

Homeostatic Synaptic Plasticity of Miniature Excitatory Postsynaptic Currents in Mouse Cortical Cultures Requires Neuronal Rab3A

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eLife Assessment

This study presents **valuable** findings on the role of the small GTPase Rab3A in homeostatic plasticity. While the study demonstrates that Rab3A is required for homeostatic scaling, the evidence supporting the model put forward by the authors is **incomplete**. The work will be of interest to researchers in the field of synaptic transmission and plasticity.

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Abstract

Following prolonged activity blockade, amplitudes of miniature excitatory postsynaptic currents (mEPSCs) increase, a form of plasticity termed “homeostatic synaptic plasticity.” We previously showed that a presynaptic protein, the small GTPase Rab3A, is required for full expression of the increase in miniature endplate current amplitudes following prolonged blockade of action potential activity at the mouse neuromuscular junction *in vivo* (Wang et al., 2011), but it is unknown whether this form of Rab3A-dependent homeostatic plasticity shares any characteristics with central synapses. We show here that homeostatic synaptic plasticity of mEPSCs is impaired in mouse cortical neuron cultures prepared from Rab3A^{-/-} and mutant mice expressing a single point mutation of Rab3A, Rab3A Earlybird mice. To determine if Rab3A is involved in the well-established homeostatic increase in postsynaptic AMPA-type receptors (AMPA receptors), we performed a series of experiments in which electrophysiological recordings of mEPSCs and confocal imaging of synaptic AMPAR immunofluorescence were assessed within the same cultures. We found that the increase in postsynaptic AMPAR levels was more variable than that of mEPSC amplitudes, suggesting other factors may contribute to the homeostatic increase in synaptic strength. Finally, we demonstrate that Rab3A is acting in neurons because only selective loss of Rab3A in neurons, not glia, disrupted the homeostatic increase in mEPSC amplitudes. This is the first

demonstration that a protein thought to function presynaptically is required for homeostatic synaptic plasticity of quantal size.

Introduction

One of the most studied phenomena triggered by prolonged activity blockade is the increase in amplitudes of miniature excitatory postsynaptic currents (mEPSCs) in neurons. First demonstrated in cultures of dissociated cortical neurons (Turrigiano et al., 1998) and spinal cord neurons (O'Brien et al., 1998), the compensatory response to prolonged loss of activity has been dubbed “homeostatic synaptic plasticity” (Turrigiano and Nelson, 2004; Pozo and Goda, 2010). These first two studies provided evidence that glutamate receptors were also increased after prolonged inactivity, via an increased response to exogenously applied glutamate (Turrigiano et al., 1998) and increased immunofluorescent labeling of GluA1 receptors at synaptic sites (O'Brien et al., 1998). The accompanying increase in synaptic AMPA receptors has been confirmed with immunofluorescence in multiple studies of hippocampal and cortical cultures treated with TTX, including (Wierenga et al., 2005; Hou et al., 2008; Ibata et al., 2008; Jakawich et al., 2010; Tatavarty et al., 2013; Xu and Pozzo-Miller, 2017; Dubes et al., 2022). It is now well accepted that the homeostatic increase in mEPSC amplitudes in neurons is due to an increase in postsynaptic AMPA receptors.

In our previous work, we found that a TTX cuff applied for 48 hrs around the sciatic nerve in mice led to an increase in the amplitude of the miniature endplate currents (mEPCs) recorded in tibialis anterior muscles. Surprisingly, and unlike at central synapses, we could find no evidence that the acetylcholine receptors levels at the neuromuscular junction (NMJ) were increased in the TTX-cuff treated mice (Wang et al., 2005). This result led us to search for presynaptic molecules that might homeostatically regulate the presynaptic quantum. In previous studies in chromaffin cells, we identified the small GTPase Rab3A, a presynaptic vesicle protein, as a regulator of synaptic vesicle fusion pore opening (Wang et al., 2008), so we examined whether deletion of Rab3A (Rab3A^{-/-}) might prevent homeostatic upregulation of mEPC amplitude at the NMJ. The results were clear: the homeostatic increase in mEPC amplitude induced by the TTX cuff was strongly reduced in the Rab3A^{-/-} mouse, and was completely abolished in the Earlybird mutant (Rab3A^{Ebd/Ebd}), which has a single point mutation in Rab3A that causes a shift towards early awakening due to a shorter circadian period, that is more dramatic than that of the Rab3A deletion mouse (Kapfhamer et al., 2002; Wang et al., 2011). These results led us to conclude that the homeostatic increase in mEPC amplitude at the NMJ is a presynaptic phenomenon.

