCCTGCTGGAGTTCGTG -3'.

### Immunocytochemistry

Neurons were fixed using 4% paraformaldehyde (in PBS, pH = 7.4), either for 10 minutes (for detection of Munc13-1) or 2 min (for detection of Munc13-2-EYFP and Munc13-3-EGFP) at room temperature. Cells were permeabilized in 0.2% Triton X-100 for 15 min and blocked in 10% BSA for 60 min. Primary antibodies were applied overnight in 3% BSA at 4 °C, and detected using fluorescent secondary antibodies applied for 60 min in 3% BSA at room temperature. Primary antibodies: Mouse anti-tyrosine hydroxylase (1:2000, Synaptic Systems, Cat. 213 111), rabbit anti-tyrosine hydroxylase (1:1000, Synaptic Systems, Cat. 213 102), rabbit anti-Munc13-1 (1:1000, Synaptic Systems, Cat. 126 103), guinea pig anti-bassoon (1:2000, Synaptic Systems, Cat. 141 004), chicken anti-MAP2 (1:2000, Novus Biologicals, NB300-213), mouse anti-GFP (1:1000, Millipore, MAB3580). The following secondary antibodies were used (1:1000, Thermo Fisher): Alexa Fluor 405 goat anti-chicken, Alexa Fluor 488 goat anti-mouse, Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 555 goat anti-guinea pig, Alexa Fluor 633 goat anti-rabbit, Alexa Fluor 633 goat anti-mouse.

Immunocytochemistry was always done such that controls for immunolabelling were done alongside the dopaminergic neurons of interest, i.e. WT vs Munc13-DKO neurons for Munc13-1 analysis, midbrain vs hippocampal neurons for Munc13-2 analysis, and midbrain vs cerebellar granule neurons for Munc13-3 analysis.

For quantitative analysis of immunolabelling, the intensity of anti-Munc13-1 or anti-GFP immunolabelling at bassoon-positive puncta was measured. Therefore, regions of interest based on bassoon labelling were identified, and the mean pixel intensity of Munc13-1 or GFP fluorescence within each bassoon ROI was measured. In midbrain neuron samples, only bassoon puncta that overlapped with anti-tyrosine hydroxylase labelling were selected in order to exclude contamination of Munc13-1 or GFP signals from non-dopaminergic cells.

For statistical analysis, the mean intensity of Munc13-1 or GFP immunolabelling was calculated for puncta from each image. To account for inter-experimental variation in immunolabelling intensity, image means were normalized by dividing the mean by the average intensity of all measured puncta within the experiment. The experimental mean for each neuron type was then calculated by averaging the normalized image means. Experimental means were then used for statistical comparisons.

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