

Supplementary Material for “Acute knockdown of AMPA receptors reveals a trans-synaptic signal for presynaptic maturation” (Tracy et al.)

Materials and Methods

DNA constructs

Each GluA-shRNA sequence was inserted into pSuper-Retro-GFP (Oligogene) according to the manufacturer's protocol. The GluA1 shRNA targeted nucleotides 2276-2294 in the flop isoform of rat GluA1. The sense sequence was 5'cagtaaacctggcagtggt3' and the antisense sequence was 5'aacactgccaggtttactg3'. The GluA2 shRNA targeted nucleotides 400-418 of rat GluA2 and was previously used by Passafaro et al. (2003) and Saglietti et al. (2007). The sense sequence was 5'ggagcactccttagcttga3' and the antisense sequence was 5'tcaagctaaggagtgtcc3'. The GluA3 shRNA targets nucleotides 1280-1298 of rat GluA3. The sense sequence was 5'cacatattgatgtataa3' and the antisense sequence was 5'ttatacatcacatattgtg3'. To generate the GluA1 rescue construct, a silent point mutation (N761) was introduced to a GFP-GluA1 construct using PCR mutagenesis. The same method was used for the GluA2 rescue construct (L136). GFP-GluA1, GFP-GluA2 and GFP-GluK2 constructs were in pCI-Neo (Promega). HA tagged NL1 was in pNice. The N-cadherin shRNA was previously used by Saglietti et al. (2007).

Cell cultures and transfection

Primary hippocampal cultures were prepared from embryonic day 22 rat brains and plated at a density of 100×10^3 cells/ml for electrophysiology and 50×10^3 cells/ml for immunostaining.

The neuronal cultures were maintained in serum-free Neurobasal media with B27-supplement (Life Technologies) and Glutamax (Invitrogen, Grand Island, NY). Neurons were transfected using calcium phosphate at 5-7 DIV. DNA constructs were allowed to express for 5 days before the cultures were used for experiments. HEK293 cells maintained in DMEM (GIBCO) with 10% FBS were transfected using calcium phosphate. After one day of expression, the cells were gently resuspended in neurobasal media and plated at low density onto hippocampal neurons at 9 DIV. Three days later, the co-cultures were used for immunocytochemistry.

Antibodies

The following mouse monoclonal antibodies were used: Syt1 (Synaptic Systems), GFP and actin (Millipore), GluN1 (BD Pharmingen), PSD-95 (Affinity Bioreagents), syntaxin I (Santa Cruz) and N-cadherin (BD Biosciences). The following rabbit (or otherwise stated) polyclonal antibodies were used: GFP (Abcam), GluA2/3, GluA1, GRIP1 and MAP2 (Millipore), bassoon and Piccolo (Synaptic Systems), HA (Abcam), and guinea pig anti-VGluT1 (Millipore). The pan-synapsin and liprin- α antibodies are gifts from Thomas Südhof's lab.

Statistical analysis

Single-factor ANOVA was used for all statistical analysis. Values are presented as mean \pm SEM in the figures.

References

Passafaro M, Nakagawa T, Sala C, Sheng M (2003) Induction of dendritic spines by an extracellular domain of AMPA receptor subunit GluR2. *Nature* **424**(6949): 677-681

Saglietti L, Dequidt C, Kamieniarz K, Rousset MC, Valnegri P, Thoumine O, Beretta F, Fagni L, Choquet D, Sala C, Sheng M, Passafaro M (2007) Extracellular interactions between GluR2 and N-cadherin in spine regulation. *Neuron* **54**(3): 461-477

Figure legends for supplementary figures

Supplementary Figure 1. AMPAR knockdown reduced excitatory synaptic strength in cultured neurons. Graph depicting the mean AMPAR eEPSC amplitude recorded from neurons in response to increasing extracellular stimulus strength. For each neuron the average amplitude of five responses was calculated at every specified stimulus intensity. The stimulus duration was kept constant at 1ms. (n=18-21 cells/group; ***, $p < 0.001$).

Supplementary Figure 2. The majority of functional NMDARs on the cell surface are synaptically localized. (A) Example traces of whole-cell currents in response to a 3-second local application of NMDA (1mM). The NMDA-evoked response was recorded from the neuron followed by MK-801 (10 μ M) bath perfusion and 50 stimuli administered at 0.5Hz. Having thus blocked synaptic NMDARs, the remaining NMDA-evoked response of the neuron was measured. Scale bars: 0.5 nA, 0.5 s. (B) Graph of the NMDA-evoked response size recorded from each neuron before and after the MK-801 blockade of synaptic NMDARs (n = 18 cells/group).

