Trans-synaptic molecular context of NMDA receptor nanodomains

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SUPPLEMENTAL INFORMATION

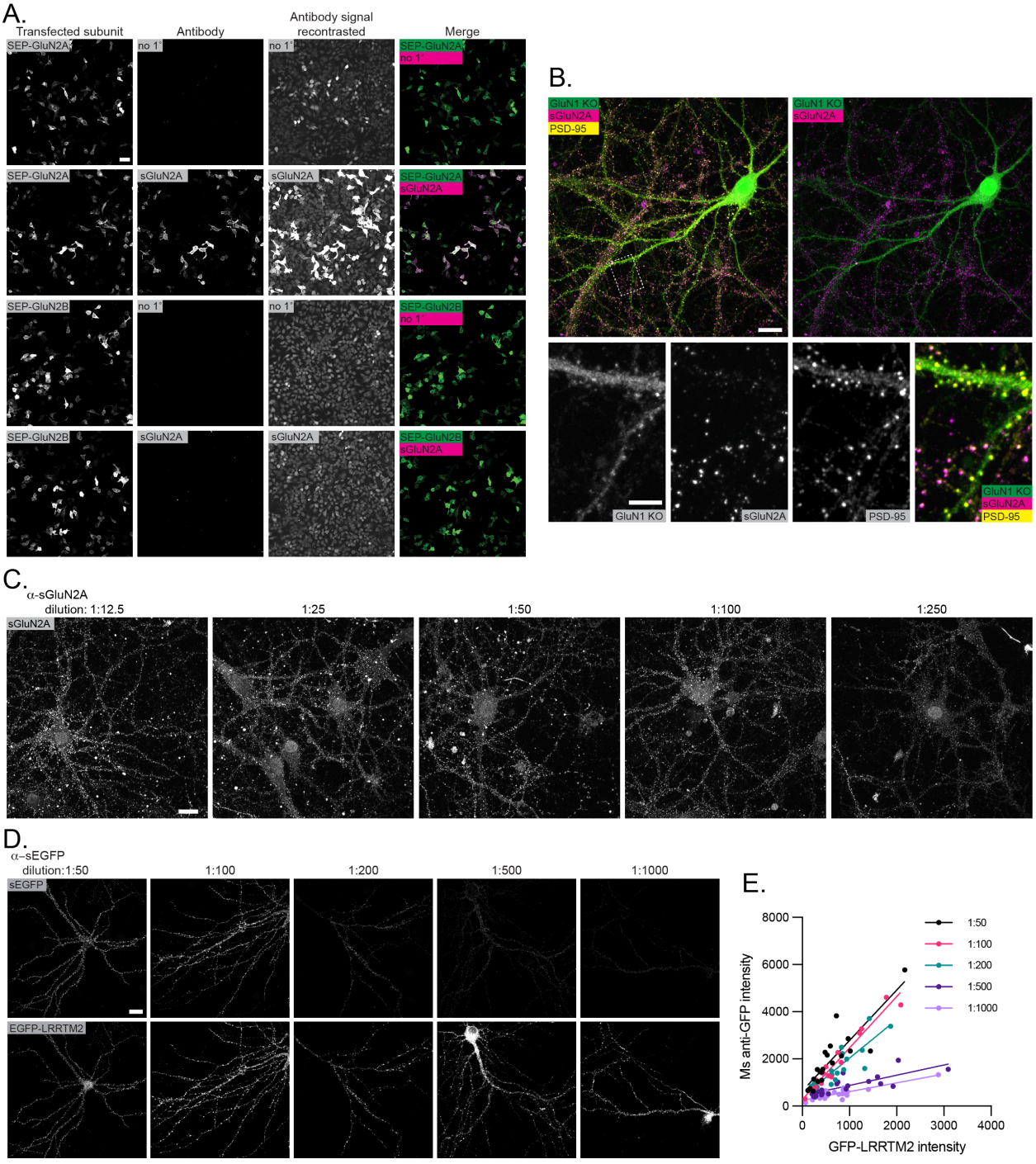

Figure S1. Antibody validation

a) Anti-GluN2A is specific for GluN2A over GluN2B. HEK cells expressing SEP-GluN2A or SEP-GluN2B were surface labeled with anti-GluN2A, and only cells expressing SEP-GluN2A showed anti-GluN2A labeling over background, no primary labeling. Images in the first column are contrast-normalized to the top row image, and in the second column to the second-row image. In the third column, the same images from the second column have been re-contrast-

normalized to the image in the first row to show staining background levels. The fourth column is the merge of columns 1 and 2. Scale bar 50 μm. **b)** Anti-GluN2A staining is not present in NMDAR knockout neurons. Neurons lacking NMDA receptors due to CRISPR-mediated knockout were not labeled by anti-GluN2A. **(Top)** Images of a neuron expressing EGFP to indicate CRISPR Grin1 KO (green) and stained for surface GluN2A (magenta) and PSD-95 (yellow). Scale bar 20 μm. **(bottom)** Zoom-in of boxed region from **top** where Grin1 KO dendritic spines lacked GluN2A staining but had PSD-95 staining to indicate synapses. GluN2A/PSD-95 positive puncta can be seen on neighboring, wild-type cells in both the zoom and overview images. Scale bar 5 μm. **c)** Anti-GluN2A dilution series. Images are contrasted to the 1:12.5 dilution. Large aggregates were apparent in dilutions of 1:50 or higher; thus 1:100 was chosen for experiments in this work. Scale bar 20 μm. **d)** Anti-EGFP dilution series. GFP-LRRTM2 knockdown/rescue-transfected neurons were surface-stained with mouse anti-EGFP at the indicated dilutions. Images are scaled to the 1:50 dilution for both EGFP and GFP-LRRTM2. Scale bar 20 μm. **e)** Quantification of total EGFP and anti-EGFP intensity per cell revealed a linear relationship for all dilutions. The saturating 1:50 dilution was chosen for experiments in this work.

