Direct visualization of triheteromeric NMDA receptors

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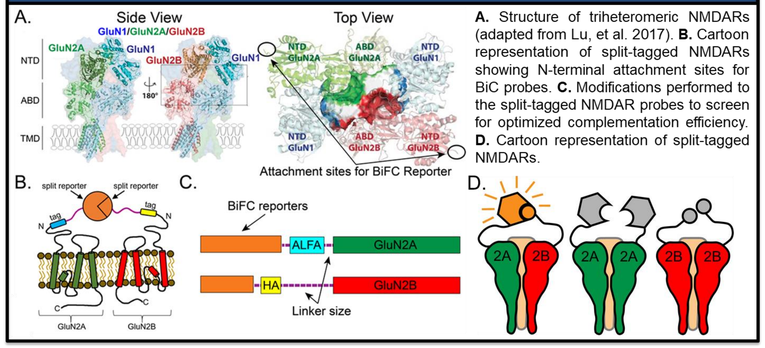
1. Introduction

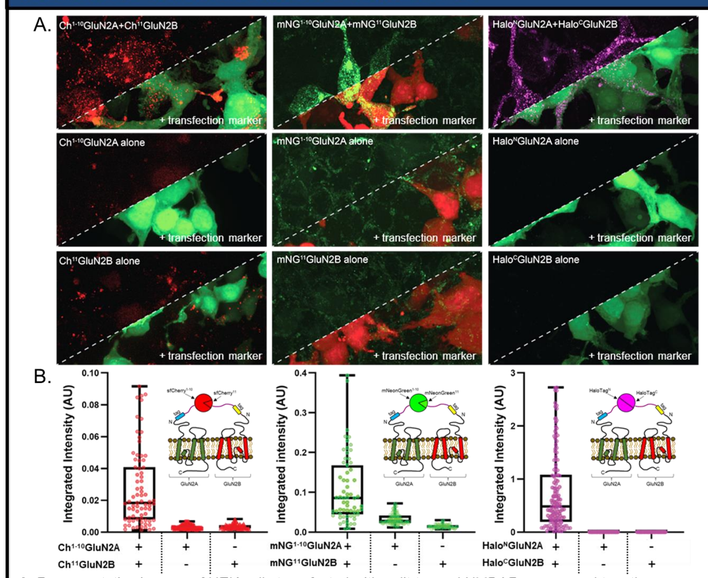
Glutamatergic signaling via NMDA receptors (NMDARs) is critical in synaptic plasticity, excitotoxicity, and numerous degenerative and cognitive disorders. Each NMDAR comprises four subunits: two obligatory GluN1 subunits and two GluN2A-D or GluN3A-B subunits. Notably, NMDARs with different subunit compositions display different channel properties and protein interactions, supporting the widely held notion that subunit composition dictates NMDAR function. Though many NMDARs contain GluN1 and two identical GluN2 subunits, in many brain regions, NMDARs commonly are “triheteromeric” receptors containing both GluN2A and GluN2B with GluN1. However, despite their deduced importance, there are no methods to unambiguously distinguish triheteromeric NMDARs from other subtypes in neurons, and thus their distribution and subcellular trafficking remain mysterious. To overcome this, we have designed a method for direct visualization of triheteromeric NMDARs using bimolecular complementation (BiC). We tagged GluN2A and GluN2B subunits with two parts of a modified fluorescent protein that complement to produce fluorescence only when an NMDAR is assembled containing both the GluN2A and GluN2B subunits (split-tagged NMDARs). To extend this toolbox, We have also created a version of split-tagged NMDARs that relies on a split HaloTag enzyme which can interact with specialized dyes with various properties. Utilizing these tools, we can now answer longstanding questions surrounding triheteromeric NMDAR trafficking and function.

