

1 **A versatile synaptotagmin-1 nanobody provides perturbation-free live** 2 **synaptic imaging and low linkage-error in super-resolution microscopy**

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24

25 **Abstract**

26 Imaging of living synapses has relied for over two decades on the overexpression of synaptic
27 proteins fused to fluorescent reporters. This strategy changes the stoichiometry of synaptic
28 components and ultimately affects synapse physiology. To overcome these limitations, here
29 we introduce a nanobody that binds the calcium sensor synaptotagmin-1 (NbSyt1). This
30 nanobody functions in living neurons as an intrabody (iNbSyt1) and is minimally invasive,
31 leaving synaptic transmission almost unaffected, as demonstrated by the crystal structure of the
32 NbSyt1 bound to synaptotagmin-1 and by our physiological data. Its single-domain nature
33 enables the generation of protein-based fluorescent reporters, as we showcase here by
34 measuring spatially-localized presynaptic Ca²⁺ with an NbSyt1-jRCaMP8 chimera. Moreover,
35 its small size makes the NbSyt1 ideal for various super-resolution imaging methods. Overall,
36 NbSyt1 is a versatile binder that will enable imaging in cellular and molecular neuroscience at
37 a higher precision than possible in the past, over multiple spatiotemporal scales.

38

1 Introduction

2 Neuronal communication strongly relies on the molecular machinery responsible for
3 the accurate release of neurotransmitters. For this crucial step in neuronal communication,
4 neurotransmitter-filled synaptic vesicles (SVs) fuse to the plasma membrane upon Ca^{2+} entry
5 through activated voltage-gated channels following an action potential. The rapid fusion of the
6 membranes from SVs to the plasma membrane occurs through a set of proteins known as the
7 soluble N-ethylmaleimide-sensitive-factor attachment receptors or SNAREs. The SNAREs
8 located at the plasma membrane form a four-helix bundle together with the SNAREs present
9 at the SVs^{1,2}. The interaction of these alpha-helices is progressive, and at some point, this
10 complex provides sufficient energy for the fusion of the SVs with the plasma membrane,
11 releasing the neurotransmitters' load to the synaptic cleft. Interestingly, this SNARE-zipper
12 mechanism for SVs fusion is regulated by Ca^{2+} in a complex manner. At central synapses, the
13 putative protein responsible for the Ca^{2+} -dependent regulation of synaptic release is
14 Synaptotagmin 1 (Syt-1), which is present in several copies on each SV^{3,4}. Syt-1 has two
15 calcium-binding domains (C2A and C2B). Although the calcium dependency and Syt-1
16 involvement in evoked neurotransmitter release are generally not questioned, the exact
17 molecular mechanism of how Syt-1 regulates the SNARE complex and SV fusion is a topic of
18 fervent research⁵⁻⁸.

19 Remarkably, several questions remain to be answered, including which is the exact
20 molecular target of Syt-1, what is the relative involvement of the two calcium binding domains,
21 and ultimately which are the different steps and interactors of Syt-1. It is known for example
22 that the C2 domains of Syt-1 interact with negatively charged membranes in a Ca^{2+} -dependent
23 manner^{9,10}, although also a Ca^{2+} -independent interaction has been observed *in vitro* for
24 membranes containing phosphatidylinositol¹¹. Several molecular mechanisms for Syt-1 have
25 been proposed including the formation of rings on presynaptic membranes in the absence of
26 Ca^{2+} , which might prevent SNARE-mediated SV fusion¹². One other model suggests that Syt-
27 1 might stabilize a still yet little defined partially-assembled SNARE complex between the SV
28 membrane and the plasma membrane and that this interaction with a partially-assembled
29 complex is resolved in the presence of Ca^{2+} , thus favoring fusion^{13,14}. An additional model
30 suggests that Syt-1 might specifically modulate the interaction with PIP_2 -containing
31 membranes promoting changes in membrane bending in a Ca^{2+} -dependent manner, thereby
32 promoting fusion^{9,15}.

33 We are persuaded that the heterogeneity of the experimental results obtained and the
34 difficulty in understanding the molecular function of Syt-1 depend in part on diverging findings
35 obtained *in vitro* and in cultured neurons¹⁶, and are in part related to the difficulty of studying
36 this molecule when expressed at the correct stoichiometry^{17,18}. For these reasons, here we
37 aimed to develop a custom molecular tool that would allow us to manipulate and monitor the
38 activity and the function of Syt-1 without adding a tag (e.g., EGFP) nor changing its
39 stoichiometry in the cell, following a number of criteria.

40 We set out to develop a small probe able to bind to an accessible domain of Syt-1, with
41 a strong affinity and high specificity, ideally, even if Syt-1 is engaged with the SNAREs,
42 calcium or membranes in the crowded volume of an active synaptic bouton. The probe should
43 be small to minimize the linkage error and retain its binding and specificity even after chemical
44 fixation, which is typically needed for various advanced microscopy techniques, but it should

1 also be functional in living neurons to monitor native Syt-1 location and function with minimal
2 interference on Syt-1 physiological tasks.

3 We present here the successful development and characterization of an alpaca single-
4 domain antibody, also known as nanobody (Nb), that remarkably fulfills most of our
5 abovementioned criteria, and therefore it appears as an ideal probe to study living synapses and
6 Syt-1 molecular physiology. This Nb is able to bind with high affinity and specificity to the
7 C2A domain from rat Syt-1 and after obtaining the crystal structure of the Nb bound to rat C2A
8 domain, we predicted that the Nb should significantly minimize the linkage error when directly
9 conjugated to fluorophores and it should also impose a minimal effect on native Syt-1 activity.
10 Due to these already favorable features, we decided to continue engineering this monovalent
11 nanobody as an ideal probe to stoichiometrically reveal Syt-1 in various high- and super-
12 resolution microscopy techniques like SIM, STED, DNA-PAINT, and Expansion Microscopy
13 (ExM). In addition, we tested and demonstrated its minimal perturbation when expressing this
14 Nb in living primary neurons (as intrabody), resulting in a flexible tool to label live synapses
15 and follow synaptic vesicles. Finally, we used this specific binder to position the sensitive
16 calcium sensor jRCaMP8¹⁹ directly on pre-synapses, allowing a highly localized calcium
17 detection and fast response to evoked and spontaneous activity without genetic manipulation
18 of synaptic proteins. We conclude that this Nb is a highly versatile tool for use in living
19 neurophysiology studies as in conventional and super-resolution imaging techniques. We
20 expect this Nb to be instrumental in tracking synaptic physiology and dissecting the molecular
21 role of Syt-1 in neuronal communication.

22

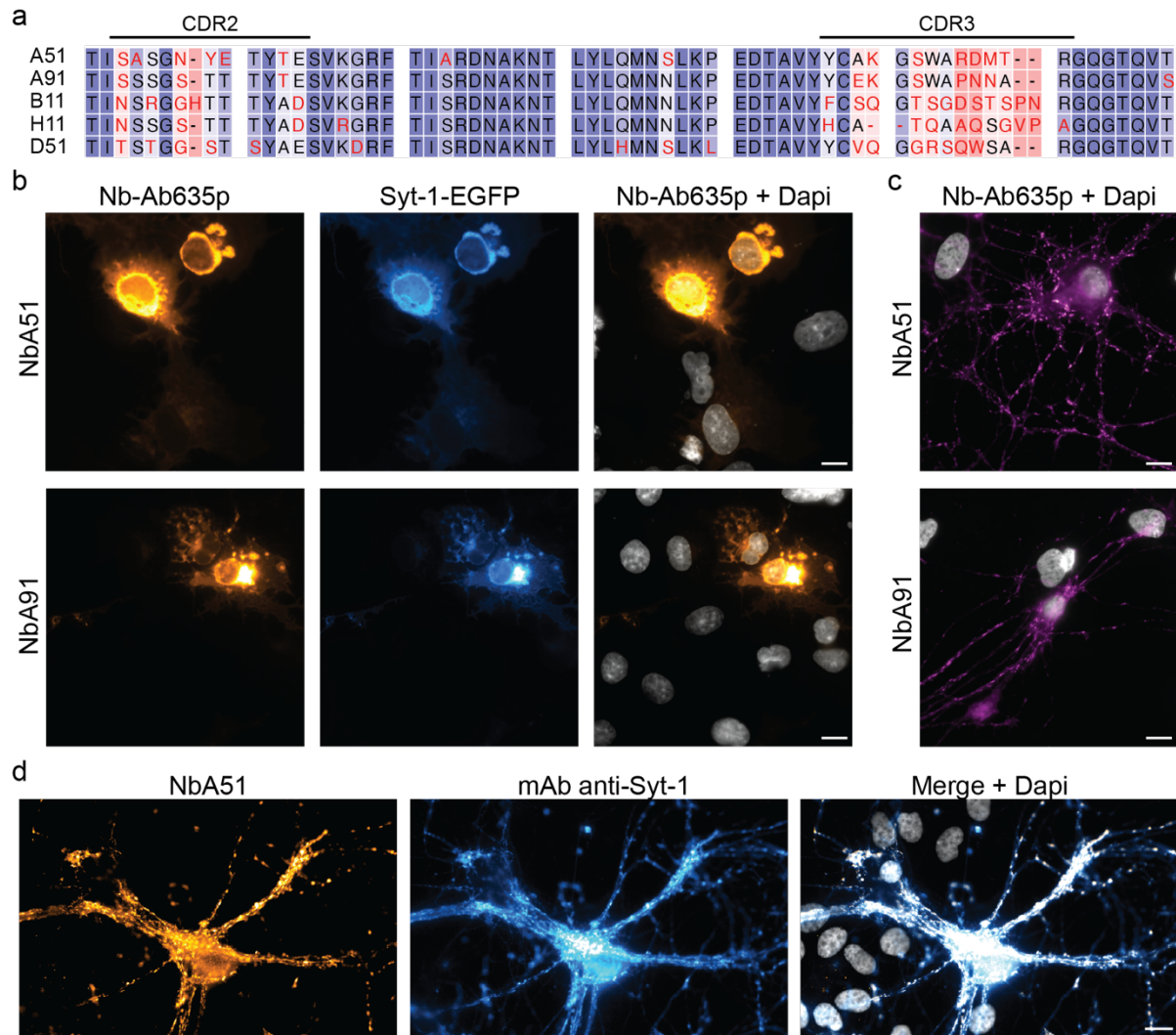
23 **Results**

24 **Selection and initial characterization of potential binders to Syt-1.**

25 For the selection of nanobodies against Syt-1 we immunized alpacas with enriched rat
26 synaptosomes. Five months after the immunization protocol, we extracted the peripheral blood
27 mononuclear cells (PBMCs) from 100 ml of blood. After total RNA extraction from the
28 PBMCs we generated a cDNA library of Nbs and cloned them into a phagemid to later screen
29 for binders using phage-display (Supp. Fig. 1a). Before performing the phage-display, we
30 verified if these animals had generated heavy-chain antibodies against the cytosolic (amino
31 acids 97-421) domain of rat Syt-1 (rSyt-1₍₉₇₋₄₂₁₎). For this, we first removed conventional
32 immunoglobulins (IgG1) that might also recognize this domain and enriched for heavy-chain
33 antibodies (IgG2 & IgG3) from the plasma of the immunized alpacas (Supp. Fig. 1b). These
34 IgG2 and IgG3 were used for the detection of rSyt-1₍₉₇₋₄₂₁₎ coated as antigen in an ELISA plate
35 (Supp. Fig. 1c). The ELISA result suggested that heavy chain antibodies binding rSyt-1₍₉₇₋₄₂₁₎
36 were present in the serum although we used a complex antigen mixture for immunization
37 (synaptosomes). These results encouraged us to proceed with phage-display screening.