It has remained somewhat of a mystery what the role of Rab3A is in synaptic transmission. The Rab3A^{-/-} mouse has minimal phenotypic abnormalities, with evoked synaptic transmission and mEPSCs essentially normal in hippocampal slices (Geppert et al., 1994). At the Rab3A^{-/-} NMJ, reductions in evoked transmission were detected, but only under conditions of reduced extracellular calcium (Coleman et al., 2007). There are some modest changes in short term plasticity during repetitive stimulus trains, with increased depression observed in response to moderate frequencies in hippocampal slices (Geppert et al., 1994) and at the mammalian NMJ (Coleman and Bykhovskaia, 2009), but increased facilitation in response to high frequency stimulation at the mammalian NMJ (Coleman and Bykhovskaia, 2010). The most dramatic effect of loss of Rab3A is the abolishment of a presynaptic form of long-term potentiation (LTP) at the mossy fiber-CA3 synapse that is induced by 25 Hz stimulation in the presence of NMDA blockers (Weisskopf et al., 1994; Castillo et al., 1997). Our result that prolonged inactivity-induced plasticity of mEPC amplitude at the mammalian NMJ is disrupted with loss of function of Rab3A further cements a role for Rab3A in activity-dependent plasticity of synaptic strength. However, a crucial question that remains is whether the TTX-cuff induced effect on the amplitude of the spontaneous synaptic current at the NMJ shares underlying mechanisms with central synapses. We set out to answer this question for the more well-studied phenomenon of homeostatic

plasticity of mEPSCs in dissociated mouse cortical neurons prepared from wild type, Rab3A^{-/-} mice and Rab3A^{Ebd/Ebd} mice treated with TTX for 48 hrs. We show here that homeostatic synaptic plasticity of mEPSC amplitude was strongly reduced in cultures from Rab3A^{-/-} mice, and completely abolished in cultures from Rab3A^{Ebd/Ebd} mice, supporting a shared mechanism with the neuromuscular junction.

Results

We previously reported that mixed cultures of cortical neurons and glia prepared from postnatal day 0-2 mouse pups responded to a block of action potential-mediated activity by a 48 hr TTX treatment with an increase in mEPSC amplitude (Hanes et al., 2020). Here, we asked the question whether cortical cultures prepared from mice lacking the small GTPase Rab3A, or, expressing a point mutation of Rab3A, Rab3A Earlybird, have an altered homeostatic plasticity response to a prolonged loss of network activity. The Rab3A Earlybird mutation was discovered in a mutagenesis screen in mice for genes involved in the rest-activity cycle (Kapfhamer et al., 2002). In that study, the authors concluded that the Earlybird mutation was likely dominant-negative, as the shift in circadian period was substantially more dramatic than that observed in the Rab3A^{-/-} mice. As described in the Introduction, the effects of loss of Rab3A have been modest, so we included experiments using the Rab3A Earlybird mutant mice to see if there would be a more robust phenotype, and in doing so, strengthen our conclusions based on the Rab3A deletion mice. To obtain Rab3A^{-/-} and Rab3A^{Ebd/Ebd} homozygotes, we established two mouse colonies of heterozygous breeders with cultures prepared from pups derived from a final breeding pair of homozygotes. Although we backcrossed Rab3A^{+/-} with Rab3A^{+Ebd} for 11 generations, clear differences in mEPSC amplitudes in untreated cultures (see below) and in calcium current amplitudes in adrenal chromaffin cells (unpublished obs.) remained. Therefore, throughout this study we keep the two strains separate and there are two Rab3A^{+/+} or ‘wild type’ phenotypes.

We found that loss of Rab3A resulted in a greatly impaired homeostatic increase after activity blockade. Example current traces of spontaneously occurring mEPSCs recorded from pyramidal neurons in untreated (CON) 13-14 DIV cortical cultures and sister cultures treated with 500 nM TTX for 48 hr prepared from wild type and Rab3A^{-/-} mice in the Rab3A^{+/-} colony are shown in **Figures 1A** and **B**, respectively. Average mEPSC waveforms from the same recordings are shown in **Figures 1C** and **D**. The mean mEPSC amplitudes for 30 control and 23 TTX-treated neurons from Rab3A^{+/+} cultures are displayed in the box and whisker plot in **Figure 1E**; after activity blockade the average mEPSC amplitude increased from 13.9 ± 0.7 pA to 18.2 ± 0.9 pA. In contrast, in cultures prepared from Rab3A^{-/-} mice, the average mEPSC amplitude was not significantly increased, for 25 untreated cells and 26 TTX-treated cells (**Figure 1F**, 13.6 ± 0.1 pA vs. 14.3 ± 0.6 pA). We found that TTX treatment also resulted in an increase in mEPSC frequency in cultures prepared from Rab3A^{+/+} mice, as shown in **Figure 1G** (CON, 2.26 ± 0.37 sec⁻¹; TTX, 4.62 ± 0.74 sec⁻¹). This TTX-induced frequency increase was strongly reduced in cultures from Rab3A^{-/-} mice, but there were a few outliers with very high frequencies still present after TTX treatment (**Figure 1H**, CON, 2.74 ± 0.49 Hz; TTX, 3.23 ± 0.93 Hz).