Supplementary Figure 3. AMPAR knockdown does not alter dendrite morphology. (A) Neurons were transfected with pSuper or GluA RNAi at 7 DIV and fixed at 12 DIV. Dendrite morphology was visualized by GFP fluorescence expressed in transfected cells. Scale bar: 20 μ m. (B) Sholl analysis was performed to investigate the complexity of dendritic branches after AMPAR knockdown (n = 19-21 neurons/group). (C) Quantification of the mean total length of dendrites (n = 19-21 neurons/group; $p > 0.7$).

Supplementary Figure 4. A decrease in synaptic release probability slows the rate of NMDAR eEPSC blockade by MK-801. (A) The progressive block of NMDAR EPSCs recorded from untransfected neurons in 10 μ M MK-801 at two concentrations of external calcium, 1 mM (black) and 2 mM (white). The amplitudes at consecutive stimuli were normalized to the first response (n = 13-14 cells/group). (B) The rate of NMDAR eEPSC blockade was fitted with a double exponential equation (*, $p < 0.05$).

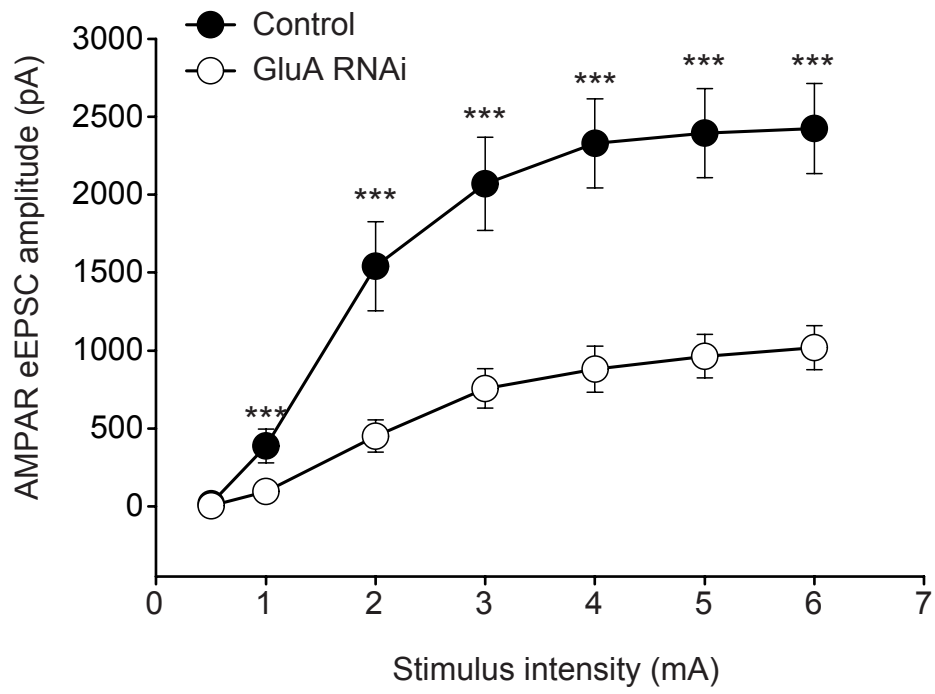
Supplementary Figure 5. Syt1 antibody uptake occurs at both glutamate and GABA releasing terminals. (A) Syt1 antibody uptake (red) was co-localized predominantly with either VGluT1 (blue) or GAD65 (green) immunolabeling. Synaptic vesicle release is evident at both excitatory synapses (arrows) and inhibitory synapses (arrowheads). Scale bar, 10 μ m. (B) Quantification of the fraction of total Syt1 puncta that co-localize with the excitatory synaptic markers, VGluT1 and VGluT2, and the inhibitory synaptic marker, GAD65. (C) and (D) Analysis of syt1 antibody uptake at neuronal synapses of untransfected neurons adjacent to those transfected with various constructs as an indication of global network activity. Percentage of functionally inactive synapses (C) and the mean intensity of Syt1 puncta co-localized with VGluT1 (D) on untransfected neurons adjacent to neurons expressing the designated constructs (n= 13-15 neurons/group; $p > 0.5$).