38 After three rounds of phage display screening (biopanning) using the whole
39 immobilized cytosolic rSyt-1₍₉₇₋₄₂₁₎, we obtained five clones with strong positive signals on an
40 ELISA. Although these clones have relatively similar complementary determining region 1
41 (CDR1), they all show significant differences in their CDR2s and CDR3s (Fig.1a, Supp. Fig.
42 1d). After subcloning all five clones into a mammalian expression vector, only two clones (A51
43 and A91) resulted in reasonable protein yields (>20 mg/L).

1 For further validation, NbA51 and NbA91 were directly conjugated with fluorophores
2 for testing them in immunofluorescence (IF) assays. Initially, COS-7 fibroblasts were
3 transfected with plasmids coding for the full-length rat Syt-1 fused to EGFP. After 24h, cells
4 were chemically fixed using paraformaldehyde and IF was performed using 50 nM of
5 fluorescent nanobodies. Both clones displayed specific signals that were detected only on
6 EGFP-positive cells, suggesting that both clones are able to detect rat Syt-1 and that their
7 epitopes seem not to be affected by aldehyde fixation (Fig. 1b). In order to understand the
8 specificity of these candidates, a dot-blot assay with purified rSyt-1₍₉₇₋₄₂₁₎ and rSyt-2₍₉₇₋₄₂₂₎
9 suggest that both candidates bind specifically to rSyt-1 and not rSyt-2 (Supp. Fig. 2). We
10 carried on an additional validation step to verify their IF performance on rat primary
11 hippocampal cultures. At this point we reasoned that if the nanobody would bind efficiently in
12 a cell context situation, where Syt-1 is in close proximity with several other interacting
13 molecules, it would thus be the one providing the higher signal-to-noise ratio (SNR) likely
14 because has a higher affinity or is able to find more epitopes. While both candidates tested
15 displayed the classical punctuated pattern of synapses expected in these primary neurons, by
16 closer inspection the candidate NbA51 provided images with negligible level of background
17 staining in cell bodies of neurons and astrocytes. Due to this, we decided to continue to work
18 with the NbA51 and rename it NbSyt1.
19



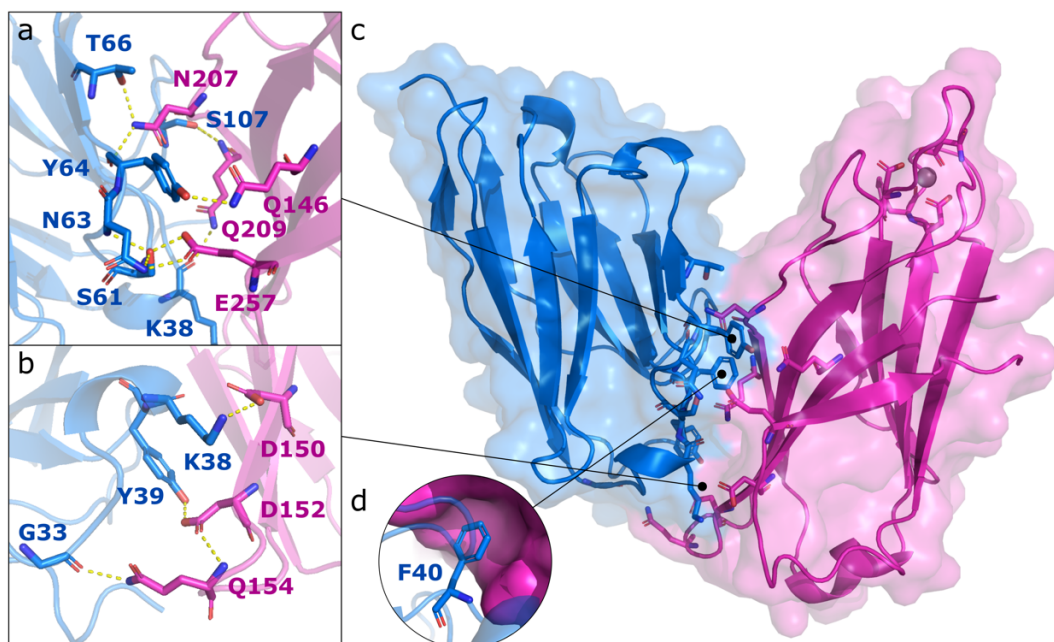
1
2 **Figure 1. Selection of nanobodies against Synaptotagmin 1.** (a) Alignment of five nanobody
3 candidates selected for binding *in vitro* to rSyt-1₍₉₇₋₄₂₁₎. Complementary determining regions (CDR) 2
4 and 3 show the maximum degree of variability. (b) Cos-7 cells transiently transfected with rat Syt-1
5 fused to EGFP (Syt1-EGFP) stained with NbA51 and NbA91 directly labeled with AbberiorStar635p
6 (Nb-Ab635p). Scale bar 10 μ m. (c) Hippocampal primary culture stained with Nb-Ab635p. Scale bar
7 10 μ m. (d) Co staining of primary neuronal culture using the NbA51-Ab635p and a conventional
8 monoclonal antibody revealed by polyclonal secondary antibody conjugated to Cy3. Scale bar 10 μ m.
9 Nuclei are stained with DAPI and depicted in greyscale.

10
11 To further confirm the specificity of NbSyt1, we performed the co-staining of primary
12 hippocampal neurons using a directly labeled version of the nanobody and a conventional
13 monoclonal antibody anti-Syt-1, which displayed virtually a complete colocalization on
14 confocal microscopy (Fig. 1d), confirming that this NbSyt1 appears to be also specific in a
15 neuronal environment. Next, we decided to understand more precisely where the NbSyt1 binds
16 within rSyt-1₍₉₇₋₄₂₁₎. For this, we produced various fragments of rSyt-1₍₉₇₋₄₂₁₎ and performed a
17 dot-blot assay. The analysis of these experiments indicated that the NbSyt1 binds to the C2A
18 calcium-binding domain of Syt-1. However, if the putative protein linker between C2A and
19 C2B is removed (fragment-4 & fragment-6, Supp. Fig. 2b) the affinity was strongly reduced or
20 completely lost. This finding motivated us to proceed with the crystallization of the complex
21 NbSyt1-Syt-1 and to resolve their interaction at high resolution.

1 The structure and interaction affinity of the complex NbSyt1 and C2A domain of rSyt-1

2 In order to perform X-ray crystallography structural studies, the NbSyt1 was expressed
3 and purified in its minimal size and without tags to be mixed in equal molar ratio with the tag-
4 free rSyt-1 C2A₍₁₄₀₋₂₆₅₎ domain. A crystal diffracting to 1.68 Å revealed the NbSyt1: C2A<sub>(140-
5 265)</sub> complex (PDB 8C5H; Supp. Table 1), burying a contact interface of approx. 662 Å². Both
6 proteins have similar folds, consisting of two antiparallel beta sheets and short alpha-helical
7 segments. Most contacts are between side chains on both the NbSyt1 and C2A₍₁₄₀₋₂₆₅₎, except
8 for S107 on the NbSyt1 and Q154 and Q209 on C2A₍₁₄₀₋₂₆₅₎, which make contacts with the
9 backbone of the neighboring chain. As shown in Figure 2d, F40 from the NbSyt1 extends into
10 a hydrophobic pocket on C2A₍₁₄₀₋₂₆₅₎ and is sandwiched between N207 and Q209. The C2A<sub>(140-
11 265)</sub> aspartate residues 172, 178 and 230 coordinate a calcium ion, and the presence of the
12 NbSyt1 does not seem to affect the calcium binding of the C2A domain. The residue E257 on
13 the crystal structure on the C2A₍₁₄₀₋₂₆₅₎ has contact with 2 amino acids on the NbSyt1.
14 Interestingly, E257 was missing in fragment-4 when performing the epitope mapping (Supp.
15 Fig. 2b), which could explain the lack of signal of NbSyt1 on fragment-4, but a strong signal
16 with fragment-6 (including E257). Next, we took the full cytosolic domain rSyt-1₍₉₇₋₄₂₁₎ and
17 determined with the microscale thermophoresis assay that the NbSyt1 binds with an affinity of
18 0.7±0.3 nM to rSyt-1₍₉₇₋₄₂₁₎ (Supp. Fig. 3).

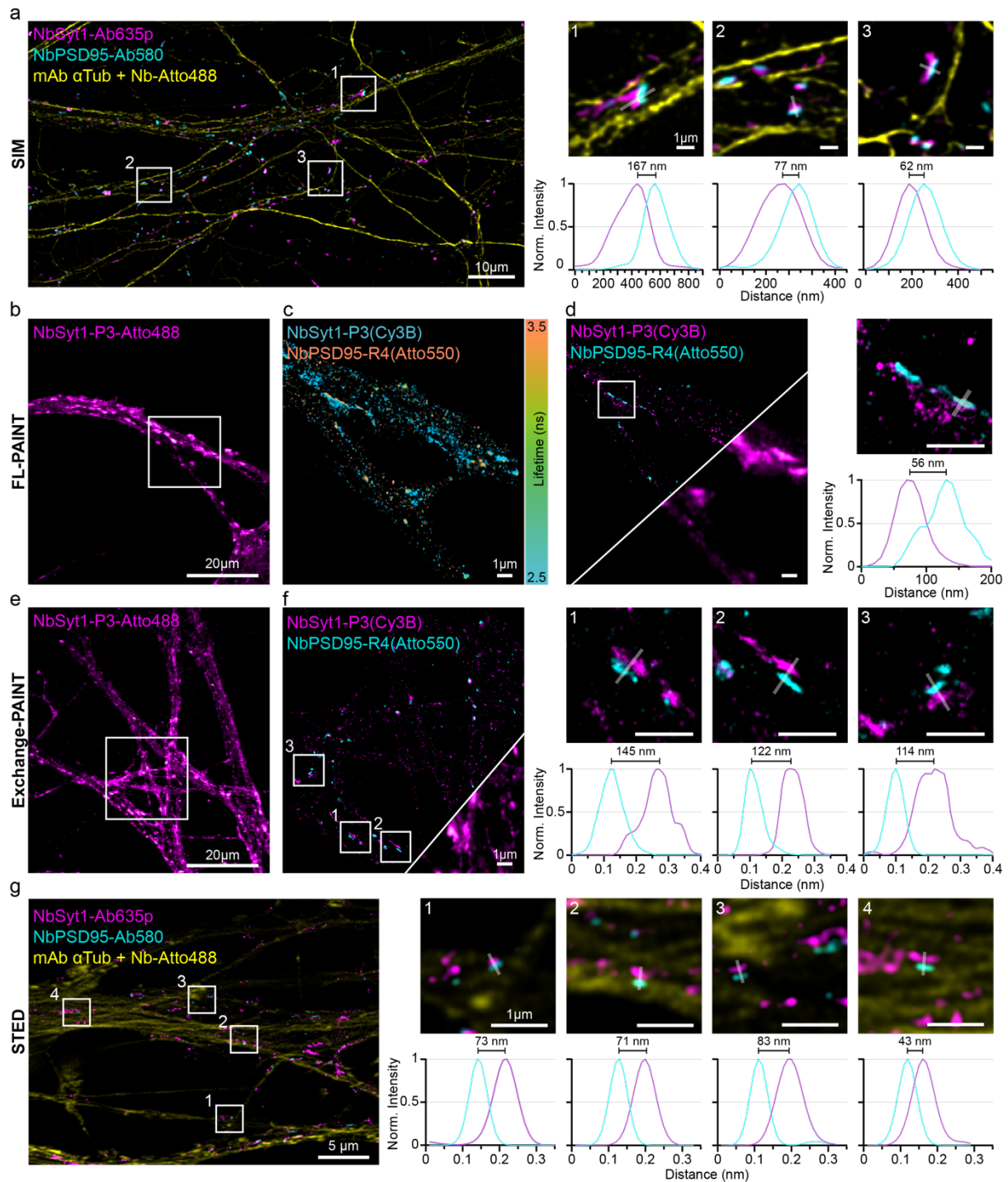
19 When analyzing the structure to understand why the NbSyt1 interacts very weakly with
20 rSyt-2, two points were observed. First, the glutamine Q209 in rSyt-1 is exchanged for
21 threonine in rSyt-2. This key interacting residue has direct contact with the backbone of K38
22 and F40 and a polar contact with side chain of residue S107 on the NbSyt1. Secondly, the
23 valine V255 in rSyt-1 is exchanged for proline in rSyt-2, which is expected to alter the
24 backbone geometry of the polypeptide chain and disrupting interactions in its proximity, e.g.
25 the interactions between E257 (conserved in rSyt-2) and the NbSyt1 (S61 & N63).