We next examined homeostatic plasticity in cultures prepared from wild type mice in the Rab3A^{+Ebd} colony. Example current traces of mEPSCs, and average mEPSC waveforms, are shown in **Figures 2A** and **C**, respectively, for cortical cultures prepared from wild type mice in the Rab3A^{+Ebd} colony. Treatment with TTX for 48 hr led to a significant increase in the average mEPSC amplitude of 23 TTX-treated cells when compared to 20 untreated cells (**Figure 2E**, CON, 11.0 ± 0.6 pA; TTX, 15.0 ± 1.3 pA). We note here that while the two wild type strains respond very similarly to activity blockade, the mean mEPSC amplitude in untreated cultures was significantly different in the two wild type strains, 13.9 ± 0.7 pA, wild type from Rab3A^{+/-} colony; 11.0 ± 0.6 pA, wild type from Rab3A^{+Ebd} colony ($p = 0.004$, Kruskal-Wallis test).

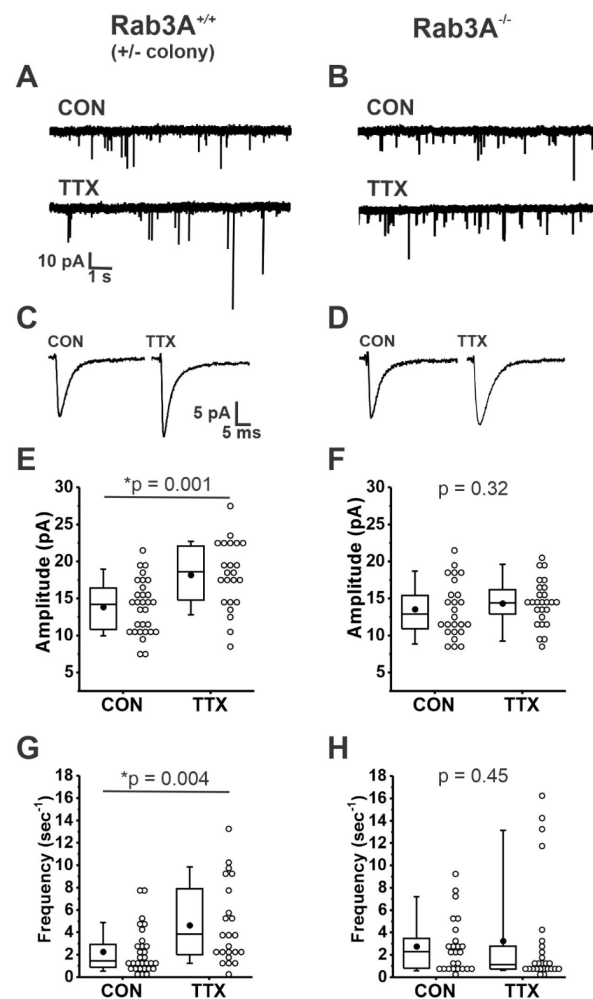


Figure 1.

Loss of Rab3A disrupted the TTX-induced increase in amplitudes of mEPSCs recorded in cultured mouse cortical neurons; the increase in frequency was more variable but also appears to be reduced.

(A) Ten second example traces recorded at -60 mV in pyramidal cortical neurons from an untreated ("CON") and TTX-treated ("TTX") neuron in cultures prepared from Rab3A^{+/+} mice from the Rab3A^{+/+} colony. (B) Ten second example traces recorded at -60 mV in pyramidal cortical neurons from an untreated ("CON") and TTX-treated ("TTX") neuron in cultures prepared from Rab3A^{-/-} mice. (C & D) Average traces for the recordings shown in A and B, respectively. (E) Box plots for average mEPSC amplitudes from untreated cells and TTX-treated cells in cultures prepared from Rab3A^{+/+} mice (Rab3A^{+/+} colony; CON, N = 30 cells, 13.9 ± 0.7 pA; TTX, N = 23 cells, 18.2 ± 0.9 pA; from 11 cultures). (F) Box plots for average mEPSC amplitudes from untreated cells and TTX-treated cells in cultures prepared from Rab3A^{-/-} mice (CON, N = 25 cells, 13.6 ± 0.7 pA; TTX, N = 26 cells, 14.3 ± 0.6 pA); from 11 cultures. (G) Box plots for average mEPSC frequency for same Rab3A^{+/+} cells as in (E) (CON, 2.26 ± 0.37 sec⁻¹; TTX, 4.62 ± 0.74 sec⁻¹). (H) Box plots for average mEPSC frequency for same Rab3A^{-/-} cells as in (F) (CON, 2.74 ± 0.49 sec⁻¹; TTX, 3.23 ± 0.93 sec⁻¹). Box plot parameters: box ends, 25th and 75th percentiles; whiskers, 10th and 90th percentiles; open circles represent means from individual cells, bin size 1 pA, 0.5 sec⁻¹; line, median; dot, mean. p values (shown on the graphs) are from Kruskal-Wallis test. For all p-values, * with underline indicates significance with p < 0.05.