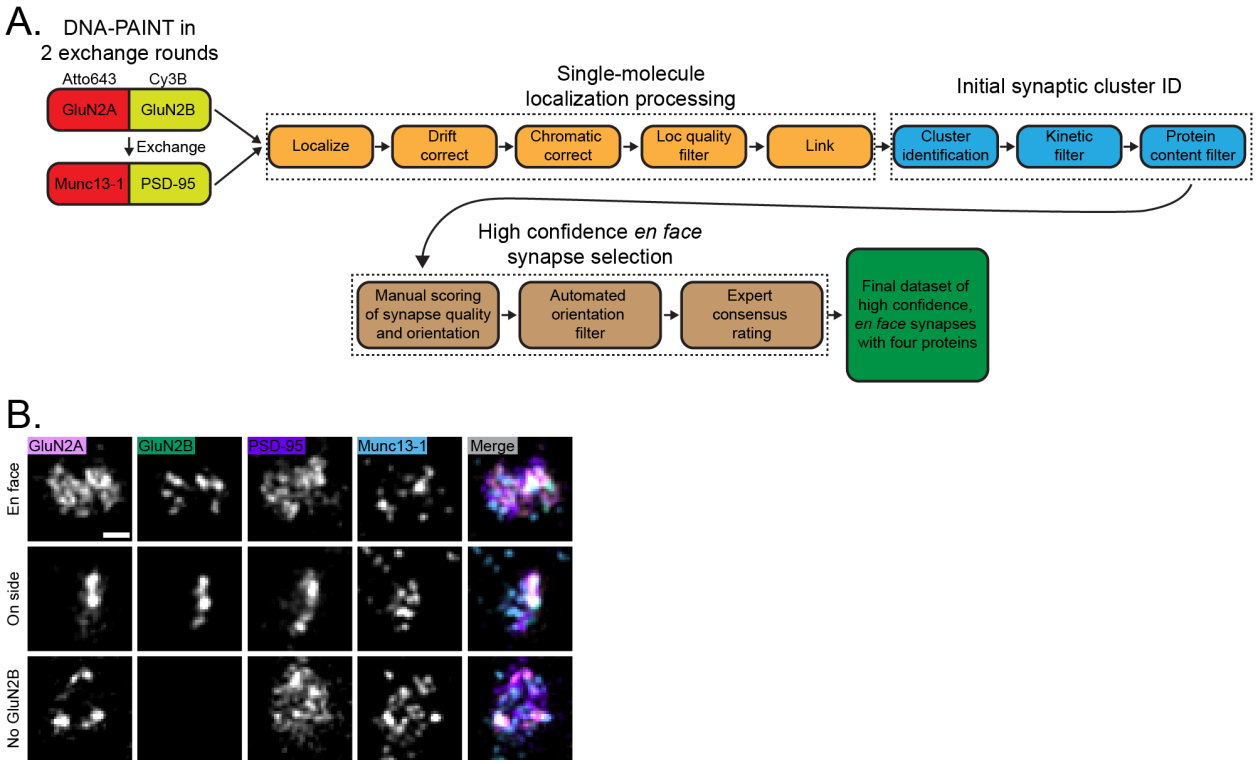

Figure S2. DNA-PAINT analysis workflow
a) Schematic describes four target DNA-PAINT acquisition and data processing workflow. Details can be found in the Supplemental Methods. **b)** Example DNA-PAINT renderings (10 nm pixels) show synapses that are oriented perpendicular to the imaging axis (ie en face) and used for analysis, or were on their side or lacked GluN2B and discarded from further analysis. Scale bar 100 nm.