26
27 **Figure 2. NbSyt1 (blue) in complex with its target rat Synaptotagmin 1 cytosolic C2A₍₁₄₀₋₂₆₅₎**
28 **domain (magenta), PDB ID: 8C5H. (a, b) Polar contacts between amino acids of the NbSyt1(blue)**
29 **and C2A₍₁₄₀₋₂₆₅₎ domain (pink), (C) Overall structure of the complex, (d) Hydrophobic interaction**
30 **between F40 in the NbSyt1 and a hydrophobic pocket in Syt-1 C2A₍₁₄₀₋₂₆₅₎ domain.**

1 **Revealing Syt-1 with minimal linkage error in various super-resolution microscopy**
2 **techniques**

3 After determining the NbSyt1 binding affinity, binding epitope and specificity to Syt-
4 1, we conjugated the NbSyt1 directly to fluorophores or to single-stranded DNA (ssDNA), to
5 fulfill some of the requirements for various advanced imaging techniques like Stimulated
6 Emission Depletion (STED), Points Accumulation for Imaging in Nanoscale Topography
7 (PAINT) and Structural Illumination microscopy (SIM). Based on the structure, we know that
8 the linkage error from the fluorophore on the N-terminus of the NbSyt1 is only ~1.2 nm to the
9 closest part of the C2A, while the fluorophore located on the C-terminus is slightly below 4 nm
10 from its target. In the case of DNA-PAINT imaging, the Atto488 fluorophore on the imager
11 strand (i.e., the ssDNA annealing to the conjugated ssDNA on the NbSyt1) will be
12 approximately ~4 nm away from Syt-1. This means that for the super-resolution imaging
13 techniques tested here, the small linkage error imposed by the NbSyt1 is neglectable. Results
14 shown on Figure 3 suggest that the commercial STED and SIM setups provided a slightly lower
15 resolution as compared to single-molecule localization-based techniques: Exchange-PAINT
16 ^{20,21} and Fluorescence Lifetime-PAINT (FL-PAINT) ²² strategies. This becomes evident when
17 looking into the gaps in intensity profiles between the Syt-1 localization peak (at the pre-
18 synapse) with the postsynaptic density marker PSD-95 peak, also detected with a directly
19 labeled nanobody (Fig. 3bc). Exchange-PAINT had the tendency, in our comparison, to
20 provide a more accurate distance between pre- (Syt-1) and post- (PSD-95) synaptic
21 compartments (i.e., ~100-150 nm^{23,24}).



1
2 **Figure 3. Imaging of NbSyt1 in various advanced imaging techniques.** Primary hippocampal
3 neurons were stained and imaged. **(a)** Full field of view from Structured Illumination Microscopy (SIM)
4 of NbSyt1 (magenta), NbPSD95 (cyan) and mAb anti- α Tub (yellow). Nbs were directly conjugated
5 with the fluorophores and mAb anti- α Tub was revealed with anti-mouse IgG1 secondary nanobody
6 (2.Nb-Atto488). White squares denote the magnified regions used for the normalized fluorescent
7 intensity line profiles of NbSyt1 (magenta) and NbPSD95 (cyan). **(b)** Diffraction-limited imaging of
8 neurons stained with NbSyt1-P3-Atto488. White square indicates the region displaying fluorescence
9 lifetime PAINT (FL-PAINT). **(c)** Fluorescence lifetime imaging of NbPSD95-R4 and NbSyt1-P3-
10 Atto488 with the P3-Cy3B and R4-Atto550 imager strands. Image is color coded based on the recorded
11 lifetimes from 2.5 to 3.5 ns (see methods and Supp. Fig. 4). **(d)** Result image of NbSyt1-P3-Atto488
12 and NbPSD95-R4 after lifetime segmentation. The white square indicates the zoomed regions with a
13 the normalized fluorescent intensity line profiles of NbSyt1 (magenta) and NbPSD95 (cyan). **(e)**

1 Diffraction-limited imaging of NbSyt1-P3-Atto488. White square indicates the region selected to
2 display **(f)** Exchange PAINT imaging of NbSyt1-P3-Atto488 (magenta) and NbPSD95-R4 (cyan) using
3 P3-Cy3B and R4-Atto550 as imager strands. The zoomed-in regions are indicated by the white squares
4 1, 2 and 3 with the normalized fluorescent intensity line profiles of NbSyt1 (magenta) and NbPSD95
5 (cyan). **(g)** Stimulated Emission Depletion (STED) imaging of NbSyt1 (magenta), NbPSD95 (cyan)
6 and mAb anti- α Tub (yellow). Nbs were directly conjugated with fluorophores and mAb anti- α Tub was
7 revealed with 2.Nb-Atto488. White squares show the chosen regions of interest for the normalized
8 fluorescent intensity line profiles of NbSyt1 (magenta) and NbPSD95 (cyan). All distanced denoted
9 between the Syt-1 and PSD-95 signals are calculated from the max value after Gaussian fit of the
10 intensity line profile.

11
12 Next, we tested if this nanobody could be used in expansion microscopy (ExM). It has
13 been suggested that nanobodies might not work in all ExM techniques²⁵. Maybe due to their
14 small size and limited numbers of fluorophores, the signal from nanobodies might not be well
15 retained in some of the hydrogels used (Fig. 4a). Although this can happen, it strongly depends
16 on the actual sequence of the Nb and the ExM technique. Some nanobodies could be more
17 efficiently retained than others. With this limitation in mind, we modified the NbSyt1 by adding
18 4 extra lysines flanking the ectopic cysteines on their N and C-termini where the fluorophores
19 are site specific conjugated (ExM cassettes). The sequence is not digested by Proteinase K
20 (used for some ExM techniques) and added residues with negative charges to compensate the
21 positive charges introduced by the lysines. We aimed that these cassettes should help retaining
22 the fluorophores to the gel following fixation for ExM. Eventually, we managed to perform
23 10X ExM²⁶ with directly labeled NbSyt1 bearing two expansion microscopy cassettes
24 (ExNbSyt1) and we imaged Syt-1 on expanded synapses together PSD-95 revealed by co-
25 staining with a nanobody anti-PSD-95 (Fig. 4d).

26

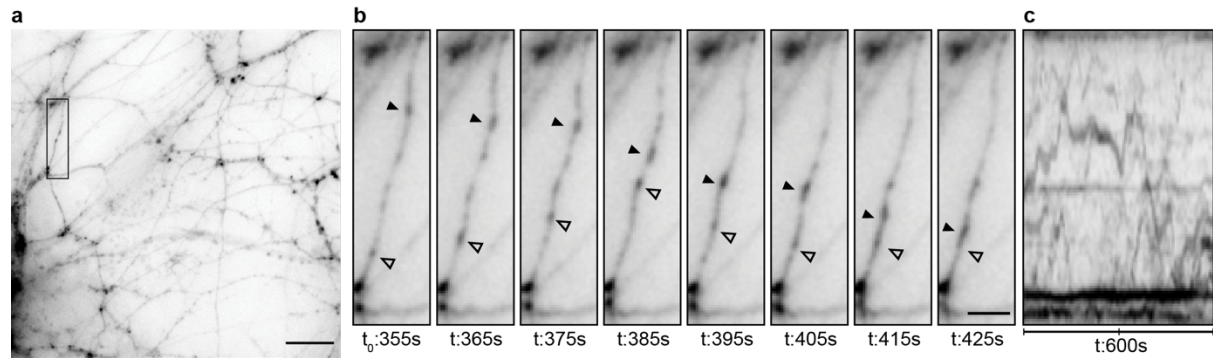


Figure 5. Live imaging of iNbSyt1-mScarlet. (a) Full field of view of a timelapse imaging (Supp. Movie 1). Area inside the black rectangle is magnified in b. Scale bar represents 10 μ m. (b) Crop region of time lapse imaging at different timepoints (t). Arrow heads denote iNbSyt1mScarlet signal moving along a neuronal process. Scale bar represents 5 μ m. (c) Ten minutes kymograph of the neuronal process.

Assessment of synaptic activity in presence of the iNbSyt1

To test whether our engineered NbSyt1 intrabody with its high specificity to the C2A domain of Syt-1 is not altering synaptic physiology, we investigated neuronal activity in primary neuronal culture. We first checked changes in synaptic vesicle release properties using the fusion of VAMP2 with the superecliptic GFP, also known as SynaptopHluorin (SpH)³¹. This approach allowed us to evaluate if the iNbSyt1 has any effect on synaptic vesicle release and SV endocytosis. First, we confirmed that the iNbSyt1 is clearly localized at presynaptic terminals where it strongly colocalized with immunostainings for Synaptophysin and Bassoon (Fig. 6a). Then, we generated a bicistronic AAV expressing the iNbSyt1 and SpH³¹. When following the SpH signal after 60 action potentials (AP) or 600 AP at 20 Hz stimulation rate, we were not able to observe differences in SpH signal traces between neurons with or without expression of the iNbSyt1 (Fig. 6b). These results directly suggest that the expected enrichment of iNbSyt1 at pre-synapses, does not affect synaptic vesicle fusion mechanisms under evoked release, nor the endocytosis and subsequent reacidification of vesicles.