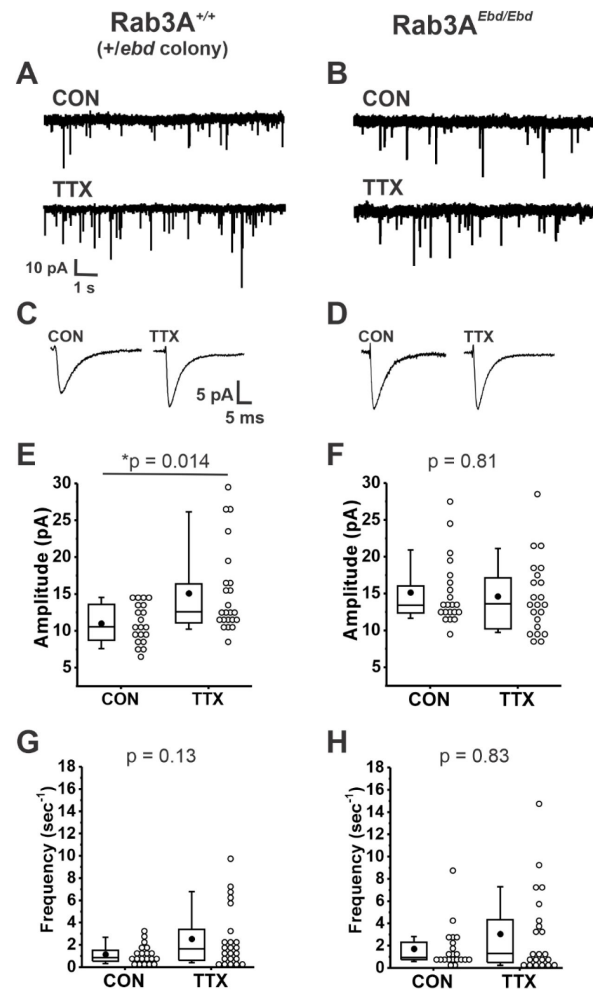


Figure 2.

Normally functioning Rab3A was required for TTX-induced homeostatic plasticity of mEPSC amplitudes in cultured mouse cortical neurons.

(A) Ten second example traces recorded at -60 mV in pyramidal cortical neurons from an untreated ("CON") and TTX-treated ("TTX") neuron in cultures prepared from *Rab3A*^{+/+} mice from the *Rab3A*^{+/Ebd} colony. (B) Ten second example traces recorded at -60 mV in pyramidal cortical neurons from an untreated ("CON") and TTX-treated ("TTX") neuron in cultures prepared from *Rab3A*^{Ebd/Ebd} mice. (C & D) Average traces for the recordings shown in A and B, respectively. (E) Box plots for average mEPSC amplitudes from untreated cells and TTX-treated cells from cultures prepared from *Rab3A*^{+/+} mice (*Rab3A*^{+/Ebd} colony; CON, N = 20 cells, 11.0 ± 0.6 pA; TTX, N = 23 cells, 15.1 ± 1.2 pA; from 6 cultures). (F) Box plots for average mEPSC amplitudes from untreated cells and TTX-treated cells from cultures prepared from *Rab3A*^{Ebd/Ebd} mice (CON, N = 21 cells, 15.1 ± 1.0 pA; TTX, N = 22 cells, 14.6 ± 1.1 pA; from 7 cultures.) (G) Box plots for average mEPSC frequency for same *Rab3A*^{+/+} cells as in (E) (CON, 1.15 ± 0.19 sec⁻¹; TTX, 2.54 ± 0.55 sec⁻¹). (H) Box plots for average mEPSC frequency for same *Rab3A*^{Ebd/Ebd} cells as in (F) (CON, 1.71 ± 0.41 sec⁻¹; TTX, 3.05 ± 0.80 sec⁻¹). Box plot parameters: box ends, 25th and 75th percentiles; whiskers, 10th and 90th percentiles; open circles represent means from individual cells; bin size 1 pA, 0.5 sec⁻¹; line, median; dot, mean. p values (shown on the graphs) are from Kruskal-Wallis test. For all p-values, * with underline indicates significance with p < 0.05.