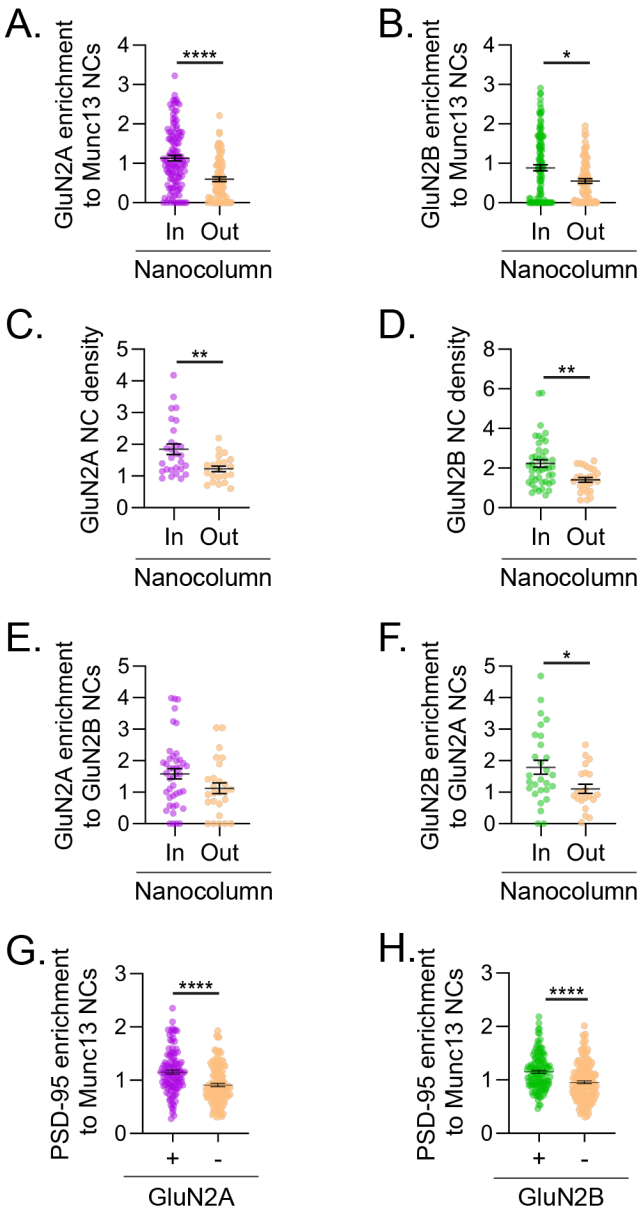

Figure S3. Enrichment indices related to Figure 5
a-h) Graphs of enrichment indices (average of the first 60 nm of the cross-enrichment plots) corresponding to the same panels in Figure 5. Points are individual nanoclusters of the indicated proteins, lines show mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ***p<0.0001.

Supplementary Table 1: DNA Constructs

Construct	Source	RRID	Figure use
pORANGE GFP-Grin2b KI	Addgene plasmid #131487; http://n2t.net/addgene:131487	Addgene_131487	Cloning intermediate
pFUGW spCas9	Addgene plasmid #131506; http://n2t.net/addgene:131506	Addgene_131506	1C,E; 2-5; S2B
pFUGW mCherry-KASH	Addgene plasmid #131505; http://n2t.net/addgene:131505	Addgene_131505	Cloning intermediate
psPAX2	Addgene plasmid #12260; http://n2t.net/addgene:12260	Addgene_12260	Lentivirus production
pMD2.G	Addgene plasmid #12259; http://n2t.net/addgene:12259	Addgene_12259	Lentivirus production
LCV2	Addgene plasmid # 82416 ; http://n2t.net/addgene:82416	Addgene_82416	Cloning intermediate
GFP-LRRTM2 knockdown/rescue	Described in ref. 14		S1D
SEP-GluN2A	Gift of Andres Barria		S1A
SEP-GluN2B	Gift of Andres Barria		S1A
GluN1-1a pcDNA3.1+	Gift of Gabriela Popescu		S1A
pFW ORANGE GFP-Grin2b KI	This manuscript		1C,E; 2-5; S2B
pFSW myr(Fyn)-EGFP-LDLRct	Described in ref. 75; specific construct this manuscript		1B
pFW	This manuscript		Cloning intermediate
LCV2 Grin1 KO	gRNA: ref. 76; construct: This manuscript		S1B

Supplementary Table 2: Primary antibodies

Primary antibodies	Source	RRID	Stock	Dilution
Monoclonal mouse (IgG2A) anti-GFP (clone 3E6)	Invitrogen A-11120	AB_221568	0.2 mg/ml	1:50
Polyclonal chicken (IgY) anti-GFP	Invitrogen A-10262	AB_2534023	2 mg/ml	1:500
Polyclonal rabbit anti-GluN2A	Gift of Rick Huganir; JH6097	n/a	0.15 mg/ml	1:100
Monoclonal mouse (IgG2A) anti-PSD95 (clone K28/43)	Neuromab 75-028	AB_2877189	0.5 mg/ml	1:80
Polyclonal rabbit anti-Munc13-1	Synaptic Systems 126103	AB_887733	0.5 mg/ml	1:250
Monoclonal mouse IgG2A anti-Bassoon (clone SAP7F407)	Enzo ADI-VAM-PS003	AB_2313990	1 mg/ml	1:500
GFP-Booster Alexa Fluor 488	Chromotek gb2AF488	AB_2827573	0.5 mg/ml	1:500