Supplementary Figure 6. Chronic blockade of AMPARs does not alter synaptic NMDAR-mediated responses. The mean amplitude of NMDAR eEPSCs recorded from untransfected

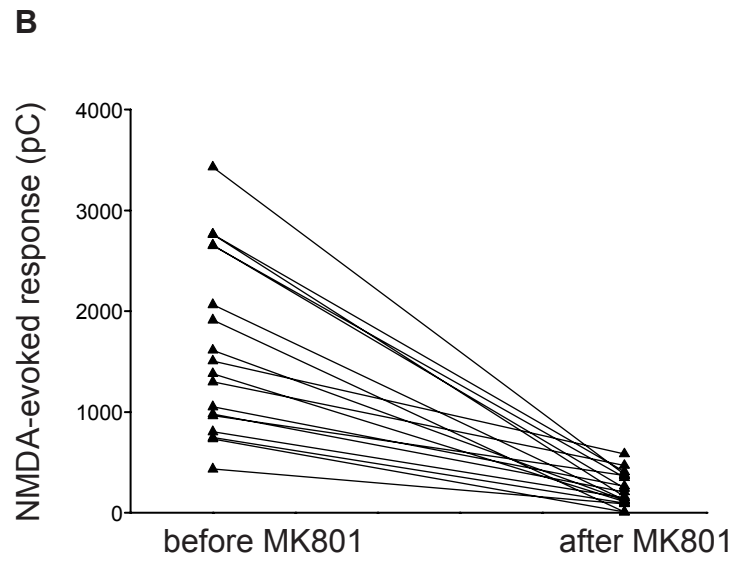
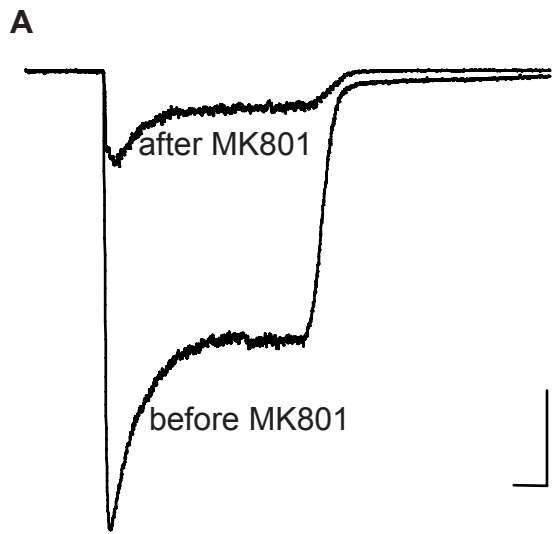
neurons that were treated with either DMSO or 10 μ M CNQX for 5 days ($n = 12$ cells/group; $p > 0.8$).

Supplementary Figure 7. HEK293 cell size and the density of synaptic contacts is unaffected by the expression of AMPARs. (A) The average size of HEK293 cells from co-culture experiments was estimated by calculating the total area of green fluorescence which delineates the morphology of each cell ($n = 24-27$ cells/group; $p > 0.15$; *, $p = 0.016$). (B) The average distance between glutamatergic synapses on HEK293 cells ($n = 24-27$ cells/group, $p > 0.3$; *, $p = 0.04$).