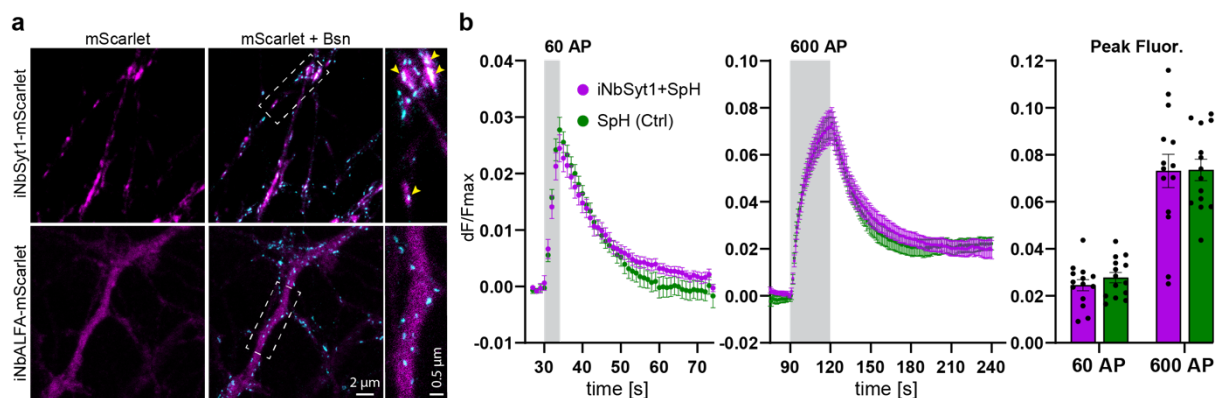
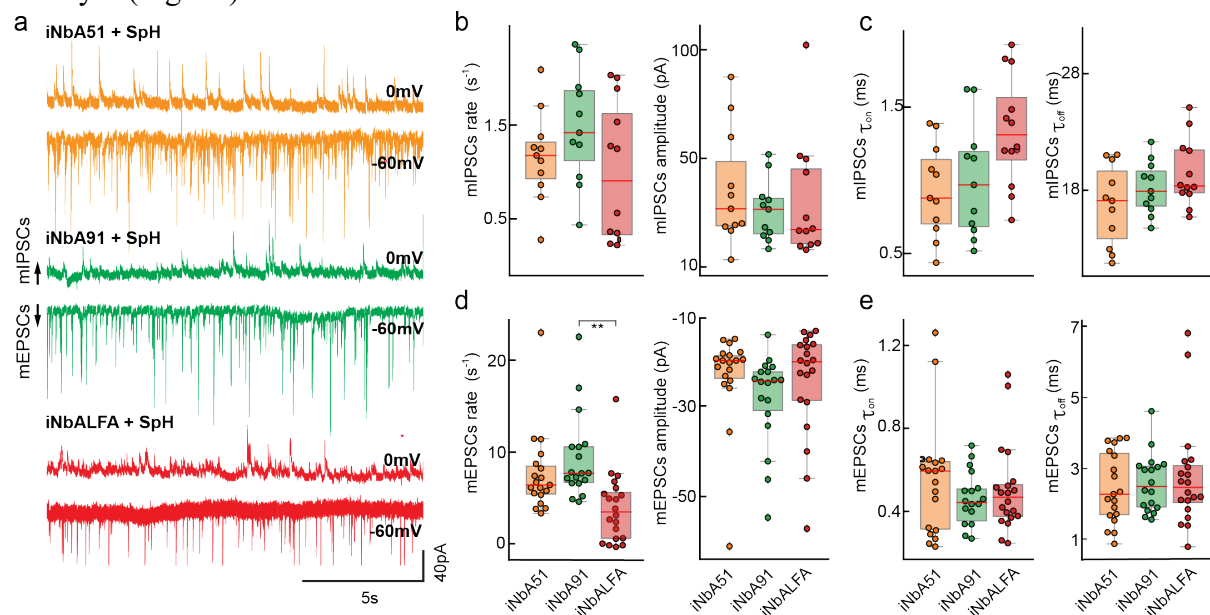


Figure 6. Intrabody iNbSyt1 localization and its potential effects on exocytosis physiology monitored using SynaptopHluorin. (a) The NbSyt1 as intrabody fused to mScarlet (iNbSyt1-mScarlet) displayed in magenta gets enriched in synapses confirmed by its co-localization signal (arrow heads on zoomed region) of immunostained Bassoon (Bsn) imaged under STED microscopy showed in cyan. Expression of the NbALFA fused to mScarlet as intrabody (iNbALFA-mScarlet) provides a diffused signal distribution throughout neuronal processes. Scale bar represents 2 μ m and 0.5 μ m in zoomed region. (b) Neurons AAV infected with a bicistronic expression of iNbSyt1 and SynaptopHluorin (SpH) enabled to monitor the iNbSyt1 potential effect on synaptic vesicle fusion competency and dynamics. The expression of only SpH as control, resulted in no significant difference in peak fluorescence nor in rise or decay time of pHluorin signal after electric field stimulation with 60

1 action potentials (AP) or 600 AP at 20 Hz. Plots in (b) display the average and the SEM from 14
 2 independent experiments (n=14), approximately 200 ROIs automatically were selected and analyzed
 3 per experiment with a total of 3323 ROIs for iNbSyt1 and 2860 for NbALFA.

4
 5 Next, we looked for changes in the spontaneous release rate that could potentially arise
 6 from expression of our Syt-1 intrabodies. We therefore recorded non-evoked excitatory and
 7 inhibitory postsynaptic currents in the presence of tetrodotoxins in primary neuronal culture
 8 (miniature EPSCs and IPSCs) (Fig. 7a) for both, the iNbSyt1 and the original candidate
 9 iNbA91. We observed no significant differences in the amplitudes (quantal release of
 10 neurotransmitters) nor in τ_{on} and τ_{off} kinetics between cultures infected with iNbSyt1
 11 and iNbALFA³² (used as a control intrabody since it has no target molecules in neurons). For
 12 the mEPSC rate, we observed a trend for both iNbSyt1 (iNbA51) and iNbA91 with a slight
 13 increase in rate of miniature postsynaptic currents, however not statistically significant for
 14 iNbSyt1 (Fig. 7d).

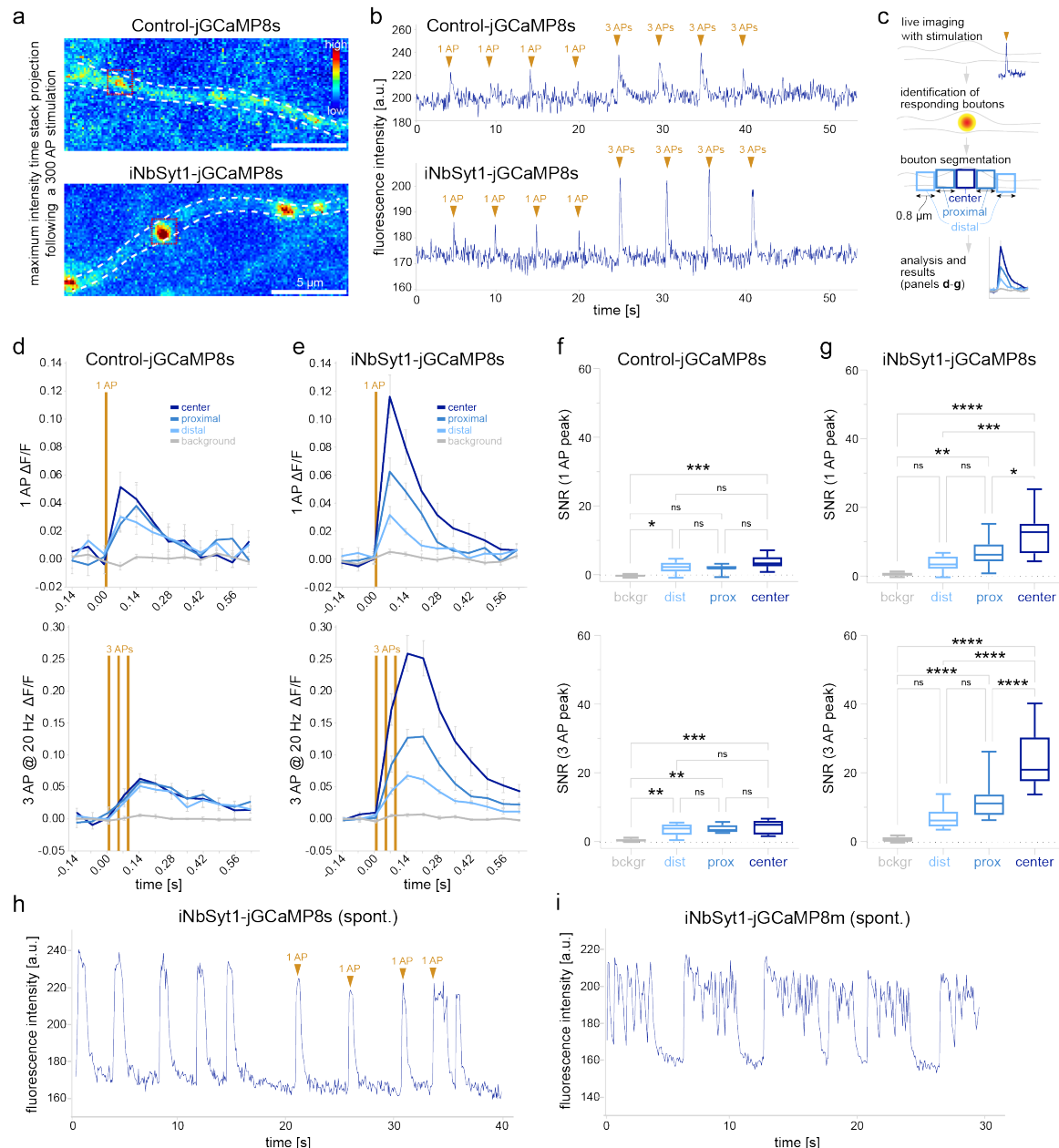


15
 16 **Figure 7. Electrophysiological analysis of mini EPSCs and IPSCs on primary hippocampal**
 17 **neurons.** (a) Exemplary traces of mEPSCs and mIPSCs of neurons in a whole-cell configuration
 18 clamped at -60 and 0 mV, respectively. Cultures were infected using AAVs containing sequences of
 19 the respective intrabody and SpH. Upper traces represent mIPSCs and lower traces mEPSPs. (b-d)
 20 Boxplots summarizing mIPSCs and mEPSCs properties such as rate and amplitude (b and d) and τ_{on}
 21 and τ_{off} (c and e). For statistics, mIPSCs, n = 10-12 cells per group; mEPSCs, n = 18-21 cells per
 22 group, a one-way ANOVA with multiple comparison was applied with Tukey correction. P-values:
 23 ** = $p < 0.01$.

24
 25 In light of our structural data that shows binding of NbSyt1 to the C2A domain of Syt-
 26 1, these findings are in line with the observations made by Courtney et al.⁸. They also observed
 27 that after genetically removing the C2A domain from Syt-1, a minimal effect on evoked release
 28 and a slight increase in the spontaneous miniature release could be observed. On the contrary,
 29 they also show that if the C2B domain is truncated, the action potential triggered synaptic
 30 vesicle fusion to the plasma membrane is drastically impaired⁸. Therefore, our data suggest
 31 that the iNbSyt1 bound to the C2A has no detrimental physiological effect in evoked activity
 32 on the synaptic physiology, and potentially neglectable effects in facilitating spontaneous
 33 release.