We found a complete disruption of homeostatic plasticity in cortical cultures prepared from $Rab3A^{Ebd/Ebd}$ mice, as can be seen in viewing example mEPSC traces and average mEPSC waveforms (**Figures 2B** and **D**, respectively). The lack of TTX effect was confirmed in a comparison of mEPSC amplitude means for 21 untreated and 22 TTX-treated cells (**Figure 2F**, CON, 15.1 ± 1.0 pA vs. TTX, 14.6 ± 1.1 pA). There was a trend towards increased mEPSC frequency in TTX-treated cultures from this wild type strain, but the difference was not as robust as for the wild type cultures from $Rab3A^{+/+}$ colony, and did not reach statistical significance (**Figure 2G**, CON, 1.15 ± 0.19 sec⁻¹; TTX, 2.54 ± 0.55 sec⁻¹). This trend was not observed in neurons from cultures prepared from $Rab3A^{Ebd/Ebd}$ mice, likely due to an increase in frequency in untreated cells—frequencies remained high in TTX-treated cells (**Figure 2H**, CON, 1.71 ± 0.41 sec⁻¹; TTX, 3.05 ± 0.80 sec⁻¹). Our results show that the homeostatic increase in mEPSC amplitude after activity blockade is disrupted in both $Rab3A^{-/-}$ and the $Rab3A^{Ebd/Ebd}$ cortical neurons, strongly supporting a crucial role of functioning Rab3A in this process.

The disruption in homeostatic plasticity differs for $Rab3A^{-/-}$ and $Rab3A^{Ebd/Ebd}$. In the $Rab3A^{-/-}$ data set, mEPSCs from untreated cultures were indistinguishable from mEPSCs from $Rab3A^{+/+}$ untreated cultures, demonstrating loss of Rab3A has no impact on basal activity mEPSC amplitudes. In the $Rab3A^{Ebd/Ebd}$ data set, mEPSC amplitudes from untreated cultures were significantly larger than those of untreated cultures from wild type mice, as can be seen comparing the CON values in **Figures 2E** and **F** (CON, $Rab3A^{+/+}$, 11.0 ± 0.7 pA, vs. CON, $Rab3A^{Ebd/Ebd}$, 15.1 ± 1.0 pA, $p = 0.0027$, Kruskal-Wallis test). The increase in mEPSC amplitude in cultures from $Rab3A^{Ebd/Ebd}$ mice is consistent with the increase in mEPSC amplitude we observed at the $Rab3A^{Ebd/Ebd}$ NMJ (Wang et al., 2011).

The small GTPase Rab3A is generally thought to function presynaptically to regulate synaptic vesicle trafficking, possibly in an activity-dependent manner (Castillo et al., 1997; Lonart et al., 1998; Leenders et al., 2001; Schluter et al., 2006; Coleman and Bykhovskaia, 2009; Tian et al., 2012). In contrast, homeostatic plasticity of mEPSC amplitude has been attributed to an increase in postsynaptic receptors on the surface of the dendrite (O'Brien et al., 1998; Turrigiano et al., 1998). At the NMJ in vivo, we could find no evidence for an increase in AChRs after TTX block of the sciatic nerve in vivo (Wang et al., 2005). Is Rab3A required for the homeostatic increase in surface AMPA-type glutamate receptors that has been confirmed by multiple studies (see Introduction)?

We explored whether Rab3A might be acting to increase postsynaptic AMPA receptors. In some studies of homeostatic synaptic plasticity it has been found that application of a Ca²⁺-permeable AMPA receptor-specific inhibitor reversed the homeostatic increase in mEPSC amplitude (Ju et al., 2004; Thiagarajan et al., 2005; Sutton et al., 2006). **Figure 3A** shows that the TTX-induced increase in mean mEPSC amplitude was nearly identical in a set of 11 CON and 11 TTX-treated cells before and after treatment with 1-Naphthyl acetyl spermine trihydrochloride (NASPM), a synthetic analogue of the Ca²⁺-permeable AMPA receptor inhibitor philanthotoxin (before NASPM, CON 12.9 ± 3.5 pA; TTX, 17.5 ± 3.1 pA; after NASPM, CON 11.9 ± 2.6 pA; TTX 16.1 ± 3.5 pA). This result indicates that the TTX-induced increase in mEPSC amplitude does not depend on Ca²⁺-permeable receptors, since the effect of TTX clearly remained when their presence was removed by acute NASPM application. We do not think that there was any technical issue with the NASPM application, because overall, mEPSC amplitudes were reduced modestly in both untreated and TTX-treated cultures (**Figure 3B**, CON, before NASPM, 12.9 ± 3.5 pA; after NASPM, 11.9 ± 2.6 pA; TTX, before NASPM, 17.5 ± 3.1 pA; after NASPM, 16.1 ± 3.5 pA). Furthermore, NASPM consistently reduced mEPSC frequency (**Figure 3C**, CON, before NASPM, 1.84 ± 0.55 sec⁻¹; after NASPM, 1.56 ± 0.53 sec⁻¹; TTX, before NASPM, 4.40 ± 3.51 sec⁻¹; after NASPM, 2.68 ± 2.25 sec⁻¹). Similar to the data in **Figure 1**, frequency is significantly increased after TTX treatment ($p = 0.04$), but this difference is no longer significant after NASPM application ($p = 0.20$). One explanation for the effect on frequency is that there are synaptic sites that express only homomers of Ca²⁺-permeable AMPA receptors (GluA2-lacking AMPARs); mEPSCs from these sites would be expected to be