2. Probe design to detect triheteromeric NMDARs



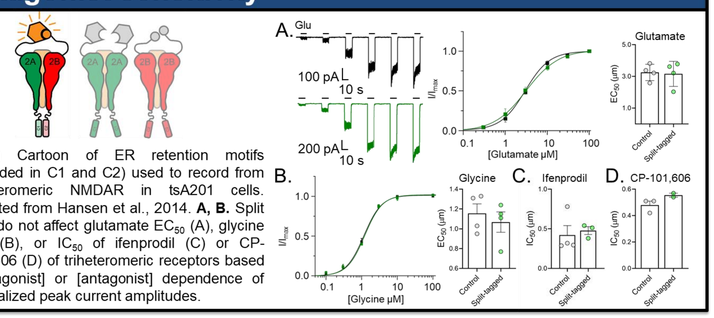
A. Structure of triheteromeric NMDARs (adapted from Lu, et al. 2017). **B.** Cartoon representation of split-tagged NMDARs showing N-terminal attachment sites for BiC probes. **C.** Modifications performed to the split-tagged NMDAR probes to screen for optimized complementation efficiency. **D.** Cartoon representation of split-tagged NMDARs.

3. Split-reporters can be used to specifically visualize triheteromeric NMDARs



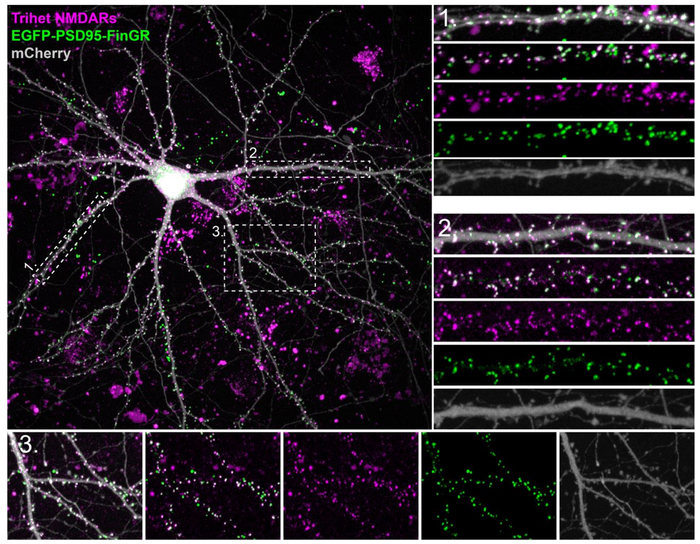
A. Representative images of HEK cells transfected with split-tagged NMDARs expressed together or separately. **B.** Mean whole-cell integrated intensity of split-tagged NMDAR signal in each condition. Note: fluorescence only occurs when two parts of BiC molecules (for sfCherry, $Ch^{1\text{-}10}$ and Ch^{11}; for mNeonGreen, $mNG^{1\text{-}10}$ and mNG^{11}; for HaloTag, $Halo^{N}$ and $Halo^{C}$) are expressed together.

4. Split-tagged NMDARs exhibit normal agonist and antagonist sensitivity



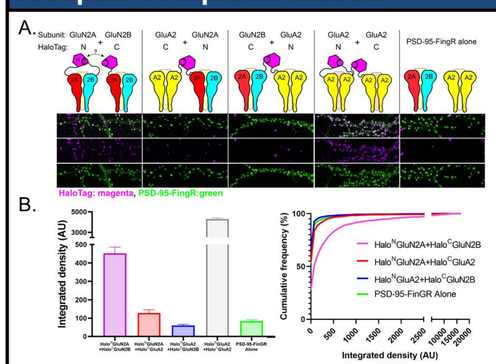
(Left) Cartoon of ER retention motifs (included in C1 and C2) used to record from triheteromeric NMDAR in tsA201 cells. Adapted from Hansen et al., 2014. **A, B.** Split tags do not affect glutamate EC_{50} (A), glycine IC_{50} (B), or IC_{50} of ifenprodil (C) or CP-101,606 (D) of triheteromeric receptors based on [agonist] or [antagonist] dependence of normalized peak current amplitudes.