1 Targeted calcium detection on pre-synapses

2 After characterizing the nanobody, its interactions and its effects on synaptic
3 physiology, we thought to use it as an ideal tool to increase the synaptic targeting of genetically
4 encoded calcium indicator, to be able to measure the Ca^{2+} changes in close proximity of the
5 SV sensor for Ca^{2+} (i.e., Syt-1). This approach would allow us to reveal active synapses in
6 living neurons, and perform calcium imaging on pre-synapses with high precision and with
7 minimal impact on neuronal physiology, as we are not overexpressing chimeric synaptic
8 vesicle proteins for the targeting. For this we generated new AAVs expressing the NbSyt1 as
9 an intrabody and fused to the calcium sensor jGCaMP8s and jGCaMP8m (iNbSyt1-
10 jGCaMP8)¹⁹. As control, we used the iNbALFA fused to the calcium sensors. As the NbALFA
11 has been shown to work as intrabody³², but has no specific binding partners in rat hippocampal
12 neurons, this would be the most appropriate control for these experiments. As expected from
13 previous results, we observed the enrichment of synaptically-targeted calcium sensors enriched
14 at synaptic boutons (Supp. Movie 3), and mostly a diffused signal with the control (Fig. 8a).
15 Both sensors showed good signal following evoked 1 and 3 APs (Fig. 8b). However, when
16 observing the traces from single boutons, the synaptically localized sensor provided more
17 reliable signals, as single action potentials were sometimes lost in the background when
18 measuring with the control sensor (Fig. 8b). To further corroborate this observation, we
19 performed a more formal analysis on several boutons, also considering if the synaptically
20 targeted sensor provides additional spatial information for the calcium signal compared to the
21 control. For this we identified the boutons at the end of each movie following a 300 AP
22 stimulus, and used this signal to select the center of the boutons synaptic proximal and distal
23 areas (Fig. 8c). Using this analysis, we were able to see that the pre-synaptically localized
24 sensors provided more precise spatial resolution and better signal to noise ratio (SNR) when
25 compared to the control (Respectively Fig. 8d, e and Fig. 8f, g). Finally, having this highly
26 sensitive and localized calcium sensor, allowed us to follow spontaneous network activity and
27 observe bursts on primary hippocampal neurons using the slow and medium speed sensors
28 (Fig. 8h, i).
29



1
2 **Figure 8. A synaptically localized calcium sensor to precisely detect calcium changes with**
3 **improved intensity and spatial resolution.** (a) Expression of the iNbSyt1 fused to jGCaMP8s shows
4 clear presynaptic localization when compared to the control (iNbALFA). Scale bar 5 μm . (b) Exemplary
5 traces from single boutons following trains of 1AP or 3AP applied at defined time intervals. Note that
6 in some cases, due to low signal-to-noise ratio (SNR), it is difficult to observe 1AP responses with the
7 control sensor. (c) Scheme summarizing the analysis performed in panels d-g. Briefly, at the end of
8 each experiment, a strong 300 AP stimulation was applied to identify responding boutons. For the
9 analysis, the central area of each bouton was considered (center) together with the proximal or the distal
10 synaptic regions, defined as small ROIs of 0.8 μm adjacent to the center as schematized. (d-e) Signal
11 expressed as $\Delta F/F$ for the different synaptic regions for either the control or for the iNbSyt1-jGCaMP8s
12 following either 1AP or 3APs. Note that the response at the center of the bouton is higher than in the
13 periphery for the iNbSyt1-jGCaMP8s (panel e). N = 80 corresponding to measures obtained for 20
14 boutons for each condition for each stimulation pattern (coming from 3 independent coverslips). (f-g)
15 SNR analyses of the data from panels d and e. The average noise was calculated for each trace at rest
16 and the SNR was measured for a background region and for the regions as schematized in panel c. The
17 data are expressed as box plots where the whiskers represent the 5-95 percentiles. For statistical
18 significance, one way ANOVA followed by Tukey's multiple comparison tests was used. *P*-values:

1 * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ and **** = $p < 0.0001$. (h-i) exemplary traces of
2 spontaneous activity (without CNQX & D-AP5) measured respectively for the iNbSyt1-jGCaMP8s (h)
3 or iNbSyt1-jGCaMP8m (i). In panel (h) also exogenous stimulation (1AP) is able to trigger network
4 responses. Note that, during bursts, the faster jGCaMP8m version might be appropriate for revealing
5 faster changes in the calcium signal as previously published¹⁹.

6 7 **Discussion**

8 Here we developed and characterized an extremely flexible affinity tool, the NbSyt1,
9 that allows the study of Syt-1 from several angles. We prove the usefulness of this Nb in
10 conventional microscopy, various super-resolution imaging modalities, and as an intrabody in
11 living neurons to follow the molecular physiology of synapses without overexpressing a tagged
12 synaptic protein.

13 We selected a camelid single-domain antibody binding with excellent specificity and
14 affinity to the C2A calcium-binding domain of Syt-1. The structural data we obtained from the
15 Syt-1-NbSyt1 complex confirms the very high binding affinity (~0.7 nM) and specificity,
16 which is a consequence of polar and hydrophobic interactions, explaining its selectivity for
17 Syt-1. NbSyt1 does not recognize the C2A domain of Syt-2, which shares a very high degree
18 of identity with Syt-1 (77,7% identity on the full protein, and 86,6% on the C2A domain).
19 Importantly, the crystal structure also shows that the binding occurs on the opposite side from
20 the calcium-binding pocket of the C2A domain, which encouraged us to use this nanobody in
21 living neurons as an intrabody, as this region is likely to be not involved in lipid or other protein
22 interactions^{10,14}.

23 The advantages of nanobodies as affinity probes for imaging over classical antibodies
24 have been thoroughly described^{24,33,34}. Some of the main benefits are their monovalency
25 (stoichiometric labeling) and their small size allowing to place the fluorophore ~2–4 nm from
26 the target. These features make NbSyt1 ideal for quantitative imaging^{35–37} and high-precision
27 nanoscopy, respectively, where conventional antibodies would place the fluorophores with a
28 linkage error comparable to or sometimes larger than the resolution that different techniques
29 attain^{24,38}.

30 Additionally, the NbSyt1 is functional when expressed in neurons as an intrabody
31 (iNbSyt1). This can be clearly observed *in vivo* as the iNbSyt1 is able to get enriched on
32 presynaptic boutons with practically no effect on SV release physiology (Fig. 6). This feature
33 results in the possibility of specifically labelling synapses relying on the endogenous
34 expression levels of Syt-1 without changing the stoichiometry of synaptic proteins by over-
35 expression or modifying their function or location by fusing them with tags like fluorescent
36 proteins. Therefore, we demonstrated using SpH (Fig. 6) and electrophysiology (Fig.7)
37 strategies that synaptic vesicle machinery appears not to be affected by the presence of
38 iNbSyt1. A not significant tendency of an increased miniature rate when expressed iNbSyt1
39 could be related to the observations made previously by Courtney et al.⁸.

40 Finally, we exploited one of its unique features of bringing a sensor directly to the
41 presynaptic compartment, and we fused the iNbSyt1 to a genetically encoded calcium sensor
42 (jCaMP8), demonstrating that it enriches at synapses. This has a simple practical implication
43 as it makes it very convenient for deciding presynaptic regions to image where Syt-1 is
44 enriched. Moreover, having a calcium sensor directly positioned where the calcium should
45 matter the most for SV-release regulation, in close proximity to its sensor, results in the precise

1 and locally-enhanced detection of a single AP with an increased signal-to-noise ratio.
2 Similarly, we used this tool for recording the spontaneous activity of hippocampal neuronal
3 cultures using two chimeras with different speeds in detecting Ca²⁺ variations (respectively the
4 slower iNbSyt1-jCaMP8s and the faster iNbSyt1-jCaMP8m). These two tools will serve the
5 community depending on different experimental needs both for the study of presynaptic
6 calcium and for the role of Syt-1 in different cellular models.

7 Overall, here we established, engineered and thoroughly characterized a nanobody
8 binding to the C2A domain of Syt-1. This versatile tool shows outstanding features for
9 quantitative and super-resolution microscopy, but it also provides exceptional properties to be
10 used in living neurons and follow or manipulate presynaptic molecular physiology. We expect
11 this bio-tool to be instrumental in understanding Syt-1's role in SV fusion and neuronal
12 physiology.

13

1 **Material and Methods**

2

3 **Synaptosomes preparations.** Rat synaptosomes were enriched as previously described³⁹
4 Briefly, rat brains were homogenized using a glass-Teflon homogenizer in precooled sucrose
5 buffer (320 mM Sucrose, 5mM HEPES, pH 7.4). Centrifugation at 1000 x g for 2 minutes was
6 performed, and the supernatant was further centrifuged at 15,000 x g for 12 minutes. Next, a
7 discontinuous Ficoll density gradient was applied. The fractions at the interface of the 9%
8 Ficoll were pooled and washed in sucrose buffer.

9

10 **Immunizations.** Two alpacas were immunized with this preparation. The procedure was
11 performed by Preclinics GmbH (Postdam, Germany). Six injections were performed weakly
12 with 500 µg rat synaptosomes (total protein determined by BCA assay). Two weeks after the
13 last immunization a single boost with 500 µg of synaptosomes was performed and 100 ml of
14 blood was taken 3 and 5 days after the boost immunization. PBMCs were isolated using Ficoll
15 gradient and Serum was stored at -80°C. Total RNA was extracted using RNA extraction
16 Qiagen kit (Qiagen).

17

18 **Enrichment of IgG2 & IgG3 from plasma.** Plasma from the 2 fully immunized animal was
19 enriched in IgG2 and IgG3 following the protocol described by Hamers-Casterman with
20 minimal modifications⁴⁰. The affinity selection steps were done using an Äkta-Prime FPLC
21 system (Cytiva). Between 5 and 10 mL of plasma was diluted in PBS in a one-to-one ratio and
22 filtered through a 0.45 µm pore size syringe filters (Stedim Minisart®, Sartorius). The plasma
23 was then injected in HiTrap protein G HP (Cytiva), the flowthrough was collected and injected
24 in HiTrap protein A (Cytiva). The bound IgGs were eluted from the HiTrap protein G column
25 with first 0.15 M NaCl, 0.58% Acetic acid, pH 3.5 to collect mainly IgG3 and a second elution
26 using 0.1 M glycine-HCl, pH 2.7 to collect mainly IgG1. The IgG bound to the HiTrap protein
27 A column were eluted with 0.15 M NaCl, 0.58% Acetic, pH 4.0 to collect the IgG2. To
28 neutralize the pH of the collected fractions, 1 M Tris-HCl pH 9.0 was added and the buffer was
29 exchanged to PBS by injection in HiTrap Desalting Columns (Cytiva). A sample of the
30 collected and desalted fractions were analyzed by denaturing PAGE. IgG2 and IgG3 fractions
31 were pooled together, and residual IgG1 were further removed by incubating the pooled sample
32 with agarose beads conjugated with anti-llama light chain (Capralogics).