completely blocked by NASPM (see cartoon description in **Figure 3D**, Left). We cannot rule out an alternative explanation, that presynaptic Ca^{2+} -permeable AMPA receptors normally enhance release probability, and NASPM prevents this action. The magnitude of the frequency reduction following acute NASPM appears to be greater after TTX treatment, suggesting that loss of activity could promote the establishment of synaptic sites that contain only Ca^{2+} -permeable receptors. However, these new sites do not appear to contribute to the increase in mEPSC amplitude (**Figure 3A**). The lack of effect of NASPM rules out the contribution of Ca^{2+} -permeable AMPA receptors GluA1, GluA3 and GluA4 (Hollmann et al., 1991; Burnashev et al., 1992; Jonas and Burnashev, 1995), as well as Ca^{2+} -permeable forms of Kainate receptors GluA5 and GluA6 (Koike et al., 1997; Sun et al., 2009) to homeostatic synaptic plasticity.

Having established that homomeric Ca^{2+} -permeable receptors are not contributing to the homeostatic increase in mEPSC amplitude, we turned to immunofluorescence and confocal imaging to assess whether GluA2 receptor expression, which will identify GluA2 homomers and GluA2 heteromers, was increased in our wild type mouse cortical cultures following 48 hr treatment with TTX. Since mEPSCs necessarily report synaptic levels of receptors, we used VGLUT1-immunofluorescence to identify synapses on pyramidal primary apical dendrites labeled with MAP-2 immunofluorescence. We focused on the primary dendrite of pyramidal neurons as a way to reduce variability that might arise from being at widely ranging distances from the cell body, or, from inadvertently sampling dendritic regions arising from inhibitory neurons. In addition, it has been shown that the increase in receptors is most dramatic in this region (Turrigiano et al., 1998). **Figure 4** (Top) shows two pyramidal neurons, one from an untreated culture on the left, and one from a TTX-treated culture on the right, prepared from Rab3A^{+/+} mice. To measure characteristics of synaptically located GluA2 receptor clusters, we zoomed in on the primary dendrite (region in white rectangle). The zoomed regions for single confocal sections of the selected area are shown below and contain pairs of VGLUT1- and GluA2-immunofluorescent clusters indicated by white trapezoidal frames. A dendrite typically required ~10 confocal sections to be fully captured, and the total number of synaptic pairs for all the sections imaged on a dendrite averaged 20 (20.4 ± 6.5 (SD); range, 11-38) so this is an atypically high number of pairs within a single section; these particular dendrites and sections were selected for illustration purposes. In addition to the synaptic pairs, we observed many GluA2-immunofluorescent clusters not associated with VGLUT1 immunofluorescence (a few are indicated with white arrows), most probably extrasynaptic receptors. There were also GluA2- positive clusters present outside of MAP2-positive dendrites, which may be located on astrocytes (Fan et al., 1999). Finally, GluA2 immunofluorescent clusters close to VGLUT1 immunofluorescence but not located along any apparent MAP2-positive neurites suggests the presence of axon-axonal contacts, although VGLUT1 has also been detected in astrocytes (Ormel et al., 2012). Only sites that contained both VGLUT1 and GluA2 immunofluorescence close to the primary MAP2-positive dendrite or a secondary branch along this primary dendrite were selected for analyses. In the experiment from which these images were collected, we analyzed the distance from the cell body for each synapse. The average distance from the cell body, for dendrites from the untreated cell, was $38.5 \pm 2.8 \mu\text{m}$; for the TTX-treated cell, it was $42.4 \pm 3.2 \mu\text{m}$ ($p = 0.35$, Kruskal-Wallis test). Since the greatest distance from the cell body is set by the outer limit of the zoom window, and that window was placed adjacent to the cell body, distances in other experiments would be in this same range.

Variability in the magnitude of the homeostatic response from culture to culture is averaged out in physiological experiments by the pooling of data from many cells across many cultures. To reduce the necessity for many cultures, we chose to pair experiments in the same cultures by recording mEPSCs from one set of coverslips, and processing another set of coverslips from the same culture for immunofluorescence. We also wanted to determine if the homeostatic response of mEPSC amplitudes varied from culture to culture, and if so, whether the GluA2 receptor levels varied in parallel. We completed this matched paradigm of physiology and immunofluorescence for 3 cultures prepared from Rab3A^{+/+} mice. We first present the results as means for each culture, for control and TTX-treated dishes, for mEPSC data (**Figure 5A**) and imaging data (**Figure 5B**).