5. Split-tagged NMDARs colocalize with synaptic markers



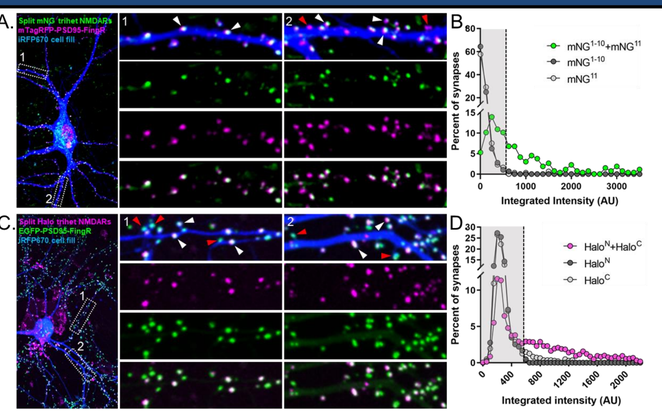
Cultured primary hippocampal neurons cotransfected with split-Halo tagged NMDARs, EGFP-PSD95-FingR and mCherry. Surface split-tagged NMDARs were labeled with cell membrane impermeable HaloTag ligands to visualize triheteromeric NMDAR subcellular distribution..

6. Split-tagged NMDARs do not undergo inter-receptor complementation



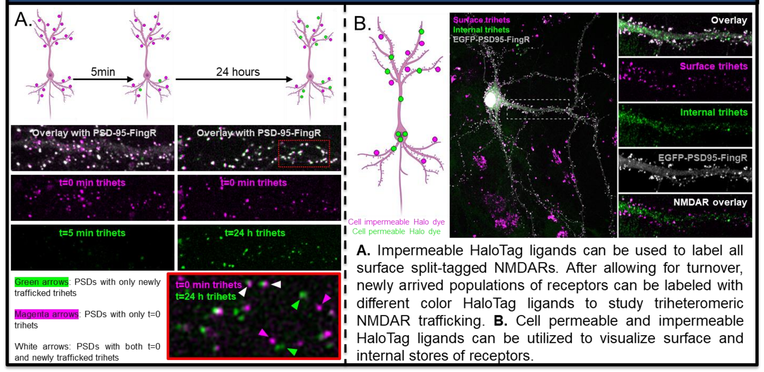
To test whether signal from complemented split-tagged NMDARs could reflect binding between separate receptor oligomers, we created split-tagged AMPAR subunits and tested whether they complement with co-expressed, complementary GluN2 subunits in neurons. **A.** Representative images of neurons transfected with EGFP-PSD95-FinGR and the indicated pairs of GluN2 or GluA2 subunits tagged with split-Halo as indicated. **B.** Mean integrated density of HaloTag signal within PSDs defined by PSD-95-FinGR puncta.

7. Variability of synaptic content of triheteromeric NMDARs



A, C. Representative images of neurons transfected with complementing split mNG **(A)** or complementing split Halo **(C)** tagged NMDARs. **B, D.** Frequency distribution of the synaptic split-tagged NMDAR signal from cultured hippocampal neurons transfected with complementing or non-complementing split-tagged NMDARs.

8. Using Halo ligands to measure triheteromeric NMDAR trafficking



A. Impermeable HaloTag ligands can be used to label all surface split-tagged NMDARs. After allowing for turnover, newly arrived populations of receptors can be labeled with different color HaloTag ligands to study triheteromeric NMDAR trafficking. **B.** Cell permeable and impermeable HaloTag ligands can be utilized to visualize surface and internal stores of receptors.

8. Conclusions

We developed a bimolecular complementation approach to label triheteromeric NMDARs in neurons.

- These split-tagged NMDARs retain normal sensitivity to agonists and antagonists, display no signs of interreceptor complementation of BiC molecules, and traffic avidly into synapses.
- Triheteromeric NMDARs can be tagged with a variety of BiC molecules including split FPs and split HaloTag, enabling diverse experiments.

This approach for the first time reveals subcellular distribution and dynamics of triheteromeric NMDARs.

- We observed striking heterogeneity between synapses in their triheteromeric NMDAR content and turnover in 24hrs, suggesting unique neuronal mechanisms controlling these mechanisms.

In summary, dynamic exchange of NMDARs into and out of synapses establishes basal transmission and forms the basis for diverse forms of plasticity, yet triheteromeric NMDAR trafficking and mobility have not been measured. Our tools for clarifying these unknown aspects of triheteromeric NMDAR dynamics will substantially broaden our understanding of how NMDARs impact synaptic development, transmission, and plasticity.

Acknowledgements

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