Supplementary Table 3: Secondary reagents

Secondary reagents	Source	RRID	Stock	Dilution
FluoTag-XM-QC anti-mouse IgG kappa light chain single-domain antibody (sdAb) (clone 1A23) + docking site F1	Massive Photonics (custom)	n/a	5 µM	n/a
FluoTag-XM-QC anti-rabbit IgG sdAb (clone 10E10) + docking site F2	Massive Photonics (custom)	n/a	5 µM	n/a
FluoTag-XM-QC anti-rabbit IgG sdAb (clone 10E10) + docking site F3	Massive Photonics (custom)	n/a	5 µM	n/a
FluoTag-XM-QC anti-mouse IgG kappa light chain sdAb (clone 1A23) + docking site F4	Massive Photonics (custom)	n/a	5 µM	n/a
ChromPure mouse IgG, Fc fragment	Jackson Immunoresearch 015-000-008	AB_2337191	Variable	n/a
ChromPure rabbit IgG, Fc fragment	Jackson Immunoresearch 011-000-008	AB_2337121	Variable	n/a
Affinipure donkey anti-rabbit IgG	Jackson Immunoresearch 711-005-152	AB_2340585	Variable	n/a
Donkey anti-chicken Alexa Fluor 488	Jackson Immunoresearch 703-545-155	AB_2340375	1.25 mg/ml	1:500
Donkey anti-mouse (IgG2A) Alexa Fluor 647	Jackson Immunoresearch 115-605-206	AB_2338917	1.25 mg/ml	1:500

Donkey anti-rabbit Cy3B	In house	n/a	~1.25 mg/ml	1:500
Donkey anti-rabbit Alexa Fluor 647	Jackson Immunoresearch 711-605-152	AB_2492288	1.25 mg/ml	1:500

Supplementary Table 4: Detailed antibody use in figures

Figures	Antibodies used	Imager used
1b	(surface) Rb anti-GluN2A + preinc anti-Rb sdAb F2 (total) Rb anti-GFP + preinc anti-Ms sdAb F3 (total) Ms anti-PSD-95 + preinc anti-Ms sdAb F1 (total) Ms anti-Bassoon + preinc anti-Ms sdAb F4	1 nM F2-Cy3B (rd 1) 0.5 nM F3-Cy3B (rd 1) 1 nM F1-Cy3B (rd 2) 2 nM F4-Atto643 (rd 2)
1c	(surface) GFP-Booster AF488 (surface) Rb anti-GluN2A + Dk anti-Rb Cy3B (total) Ms anti PSD-95 + Dk anti-Ms AF647	
1e 2-5, S2b	(surface) Ms anti-GFP + preinc anti-Ms sdAb F1 (surface) Rb anti-GluN2A + preinc anti-Rb sdAb F2 (total) Ms anti-PSD-95 + preinc anti-Ms sdAb F4 (total) Rb anti-Munc-13 + preinc anti-Rb sdAb F3 (total) GFP-Booster AF488	2 nM F1-Cy3B (rd 1) 1 nM F2-Atto643 (rd 1) 1 nM F4-Atto643 (rd 2) 1 nM F3-Cy3B (rd 2)
S1a	(surface) Rb anti-GluN2A + Dk anti-Rb AF647 (total) Ck anti GFP + Dk anti-Ck AF488	
S1b	(surface) Rb anti-GluN2A + Dk anti-Rb Cy3B (total) Ms anti-PSD-95 + Dk anti-Ms AF647 (total) GFP-Booster AF488	
S1c	(surface) Rb anti-GluN2A + Dk anti-Rb AF647	
S1d	(surface) Ms anti-GFP + Dk anti-Ms AF647 (total) GFP-Booster AF488	

Supplemental Note 1

Single-molecule analysis pipeline details:

1. **Separate emission channels:** Merge multi-page TIFF files from each acquisition in FIJI, then crop the image to separate red and far-red emission and save each as .raw using the ImageJ raw-yaml-export plugin (https://github.com/jungmannlab/imagej-raw-yaml-export).
2. **Localize each emission channel:** Localize spots in each .raw file using the Picasso *localize* command line function (LQ-GPU fit, box size 7, min net gradient set separately for each channel but usually 8000-15000)
3. **Drift correction:** Drift correct images with redundant cross correlation (RCC) using the Picasso *undrift* command line function (segment 1000)
4. **Generate dual view correction t-form:** First roughly align red and far-red sides of the TetraSpeck image by minimizing a nearest neighbor search between bead localizations, then pair localizations between the sides and calculate a t-form using the *fitgeotrans* function in MATLAB with a 2nd degree polynomial. Residual deviation between bead pair positions was estimated as <12 nm after correction.
5. **Recombine acquisitions:** Combine the localization files from each channel of each split acquisition, and correct for the dual view using the t-form and MATLAB's *transformPointsInverse* function to shift localizations into the red emission space. At this point, each region consists of four drift and dual view corrected localization files (for each of four proteins imaged), and operations are performed per region.
6. **Cross-correlate to correct residual offset:** Correct any residual, remaining linear offset between proteins in a region by cross-correlating the images to the GluN2B acquisition. In testing, the gold nanoparticles and overall shape of the image dominated the cross-correlation, and the presence of synapses had little effect. This was performed using a custom MATLAB function based on normalized cross correlation (Tang et al., 2016), with added image smoothing to remove local high density peaks.

7. **Filter localizations:** For each protein, remove any localizations 1) with fewer photons than the photon mode, 2) standard deviation of the fit >2 pixels (320 nm), 3) localization error >20 nm.
8. **Link localizations:** Link localizations temporally using Picasso *link* command line function with radius 0.3 pixels (48 nm) and 5 dark frames allowed. These parameters were empirically determined to be optimal to link localizations that persisted for more than one frame without linking nearby localizations from another imager strand, and accounting for long-lived localizations that may drop below the detection threshold for several frames.
9. **Cluster identification:** Use Picasso *dbscan* command line function with radius 0.3 pixels (48 nm) and minimum points 10 on each protein in a region to identify densely localized synaptic clusters.
10. **Kinetic cluster filtering:** Using a custom function in MATLAB, filter clusters based on their blinking kinetics (Sun et al., 2021). Real DNA imager binding sites will be localized constantly throughout the acquisition, and therefore have a mean frame number close to the total frames/2 (25,000) with a large standard deviation of frame number, while non-specific binding events occur for a few frames then never again, resulting in a low standard deviation of their frame number and a mean frame number that may deviate from total frames/2. Therefore, clusters were removed if their mean frame number was greater or less than the mean +/- 2x the standard deviation of a gaussian fit of the mean frame number of all clusters or if the standard deviation of the frame number was <5000 or >20000 (empirically determined from the images to remove clusters that were not real synapses).
11. **Protein-based cluster filtering:** Using a custom function in MATLAB, PSD-95 clusters were removed from the dataset if they did not have overlap with GluN2B (ie, not a knockin synapse or came from a neighboring cell) or Munc-13 (ie, not a synapse).
12. The result of this process is a drift corrected, dual-view corrected dataset of 4 proteins per region, filtered for high quality localizations, where most non-synaptic and non-KI cell clusters have been removed.
13. **Synapse picking:** To obtain a high-confidence set of near-en-face synapses for analyses:
    a. Each filtered PSD-95 cluster above was manually inspected for the presence of the other imaged proteins in sufficient density for analysis and to confirm that the kept cluster was a synapse. Synapses were kept based on disc like shapes with overlap of pre- and post-synaptic proteins and a size range of ~100 – 800 nm diameter, and their position near a dendrite, then scored as “en face”, “side view” or “intermediate”, ie, somewhere between en face and side.
    b. Synapses were further filtered for en face synapses by removing those from the previously judged “en face” group where the long/short axis ratio of the PSD-95 cluster was > 2.
    c. Finally, 3 expert raters evaluated each synapse as en face and manually adjusted synaptic borders as necessary (only when DBSCAN was obviously incorrect), and in the case of extrasynaptic analysis, manually removed other synapses from within a 500 nm distance of the synapse in question. Synapses were included in the high confidence dataset only when all 3 raters agreed.