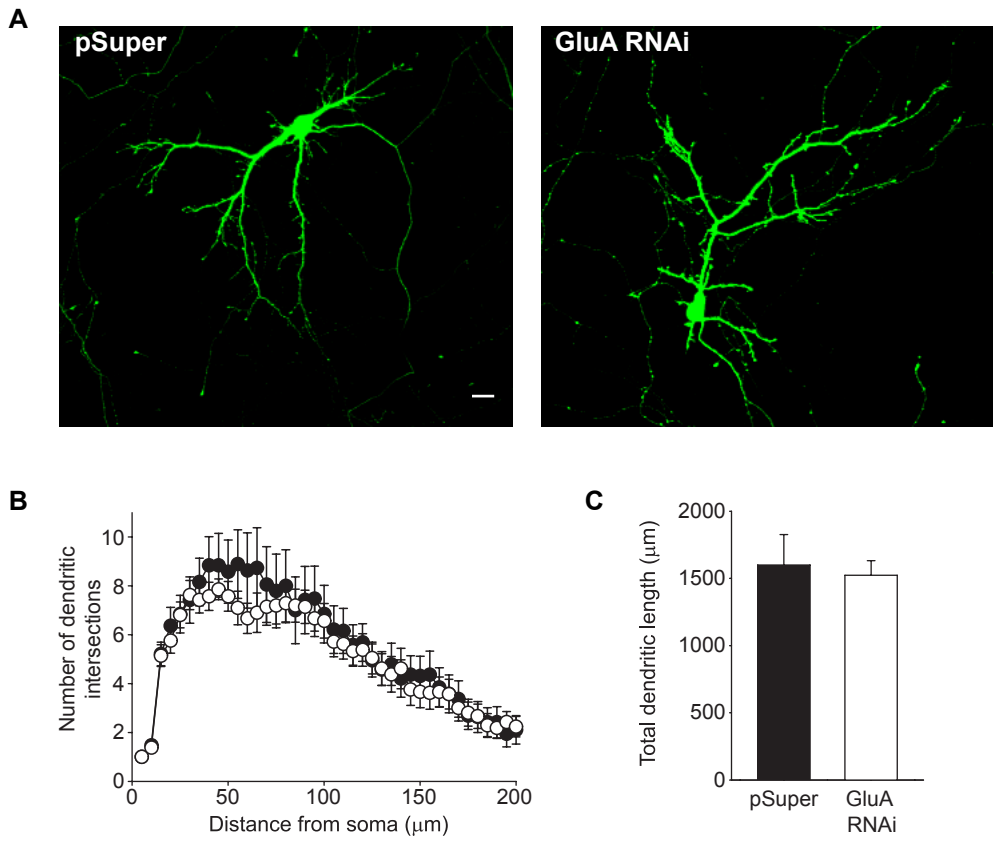
Supplementary Figure 8. Validation of GluA2 ecto expression to investigate the role of the AMPAR ectodomain in synapse maturation. (A) HEK293 cells expressing GFP alone or GFP-GluA2 ecto, where the GFP is tagged on the extracellular domain of GluA2. Incubation of live cells with media containing a GFP antibody only immunolabeled GFP-GluA2 ecto expressed on the cell surface (red). Scale bar, 10 μ m. (B) Hippocampal neurons expressing GFP-GluA2 or GFP-GluA2 ecto, both with a GFP tagged on the extracellular domain. A GFP antibody applied to live neurons only immunolabeled GFP-GluA2 expressed on the cell surface (red). Although GFP-GluA2 ecto was indeed expressed by neurons indicated by the expression of GFP, the lack of surface immunostaining of GFP (red channel) suggests that this construct is not trafficked properly in neurons. Scale bar, 10 μ m.



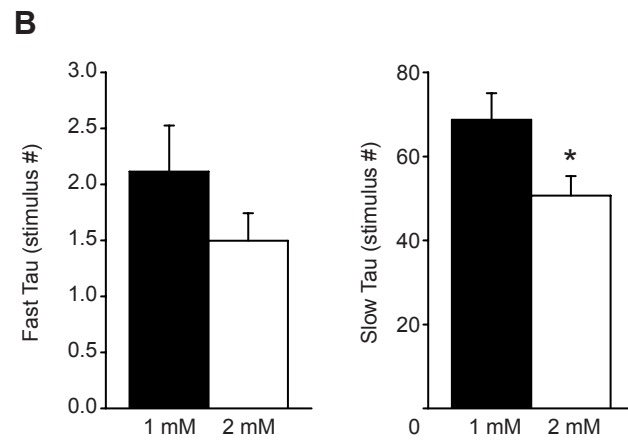
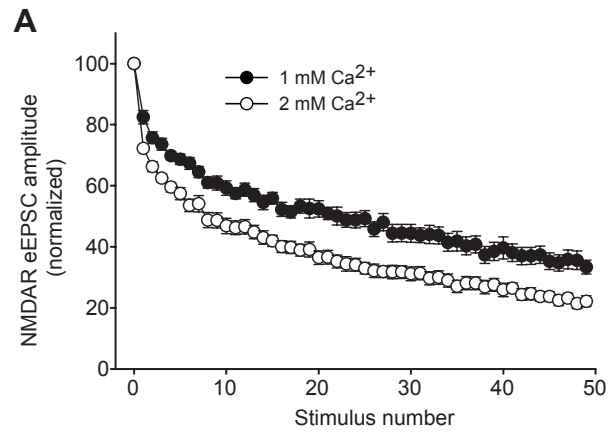
Tracy et al., Supplementary Figure 1