33

34 **Plasma-ELISA.** Purified antigen was immobilized on a 96 well immunosorbent plate (Nunc).
35 All the following steps were done by gentle shaking on an orbital shaker. 30 nmol of purified
36 Syt-1₍₁₉₇₋₄₂₁₎ was diluted in 200 µL of 100 mM Tris, 150 mM NaCl (pH 8.0) were coated
37 overnight at 4°C. The plate was then washed with PBS and blocked with 5% (w/v) skim Milk
38 in PBS for 3 h at RT. The enriched IgG2-IgG3 were added to the wells in a concentration of
39 0.5 mg/mL and incubated for 2h at RT. A series of three washes with PBS was performed. The
40 presence of bound IgG2 and IgG3 was revealed with the mouse anti-Camelid antibody coupled
41 to HRP (Preclinics, clone: P17Ig12) diluted 1:2000 in PBS. The antibody was incubated for 2
42 h at RT, then washed 3 times with PBS. The ELISA was revealed by addition of 100 µL of
43 TMB substrate (ThermoFischer) until the blue color was stable. The reaction was quenched by

1 addition of 100 μ L of 2 M sulfuric acid. The absorbance was then read at 430 nm using a plate
2 reader (BioTek).

3
4 **Nanobody library generation.** Total mRNA was extracted from the PBMC obtained from 2
5 alpacas using standard RNA extraction kit (Qiagen). Recovered mRNA was retrotranscribed
6 to cDNA by using Superscript IV (ThermoFischer) and the Cal 0001/2 primers as described
7 before⁴¹. Next, a second PCR was performed to introduce the Gibson cloning overhangs for
8 further insertion in the phagemid. The final PCR product was diluted to 5 ng/ μ L and some
9 loaded on a 1.5 % Agarose gel to confirming the right size of the PCR product. Fragments were
10 cloned into the phagemid using Gibson assembly. The phagemid backbone used was obtained
11 from the pHen2 plasmid. After Gibson cloning, the obtained construct was purified by PCR
12 purification kit (Qiagen) and the concentration was measured by Nanodrop. The constructs
13 were then electroporated in TG1 bacteria (Biocat). For the transformation, 65 ng of DNA were
14 added to 50 μ L of TG1. This process was repeated 20 times. The reactions were left 1 hour at
15 37°C for initial growth and then pooled in 400 mL of 2YT medium (ThermoFischer)
16 supplemented with antibiotics and grown overnight at 37°C. Next day, bacteria were pelleted
17 and resuspended in 25 mL LB medium (ThermoFischer) and 25 % Glycerol. The
18 Synaptosome-library was aliquoted, snap frozen and stored at -80 °C.

19
20 **Phage-Display.** The procedure was performed as described in Maidorn et al.⁴² with some
21 modifications. To start the process, a 1 mL the synaptosome nanobody library was diluted in
22 500 mL of 2YT supplemented with antibiotic and grow at 37°C until OD600 reached ~0.5.
23 Next, $\sim 0.1 \times 10^{13}$ M13KO7 Helper Phages (NEB) were added to the culture and let the infection
24 for 45 minutes at 37°C. Bacteria were then pelleted and resuspended in 500 mL 2YT medium
25 supplemented with the necessary antibiotics. Infected bacteria were incubated overnight at
26 30°C to produce the phages. The next day culture supernatant was incubated with 4 % (w/v)
27 PEG-8000 and let on ice for <2 hours to allow phages to precipitate. After several washings
28 steps in PBS, phages were filtered with a 0.45 μ m syringe filter (Sartorius). Purified Syt-1₍₉₇₋
29 421) was conjugated to desthiobiotin-N-Hydrosuccimide Ester (Beryy and Associates). Excess
30 of dt-Biotin-NHS was removed using Nap10 column (Cytiva). Between 1 and 3 nmol of
31 antigen was bound pre-equilibrated Dynabeads MyOne Streptavidin C1 (ThermoFischer) for
32 1 hour at RT. After binding of Syt-1₍₁₉₇₋₄₂₁₎ to the beads, the beads were washed 5 times with
33 PBS supplemented with 0.01 % (v/v) Tween (PBS-T). The purified phages were mixed with
34 the beads loaded with Syt-1₍₁₉₇₋₄₂₁₎ and incubated for 2 hours at RT. Bead were thoroughly
35 washed in PBS-T. The elution of Syt-1₍₁₉₇₋₄₂₁₎ and bound phages was carried out using 50 mM
36 Biotin in PBS. Eluted phages were then used to reinfect TG1 cells and initiate another cycle of
37 panning (a total of 3 panning rounds were performed). After the last panning round, bacteria
38 were plated in LB agar supplemented with antibiotics. The next day 96 colonies were picked
39 and grew in 96-deep-well plates. A copy of the plate was stored at -80°C after adding 50%
40 glycerol.

41
42 **Phage-ELISA.** Each clone in the 96-deep-well plate was infected with helper phages and let
43 overnight producing phages. Bacteria are centrifuged down and supernatants full with phages
44 can be use directly. The desthio-biotinylated Syt-1₍₉₇₋₄₂₁₎ was immobilized at 4°C overnight on

1 a flat-bottom Nunc MaxiSorp™ 96-well plate (ThermoFischer). The plate was washed 3 times
2 with PBS-T and blocked using 5% skim Milk and 1% BSA in PBS-T for 2h at RT. Next, 25 µl
3 of phages produced by the picked colonies were incubated on the well with 75 µL 5% skim
4 milk in PBS-T for 1 hour at RT. The unbound phages were washed six times with PBS-T, and
5 bound phages were detected with anti-major coat protein M13-HRP (Santa Cruz, #sc-53004-
6 HRP) diluted 1:1000 in 100 µL PBS-T. The antibody was let for 1 hour at RT and excess was
7 thoroughly washed away with PBS-T. TMB substrate (3,3',5,5'-tetramethylbenzidine, 1-
8 Step™ Ultra TBM-ELISA, Thermo Scientific) was added to each well and the colorimetric
9 reaction was stopped by addition of 100 µL of 2M sulfuric acid. The absorbance was read at
10 450 nm throughout the plate (Cytation™ 3: BioTek™ Instruments, Inc.).

11
12 **Protein expression and purification.** Syt-1 fragments were produced in NEB-Express (NEB),
13 while nanobodies were produced in SHuffle® Express (NEB). Bacteria were grown on terrific
14 broth supplemented with kanamycin at 37°C for Syt-1 and 30°C for nanobodies. When OD600
15 reached ~2-3, 0.4 mM IPTG was added and temperature was set to 30°C. Induction was
16 allowed for ~16h (overnight). Fully grown cultures were centrifuged and pellet resuspended in
17 cold lysate buffer (LysB: 100 mM HEPES, 500 mM NaCl, 25 mM imidazole, 2.5 mM MgCl₂,
18 10% v/v glycerol, 1 mM DTT, pH 8.0) supplemented with DNase (1:250), lysozyme (1:250)
19 and 1 mM PMSF. After 30 minutes incubation and disruption by sonication, lysate was
20 centrifuged at ~11,000g for 1h at 4°C. Supernatant was incubated with LysB equilibrate Ni⁺
21 beads (Roche cOmplete Resin) for 1h at RT. Beads were washed with 3 column volumes using
22 LysB buffer, 5 CV with high salt buffer (HSB; 50 mM HEPES, 1.5 M NaCl, 25 mM imidazole,
23 2.5 mM MgCl₂, 5% v/v glycerol, 1 mM DTT, pH 7.5). Finally, before the elution, beads were
24 washed in the buffer of choice for the next application. Elution was carried out using self-
25 produced SUMO protease cleaving on column the HisTag-SUMO domain, releasing the
26 protein of interest. Fractions were collected until absorbance a 280 nm drops to baseline. Eluted
27 proteins were initially evaluated on a PAGE.

28
29 **Fluorophore conjugation to nanobodies.** Purified nanobodies bearing one or two ectopic
30 cysteines (at their C-terminus or N- and C-termini) were reduced for 1 h using 10 mM of tris(2-
31 carboxyethyl)phosphine (TCEP), pH 7. The excess of TCEP was removed using a gravity
32 NAP5 column (Cytiva) previously equilibrated with degasses PBS pH 7.4. Freshly reduced
33 nanobodies were immediately mixed with ~3-5 molar excess of maleimide functionalized
34 fluorophore and incubated for 1h. The excess of dye was removed by using Superdex™ 75
35 increase 10/300 GL column (Cytiva) on Äkta FPLC system.

36
37 **Oligonucleotide conjugation to nanobodies.** NbSyt1 was produced having one ectopic
38 cysteine on its C-terminus, while secondary anti-mouse IgG1 nanobody and anti PSD-95
39 nanobody carrying an ectopic cysteine were obtained from NanoTag Biotechnologies GmbH
40 (#N2005 and #N3705). Nanobodies used for DNA-PAINT imaging were coupled with
41 different docking single-DNA-strands (Biomers GmbH, Ulm, Germany) as described
42 earlier^{21,22}. In brief, nanobodies were reduced with 5 mM TCEP, pH: 7, (Sigma-Aldrich,
43 #C4706) for 2 h. After removal of TCEP via 10 kDa molecular weight cut-off (MWCO)
44 Amicon spin filters (Merck, #UFC500324), the reduced nanobodies were coupled using 10-

1 fold excess of maleimide-DBCO crosslinker (Sigma-Aldrich, #760668). The excess of
2 crosslinker was removed using 10 kDa MWCO Amicon spin filters. Azide functionalized
3 DNA-strands (Biomers) were mixed in excess with nanobody-DBCO to promote the azide-
4 alkyne cycloaddition (click) reaction. The excess Azide docking DNA-strands were removed
5 by size exclusion chromatography using an Äkta pure 25 system (Cytiva) equipped with the
6 Superdex® Increase 75 column (Cytiva). The sequences of the docking strands P3 (3'-
7 TTTCTTCATTATTTT-5') and R4 (3'-ACACACACACACACACACA-5')^{43,44}

8

9 **Dot blot assays**

10 Target proteins and negative control (BSA) were spotted (~1 µg and ~5 µg respectively) on a
11 nitrocellulose membrane and let them dry at RT. Membranes were incubated with blocking
12 buffer (PBS supplemented with 0.05% (v/v) of Tween20 and 5% milk) under gentle shaking
13 for 2h. After blocking step, blocking buffer was removed and membranes were incubated with
14 NbA51 or NbA91 directly labeled with AbberiorStar635p in 5% milk PBS-Tween20 at a final
15 concentration of 5 nM for 1h. For specificity assay in Supp. Fig 2, one membrane was
16 incubated with 1:100 dilution of polyclonal affinity purified anti-Synaptotagmin 1/2 cytosolic
17 domain (SySy, #105 003) premixed with 3 molar excess of FluoTag®-X2 anti-Rabbit IgG
18 conjugated to AbberiorStar635p (NanoTag Biotechnologies, #N2402-Ab635P). Membranes
19 were thoroughly washed with PBS-T and fluorescence signal was detected in Amersham™
20 Imager 600.

21

22 **Cell culture.** COS-7 cells were cultured in Dulbecco's MEM supplemented with 10% FBS, 4
23 mM L-glutamine, 0.6% penicillin and streptomycin, cells were cultured at 37°C, 5% CO₂ in a
24 humidified incubator. COS-7 fibroblasts were obtained from the Leibniz Institute DSMZ—
25 German Collection of Microorganisms and Cell Culture (DSMZ Braunschweig, Germany). For
26 immunostainings, cells were plated on poly-L-lysine (PLL)-coated coverslips.