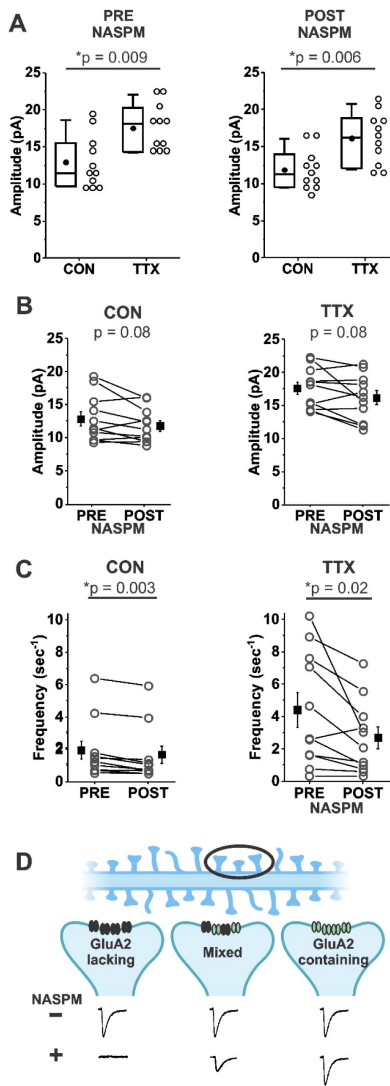


Figure 3.

Homeostatic plasticity of mEPSC amplitudes in mouse cortical cultures treated with TTX for 48 hr was unchanged by acute inhibition of Ca²⁺ permeable AMPA receptors by NASPM (20 μ m).

(A) Box plot comparison of the TTX effect on mEPSC amplitudes in the same pyramidal neurons before and after application of 20 μ M NASPM. (N = 11 cells from 3 cultures; Pre-NASPM, CON: 12.9 ± 1.1 pA; TTX: 17.5 ± 0.9 pA; post-NASPM, CON: 11.9 ± 0.8 pA; TTX: 16.1 ± 1.0 pA). (B) Line series plot of mEPSC amplitudes before and after acute perfusion with 20 μ M NASPM for untreated and TTX-treated pyramidal neurons; same cells as in (A); CON, pre-NASPM: 12.9 ± 1.1 pA; post-NASPM: 11.9 ± 0.8 pA; TTX, pre-NASPM: 17.5 ± 0.9 pA; post-NASPM: 16.1 ± 0.1 pA. (C) Line series plot of mEPSC frequency before and after acute perfusion with 20 μ M NASPM for untreated and TTX-treated pyramidal neurons; same cells as in (A); CON, pre-NASPM: 1.84 ± 0.55 sec⁻¹; post-NASPM: 1.56 ± 0.53 sec⁻¹; TTX, pre-NASPM: 4.40 ± 1.06 sec⁻¹; post-NASPM: 2.68 ± 0.68 sec⁻¹. (D) A proposed mechanism for why NASPM had a robust effect on frequency without greatly affecting amplitude. A dendrite with spines (Top) is expanded on three postsynaptic sites (Middle) to show possible types of AMPA receptor distributions: Left, at a site comprised only of Ca²⁺-permeable AMPA receptors (NASPM-sensitive, GluA2-lacking receptors (black)), NASPM would completely inhibit the mEPSC, causing a decrease in frequency to be measured in the overall population; Right, at a site comprised only of Ca²⁺-impermeable AMPA receptors (NASPM-insensitive, GluA2-containing receptors (green)), NASPM would have no effect on mEPSC amplitude; Middle, at a site comprised of a mix of Ca²⁺-permeable and Ca²⁺-impermeable AMPA receptors, NASPM would partially inhibit the mEPSC. Diagram produced in *BioRender.com* (2024). Box plot parameters: box ends, 25th and 75th percentiles; whiskers, 10th and 90th percentiles; open circles represent means from individual cells; line, median; dot, mean. p values (shown on the graphs) are from Kruskal-Wallis test. Line series plot p values are from student's paired t test. For all p-values, * with underline indicates significance with p < 0.05.