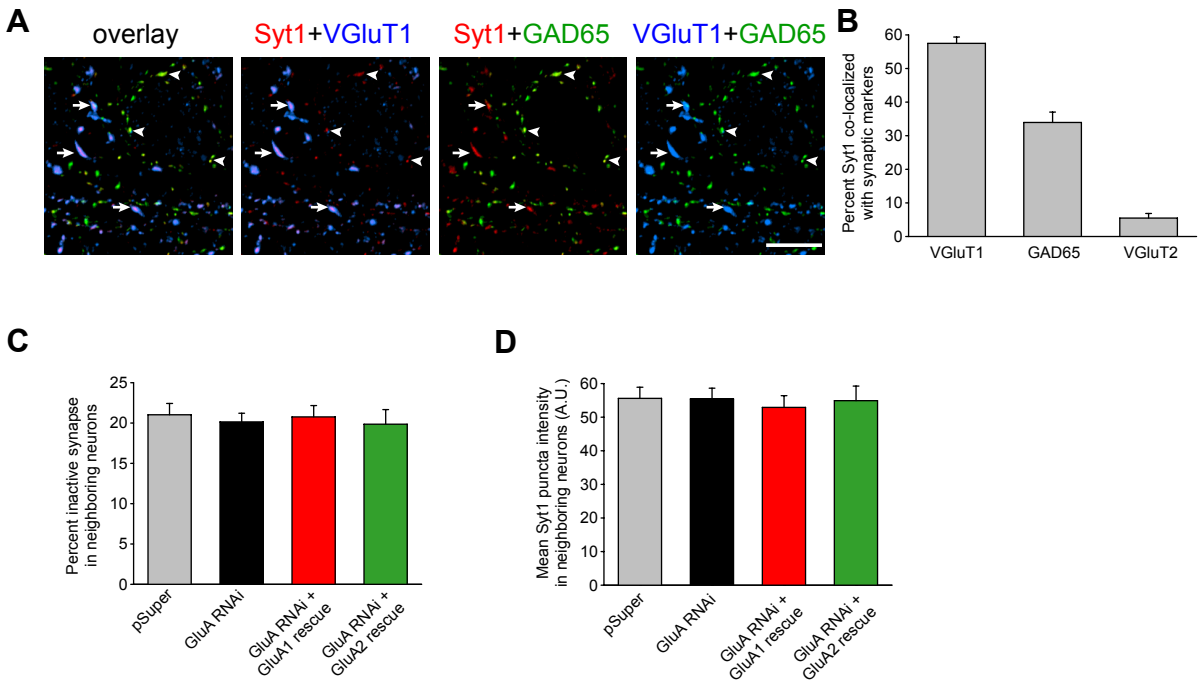
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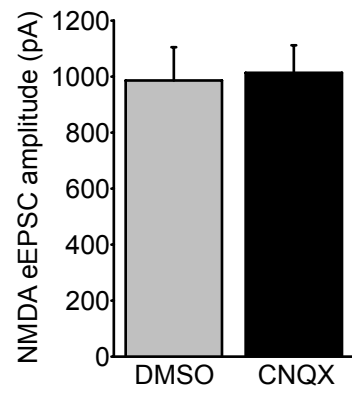
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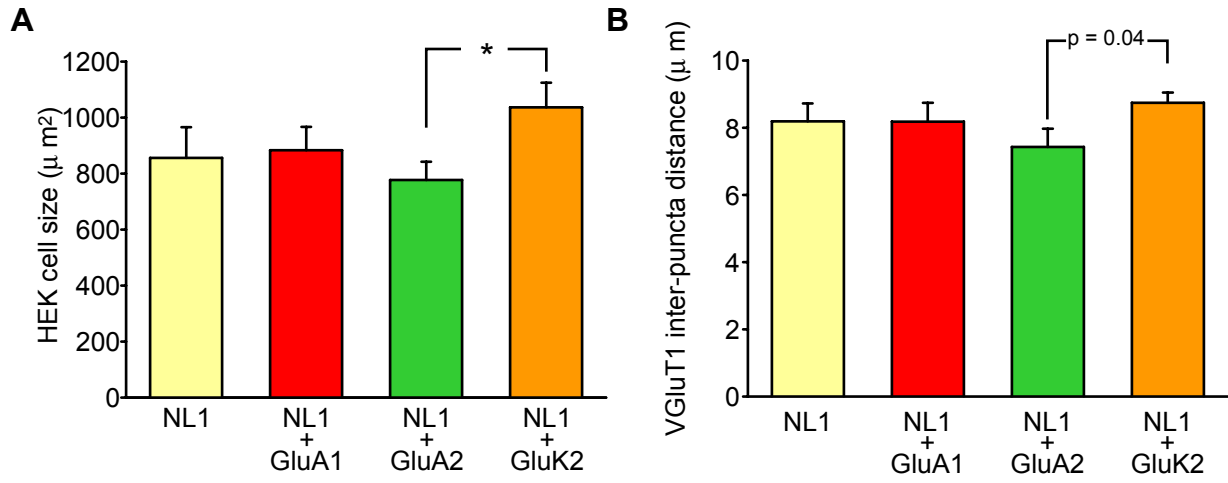
Tracy et al., Supplementary Figure 4