27 Rat primary hippocampal neuron cultures for imaging were prepared as described before²⁸. In
28 brief, the brains of P1-2 rat pups were extracted and placed in cold HBSS (ThermoFisher). The
29 hippocampi were extracted and placed in a solution containing 10 mL DMEM (ThermoFisher),
30 1.6 mM cysteine, 1 mM CaCl₂, 0.5 mM EDTA, 25 units of papain per mL of solution, with
31 CO₂ bubbling, at 37°C for 1h. The solution was removed and the hippocampi were incubated
32 in 10% FBS-DMEM, 73 µM albumin for 15 minutes. The hippocampi were triturated using a
33 10 mL pipette in complete-neurobasal medium (Neurobasal A (ThermoFisher), containing 2%
34 B27 (ThermoFisher) and 1% Glutamax-I (ThermoFisher)). Neurons were plated (12-well
35 plate) on glass coverslips coated with poly-L-lysine-hydrochloride (1 mg/ml, Merck). After 2h,
36 the plating medium was replaced with 1.25 ml complete-neurobasal medium and neurons were
37 incubated for 15 days at 37°C, 5% CO₂ in a humidified incubator. Cultures for
38 electrophysiology recordings were prepared according to Goslin and Banker⁴⁵ using embryonic
39 day 18 old rat embryos. Cells were plated in a density of 40.000 cells per 18 mm coverslip,
40 grown in 1 ml of neurobasal medium (NB, Gibco) supplemented with B27 medium. Ara-C
41 (cytosine β-D-arabinofuranoside) at a final concentration of 5 µM was included in the culture
42 medium at DIV 7 to suppress glia proliferation.

43

1 **Affinity determination.** The affinity of the NbSyt1 was measured by microscale
2 thermophoresis using the device NT.115Pico Monolith (NanoTemper). NbSyt1 was labelled
3 with Alexa647 (as described above) and diluted in MST buffer (NanoTemper) supplemented
4 with 0.05 % Tween. Fluorescent NbSyt1 was incubated with different dilutions of purified Syt-
5 1₍₉₇₋₄₂₁₎ using Premium Coated Capillaries (NanoTemper). For operation of the instrument and
6 evaluation of affinity data, the MO.Control and MO.Affinity Analysis software (NanoTemper)
7 were used.

8
9 **Immunofluorescence.** For Fig. 1b COS-7 Cells were fixed for 20 minutes using 4%
10 paraformaldehyde (PFA) in PBS at RT. After rinsing short with PBS, remaining aldehyde
11 groups were quenched for 15 minutes using 0.1 M glycine in PBS at RT. Cells were blocked
12 and permeabilized with 3% (w/v) BSA + 0.1% (v/v) Triton X-100 for 40 minutes at RT and
13 gently shaking. Nanobody candidates were applied in PBS supplemented with 1.5% BSA and
14 0.05% Triton X-100 for 2h at RT with gentle shaking. After staining, several washing steps
15 using PBS were carried out including DAPI short staining. Coverslips were shortly rinsed in
16 distilled water and mounted using Mowiol (12 ml of 0.2 M Tris buffer, 6 ml distilled water, 6
17 g glycerol, 2.4 g Mowiol 4-88, Merck Millipore). Samples were imaged directly or within the
18 next 48h, samples were kept at 4°C. Primary hippocampal neuronal cultures (15 DIV) imaged
19 in Fig. 1c and Fig.3 were first fixed with 4% PFA solution for 30 minutes at RT and quenched
20 with 0.1 M glycine (Merck) solution in PBS for 15 minutes at RT. Samples were permeabilized
21 and blocked with PB buffer (1.5% BSA and 0.05% Triton X-100 in PBS at pH 7.4) for 40
22 minutes at RT. For immunostaining, mouse monoclonal anti-Synaptotagmin 1 (#105011,
23 SySy) recognized by conventional secondary Ab conjugated to Cy3 (1:500, #115-005-166,
24 JIR; for Fig. 1) or for Fig. 3a and g, the anti- α -Tubulin primary Ab (#302211, SySy) was pre-
25 mixed with FluoTag-X2 anti mouse IgG1 coupled to Atto488 (#N2002, NanoTag
26 Biotechnologies) initially in 20 μ l of PBS and after 30 minutes at RT, 980 μ l of PBS was added
27 to bring the concentration of Ab and nanobody to 7 and 15 nM respectively. Into this pre-
28 mixture tube, 25 nM of FluoTag-X2 anti-PSD95 coupled to AbberiorStar580 (#N3702,
29 NanoTag Biotechnologies) and 25 nM NbSyt1 coupled to AbberiorStar635p were added.
30 Samples were incubated for 1 hour at RT under slow orbital stirring. Neurons were washed 3x
31 with PBS and rinsed once with high salt PBS (0.5 M NaCl). Samples were finally rinsed in
32 water and mounted on glass slide using Mowiol. For DNA-PAINT imaging (Fig. 3b, c, d, e
33 and f) immunostaining was performed using ~50 nM of the NbSyt1 functionalized with the
34 docking DNA strand P3 coupled to Atto488 on its 3'-end (NbSyt1-P3-Atto488).
35 Simultaneously, post-synapses were labelled with 50 nM of FluoTag-X2 anti-PSD95 (#N3702,
36 NanoTag Biotechnologies) functionalized with the docking DNA strand R4 on its 3'-end
37 (NbPSD95-R4). After immunostaining, samples were post-fixed with 4% PFA for 15 minutes,
38 quenched with 0.1 M Glycine and stored in PBS in 4 °C until imaged.

39
40 **Crystallization and structure determination.** NbSyt1 was incubated with the cytosolic
41 C2A₍₁₄₀₋₂₆₅₎ domain of Synaptotagmin 1 at 1:1 molar ratio and concentrated to 65 mg/ml using
42 3000 Da cut-off spin columns at 4°C, prior to setting sitting-drop crystallization plates. Crystals
43 of the NbSyt1-Synaptotagmin 1 complex suitable for data collection were found in the G8
44 condition from the JCSG+ screen (Molecular Dimensions), containing 0.15 M Di-Malic acid

1 pH 7.0, 20% PEG 3350 at 1:1 volume ratio with the protein solution. Crystals were frozen and
2 data collection was performed at the BioMAX beamline, MAX IV (Lund, Sweden)⁴⁶. The data
3 were processed using XDS⁴⁷, molecular replacement was performed with Phaser⁴⁸ using
4 structures 5T0R⁴⁹ and 6I2G³² as search models. The structure was built using Coot⁵⁰ and
5 refined in Phenix⁵¹. Data collection and refinement statistics are shown in Supp. Table 1.

6
7 **Live intrabody imaging and electrical stimulation in hippocampal neurons.** Images were
8 taken by an inverted Nikon Ti epifluorescence microscope (Nikon Corporation, Japan)
9 equipped with a Plan Apochromat 60×, 1.4 NA oil immersion objective, an IXON X3897
10 Andor camera, an Oko Touch Environmental control and NIS-Elements software.

11 *Live imaging of intrabodies:* Rat hippocampal neurons (DIV ~11) were transduced with an
12 adeno-associated virus (AAV) containing the sequence for expression of directly fluorescently
13 labelled (mScarlet or mNeonGreen) intrabodies (iNbSyt1-ALFA / iNbALFA-3xFLAG) and
14 imaged live 3-4 days post-infection. Transduced neurons were placed onto a custom-built live-
15 imaging chamber and imaged in Tyrode's solution (124 mM NaCl, 5 mM KCl, 30 mM glucose,
16 25 mM Hepes, 2 mM CaCl₂ and 1 mM MgCl₂ at pH 7.4) at ~0.2 frames per second (fps).

17 *SynaptopHluorin (SpH):* Rat hippocampal neurons (DIV ~11) were infected with an AAV
18 holding a bicistronic plasmid for expression of the respective intrabody and SpH. Live imaging
19 experiments were performed between day 3-5 post-infection. Transduced neurons were placed
20 onto a custom-built live-imaging chamber and imaged in Tyrode's solution supplemented with
21 10 μM cyanquixaline (CNQX, Tocris Bioscience, Cambridge, UK) and 50 μM 2-amino-5-
22 phosphonopentanoic acid (D-AP5; Tocris Bioscience, Cambridge, UK) to avoid spontaneous
23 network activation. Neurons underwent field stimulation at 30s (for 3s at 20Hz; i.e., 60 AP)
24 and for 90s (600 AP) while imaged at a rate of 1 fps. After electrical stimulation, 50 mM NH₄Cl
25 was applied to evaluate the total SpH pool³¹.

26 *Calcium imaging:* Rat hippocampal neurons (DIV ~10) were infected with an AAV leading to
27 expression of the respective intrabody fused to the Ca²⁺-sensor jRCaMP8s/m¹⁹. Live imaging
28 experiments were performed between day 4-6 post-infection. Transduced neurons were placed
29 onto a custom-built live-imaging chamber and imaged in Tyrode's solution. For the assessment
30 of spontaneous network activity, neurons were imaged for 60s in the absence of blockers. For
31 responses following field stimulation, the Tyrode's solution was supplemented with 10 μM
32 CNQX and 50 μM D-AP5 to avoid spontaneous network activation. Neurons underwent field
33 stimulation at 20Hz using one field stimulation (one action potential, 1AP) or 3APs. Images
34 were taken at a rate of 15 fps.

35 The analysis of images with these sensors was performed using Fiji/imageJ⁵². For synaptic
36 endocytosis, briefly, based on the total content of SVs (signal uncovered with NH₄Cl
37 treatment), regions of interest were set and fluorescence intensity measured and averaged for
38 each time point per image. These averaged time courses then were normalized to fluorescent
39 intensities directly preceding the respective stimulation trigger, bleach corrected and peak
40 aligned. For jRCaMP8s analyses, the responding boutons were identified at the end of the
41 movie applying 300 APs. The ΔF/F was calculated measuring the basal fluorescence, averaging
42 the fluorescence for at least 5 time points before each stimulation. This basal initial
43 fluorescence (F) was used to calculate the ΔF (corresponding to the fluorescence at each time

1 point from which the initial fluorescence F was subtracted), divided by F . This $\Delta F/F$ is to
2 account for possible small differences in the expression of the sensors across different cells.

3
4 **Confocal & STED microscopy.** Images from mounted samples in Mowiol and expanded gels
5 in a custom-made chamber were acquired using STED Expert line microscope (Abberior
6 Instruments). The microscopy setup was comprised of an IX83 inverted microscope (Olympus)
7 equipped with UPLSAPO 100x 1.4 NA oil immersion objective (Olympus). In addition, 488
8 nm, 561 nm and 640 nm lasers were used for confocal imaging. High-resolved images were
9 obtained from the same setup using the 595 nm and 775 nm pulsed STED depletion lasers in
10 combination. Images were analyzed in Fiji/ImageJ (v. 1.53o).