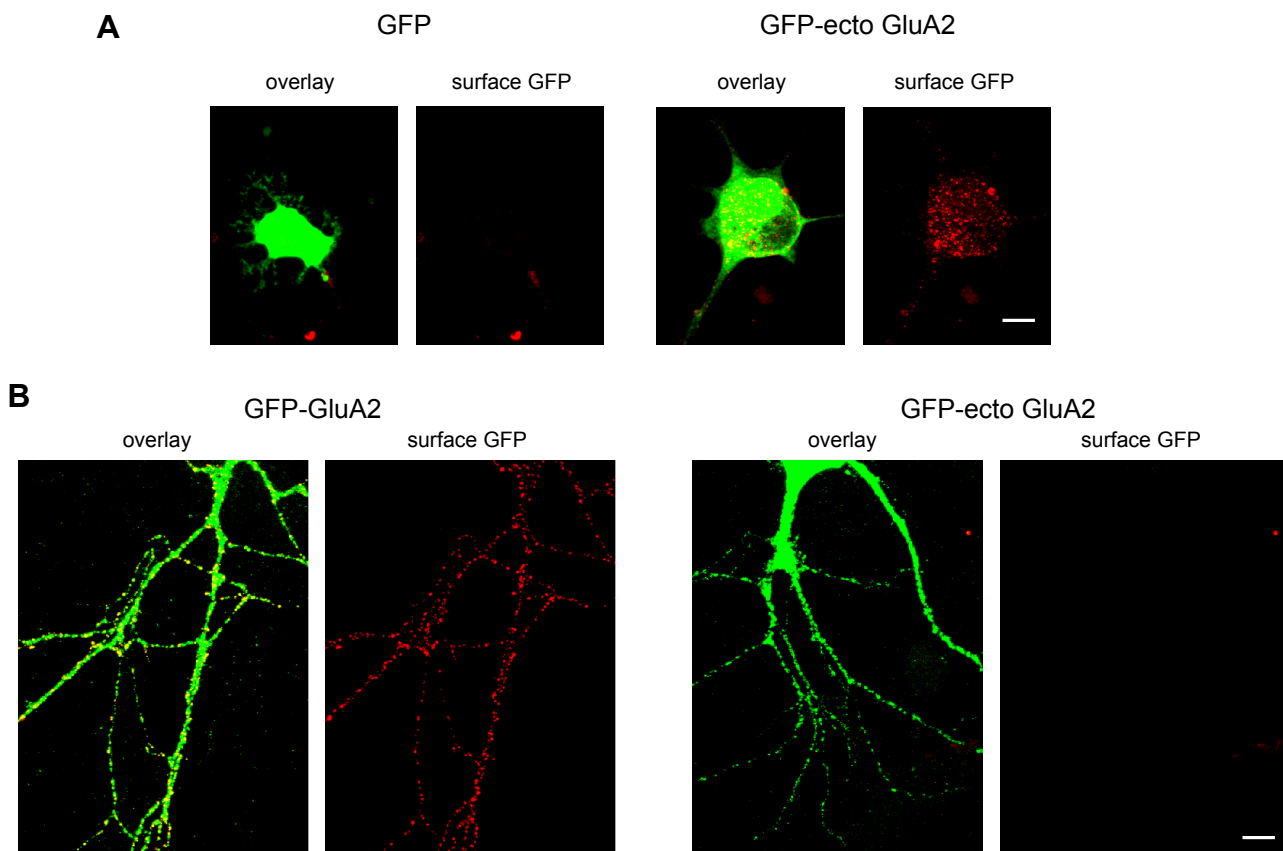
Tracy et al., Supplementary Figure 5



Tracy et al., Supplementary Figure 6



Tracy et al., Supplementary Figure 7



Tracy et al., Supplementary Figure 8