11
12 **Structured Illumination Microscopy (SIM).** Imaging was performed on the Elyra 7 (Zeiss)
13 with Lattice SIM² microscope equipped with a Plan-Apochromat 63x 1.4 NA oil immersion
14 objective (Zeiss). Highly resolved 3D 3-coloured images were acquired on 80 x 80 μm field of
15 view after overlapping 40 z-stacks. For that, glass slides of immunostained neurons mounted
16 in Mowiol were excited with 642nm, 561nm and 488nm laser wavelengths. Image
17 reconstruction and analysis took place in the ZEN software (Zeiss), after acquiring several
18 frames from each z-stack.

19
20 **Exchange-PAINT and FL-PAINT imaging and data analysis.** Both Exchange-PAINT and
21 FL-PAINT measurements were performed on a custom-built confocal setup⁵³. The imaging
22 chamber was fixed with clips to the microscope's stage and a PDMS layer was used as top
23 cover. Prior to the super-resolution imaging, an area of 80 μm \times 80 μm was scanned and then
24 a suitable region for imaging was selected based on the NbSyt1-Atto488 signal. Typically, 20k
25 scan images with virtual pixel size of 100 nm and pixel dwelling time of 2.5 μs were recorded.
26 The total acquisition time was around 45 minutes for a 20 μm \times 20 μm scan region. The laser
27 power was adjusted according to the sample brightness. In FL-PAINT experiments, a mix of
28 imager DNA strands R4-Atto550 (5'-TGTGTGT-Atto550-3'), which labeled the NbPSD95
29 and P3-Cy3B (5'-GTAATGAAGA-Cy3B-3'), which labeled NbSyt1, were used at a final
30 concentration of 0.1 nM each in PBS buffer including 500 mM NaCl. For Exchange-PAINT
31 imaging, the solutions exchange in experimental chamber has been realized using custom-built
32 microfluidics system²¹. First, R4 imager DNA strand was diluted in PBS buffer including 500
33 mM NaCl to the final imaging concentration of 0.1 nM. After imaging the first target, the
34 imager was washed away from the chamber by flushing the chamber with PBS buffer. Then,
35 the imaging buffer with the next imager was introduced into the chamber and the next imaging
36 round has been performed. In Exchange-PAINT and FL-PAINT data analysis, we chose a time
37 binning of three scanned frames, corresponding to the time bin of 0.3 s, while the scan region
38 was 20 μm \times 20 μm . The detection of emitter candidates and its sub-pixel localization were
39 performed using a cross-correlation algorithm and pixel-integrated Gaussian MLE fitting²².
40 Blinking events detected in a single frame were discarded. Out-of-focus localizations with
41 point spread function (PSF) width of more than 180 nm or/and a number of detected photons
42 in a single event smaller than 100 were rejected. For lifetime determination, we discarded the
43 first 0.1 ns after the maximum of the TCSPC histograms, and tail-fitted the remained curve of
44 a histogram with a mono-exponential function using a maximum likelihood estimator (MLE).

1 Lifetime values in the range from 0.5 to 5.0 ns were included in the analysis. Subsequently, a
2 drift correction was applied, and finally DNA PAINT images were reconstructed. The
3 following average localization precision values were calculated: 11.9 nm for Figure 3c, 13.6
4 and 8.4 nm for Figure 3f (P3 and R4 imagers). For targets crosstalk estimation in the FL-PAINT
5 imaging in Fig 3c, we fitted the lifetime histograms with two-Gaussian function and determined
6 lifetime threshold values manually to separate between the two different targets. Then, we
7 calculated the total crosstalk as the total number of localizations in-between thresholds (wrong
8 attribution), divided by the total number of localizations beyond thresholds (correct
9 attribution), as shown in Supp. Figure 4.

10
11 **Expansion Microscopy.** Primary hippocampal neurons were washed once with pre-warmed
12 PBS (37°C) and fixed using 4% PFA solution for 10 minutes at 4°C and then for 30 minutes at
13 RT. Unreacted aldehydes were quenched with 100 mM NH₄Cl in PBS for 20 minutes at RT.
14 Neurons were washed three times with permeabilization and blocking (PB) buffer (2% BSA,
15 0.1% Triton-X 100 in PBS). For immunolabeling, NbSyt1 equipped with anchoring cassettes
16 and AbberiorStar635p together with nanobody anti- PSD95 (FluoTag-X2 anti-PSD95,
17 #N3705; NanoTag Biotechnologies) conjugated to sulfo-AlexaFluor488 were used at 20 nM
18 in PB buffer for 1h at RT. Excessive probes were washed with PB buffer and then samples
19 were postfixed using 4% PFA in PBS for 10 min at RT followed by quenching with 100 mM
20 NH₄Cl in PBS for 15 minutes at RT. Next, samples were undergone 10x Expansion²⁶ with
21 some modifications. In Brief: Acryloyl-X (#A20770, ThermoFisher) stock solution (5 mg) was
22 diluted in DMSO reaching the concentration of 10 mg/ml (pH: 8) followed by further dilution
23 to 0.3 mg/ml in PBS and 500 µl was added on the cells. Samples were incubated overnight at
24 RT in the dark and washed with PBS before subjected to gelation. Fresh monomer solution
25 (MS) was prepared by adding 1.335g N,N-Dimethylacrylamide (DMAA, #274135, Sigma)
26 stock solution and 0.32g sodium acrylate (SA, #408220, Sigma) in 2.85 ml of ddH₂O.
27 Potassium persulfate solution (KPS) was freshly prepared at a stock of 36 mg/ml and added at
28 3.6 mg/ml in degassed ice-cold MS, protected from light. For the gelation reaction 4 µl TEMED
29 (#T7024, Sigma) was added to 1 ml of KPS/MS solution, vortexed and a droplet (90 µl) was
30 immediately spotted on parafilm. Thereafter, samples were placed with cells down on the
31 droplet and left for ~24h at ~23°C until the gel was fully polymerized. For Homogenization,
32 gel was detached from the parafilm, transferred into a glass petri dish and was incubated with
33 the disruption buffer (5% Triton-X and 1% SDS in 100 mM TRIS, pH 8.0) for 2 hours with
34 disruption buffer exchange at 30 min intervals. Next, samples were autoclaved for 20 minutes
35 at 121°C in disruption buffer and gels were transferred into 120mm square petri dish filled with
36 ddH₂O and expanded by exchanging the water every 30-60 minutes 4-5 times. Expanded gels
37 were left overnight in water and placed in custom-made chamber for imaging.

38
39 **Molecular biology.** Sequences for intrabody expression were synthesized (ThermoFischer)
40 fused to a C-terminal ALFA-tag and cloned into an AAV backbone⁵⁴, which additionally
41 contained an expression cassette for Synpatobrevin2-pHluorin (Synaptophluorin or SpH).
42 Both genes, the intrabody and the SpH are under the control of a hSyn1-promoter. For the
43 constructs used in calcium imaging, the first hSyn1-promoter together with the Vamp2-
44 pHluorin cassette was removed. Subsequently, the desired sequence for jGCaMP8s/m was

1 cloned in frame to NbSyt1-ALFA or NbALFA-3xFLAG by restriction sites for AgeI and AscI.
2 For live imaging of the intrabodies NbSyt1 (iNbSyt1) and NbALFA (iNbALFA), the nanobody
3 sequences were fused to mScarlet or mNeonGreen. Fluorescent proteins were amplified with
4 the following primers (forward: TCCACCGGTACCATGGTGAGCAAGGGCGAGG and
5 reverse: ATTGGCGCGCCTCTTGTACAGCTCGTCCATGCC) to introduce the restriction
6 sites AgeI and AscI and cloned into the AAV backbone containing the iNbSyt1-ALFA or
7 iNbALFA-3xFLAG.

8
9 **Adeno-associated viruses (AAVs).** The AAVs were generated as previously described^{54,55}.
10 AAVs were produced in HEK293T cells by co-transfection of pHelper plasmids (pFΔ6, pRV1,
11 p21) and pAAV target plasmid in a 4:1:1:2 molar ratio by use of Lipofectamine 2000
12 (ThermoFisher) according to company protocol. 72 h post transfection, cells were harvested
13 and lysed by 3 cycles of thawing and freezing followed by treatment with benzoase nuclease
14 (Millipore). After pelleting down cell debris (16.9 g/rcf, 30 minutes at 4°C), supernatants were
15 filtered, aliquoted and snap frozen in liquid nitrogen, before viruses were stored at -80°C until
16 used.

17 **Electrophysiology**

18 All experimental procedures were carried out in accordance with the EU Council Directive
19 2010/63/EU and were approved by the local Committee for Ethics and Animal Research
20 (Landesverwaltungsamt Sachsen-Anhalt, Germany). Electrophysiology was performed on
21 primary hippocampal cells infected with AAV encoding for the intrabody and SpH from
22 DIV14-18 under visual control using oblique illumination on a Slicecope Pro2000 (Scientifica,
23 Uckfield, East Sussex, UK). Prior to recordings, expression was verified pHluorin
24 fluorescence. Whole-cell voltage-clamp recordings were performed using a MultiClamp 700B
25 amplifier, filtered at 8 kHz and digitized at 20 kHz using a Digidata 1550A digitizer (Molecular
26 Devices). Extracellular Tyrode solution, adjusted to pH 7.4 and 300 mOsm, contained: 150
27 mM of NaCl, 4 mM of KCl, 1.25 mM of MgCl₂.6H₂O, 10 mM of glucose, 10 mM of HEPES,
28 2 mM of Ca²⁺, and 1 μM of tetrodotoxin (TTX). Intracellular pipette solution for voltage clamp
29 recordings, adjusted to pH 7.3 and 285 mOsm, contained: 115 mM of Cs-methanesulphonate,
30 10 mM of CsCl, 5 mM of NaCl, 10 mM of HEPES, 20 mM of TEA.Cl hydrate, 4 mM of
31 MgATP, 0.3 mM of GTP, 0.6 mM of EGTA, and 10 mM of QX314. Whole-cell recordings
32 were carried out at -60 mV (reversal potential of GABAA receptors) for mEPSCs and 0 mV
33 (reversal potential of AMPA and NMDA receptors) for mIPSCs. For statistical analysis (Fig.
34 7), two-tailed unpaired Mann-Whitney U test was performed. It is a nonparametric test in which
35 the sample medians are compared based on the shape of distribution between the two
36 independent groups. To correct significance level, a Bonferroni correction was then employed.
37 The resulting box plots display the median and the first and third quartile with whiskers
38 extending to 1.5 times the interquartile range above and below the upper and lower quartiles,
39 respectively ($Q1 - 1.5 * IQR$ or $Q3 + 1.5 * IQR$).

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16 **Author contributions**

17 KQZVR, NM, RR and SSI designed, performed and analyses most of the experiments. LA
18 helped with calcium imaging and its analysis, AMR and KAS planned and performed ExM. MM
19 initiated the phage-display screenings, JH produced and conjugated proteins. MMC and PS
20 planned, performed and analyzed the structural biology work. HH and MP planned, performed
21 and analyzed electrophysiology data. NO and RT helped with imaging and analysis of
22 Exchange PAINT and FL-PAINT. EFF, designed and guided experiments, performed analysis
23 and wrote the manuscript. FO conceived and lead the project, designed performed and guided
24 experiments, analyzed data, and wrote the manuscript. All authors contribute with writing.

26 **Competing interests**

27 FO is a shareholder of NanoTag Biotechnologies GmbH. All other authors declare no
28 competing interests.

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