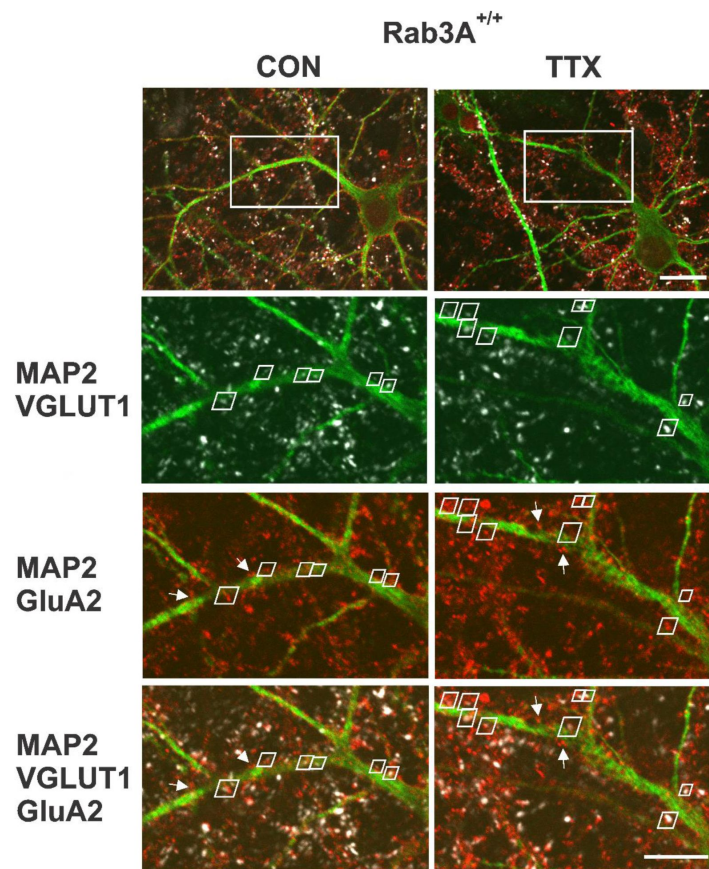


Figure 4.

Identification of synaptic GluA2 receptor immunofluorescence on primary dendrites of pyramidal neurons in high density mouse cortical co-cultures prepared from Rab3A^{+/+} mice.

(Top) Non-zoomed single confocal sections collected with a 60X oil immersion objective of recognizably pyramidal-shaped neurons, presumed to be excitatory neurons, selected for synaptic GluA2 analysis. A neuron was selected from an untreated coverslip (CON, Left) and a TTX-treated coverslip (TTX, Right) from the same culture prepared from Rab3A^{+/+} animals (= Rab3A^{+/+} Culture #2 in [Figure 5](#)). The white rectangular boxes indicate 5X zoomed areas shown in the images below. Note that the depth of the single confocal section of the non-zoomed neuron image is not at the same depth as the confocal section in zoomed dendritic image, so some features are not visible in both images. Scale bar, 20 μ m. (Bottom) 5X zoom single confocal sections selected for demonstration purposes because they had an unusually high number of identified synaptic pairs along the primary dendrite contained within a single confocal section. Synaptic pairs, highlighted with white trapezoids, were identified based on close proximity of GluA2 (red) and VGLUT1 (white) immunofluorescence, apposed to the MAP2 immunofluorescent primary dendrite (green). Some apparent synaptic pairs are not highlighted with a white trapezoid because there was a different confocal section in which the two immunofluorescent sites were maximally bright. There were GluA2 positive clusters located on the primary dendrites that were not apposed to VGLUT1-positive terminals (4 of these non-synaptic GluA2 clusters are highlighted with white arrows in the MAP2-GluA2 and MAP2-GluA2- VGLUT1 panels). Scale bar, 10 μ m.

Levels of GluA2 immunofluorescence at synaptic sites were quantified by the size of the GluA2-immunofluorescent receptor cluster, because this showed the greatest response to activity blockade; the average intensity value, and the integral of intensity values across the area of the cluster, are included in a summary of the data in **Table 1** [↗](#). Three of three cortical cultures showed an increase in mean mEPSC amplitude after TTX treatment. In contrast, one of the same three cultures showed a decrease in mean GluA2 receptor cluster size. Therefore, the decrease in receptor levels in Culture #3 was due to something other than a lack of homeostatic effect on mEPSCs in that culture. Because there was a clear trend towards increased GluA2 receptor cluster size, but the receptor response appears more variable, we performed immunofluorescence measurements on two additional cortical cultures, both of which showed modest increases after TTX treatment (**Figure 5C** [↗](#)). We confirmed that there was not an increase in GluA1 receptor levels in two matched cultures that showed robust homeostatic increases in mEPSC amplitudes (**Supplementary Figure 1A** [↗](#), culture mean mEPSC amplitudes; **Supplementary Figure 1B** [↗](#), culture mean GluA1 receptor cluster sizes).

We next pooled data from the multiple cultures to examine effects of activity blockade on GluA2 receptors at the level of cell means. Because the average number of synaptic sites identified in the primary dendrites in three cultures was 20.4, we also took 20 mEPSC samples from each cell, the 21st to the 40th mEPSC recorded, to minimize any differences in variability between GluA2 receptors and mEPSCs that might be due to a difference in sampling. In the data pooled from the 3 matched experiments, neurons from Rab3A^{+/+} cultures showed a significant increase in mean mEPSC amplitudes following activity blockade (**Figure 5D** [↗](#); **Table 1** [↗](#)). This result indicates that the homeostatic response averaged across the 3 cultures was very similar to, but slightly smaller than, that of the data set presented in **Figure 1** [↗](#). The mean for size of GluA2 immunofluorescent receptor clusters showed a trend towards higher values after activity blockade (18.1%) that was of similar magnitude to the mEPSC amplitude increase (19.7%), but did not reach statistical significance for 3 cultures (**Figure 5E** [↗](#)) or 5 cultures (**Figure 5F** [↗](#); **Table 1** [↗](#)). As expected, mean mEPSC amplitude was not increased following activity blockade in the data pooled from a new set of 3 Rab3A^{-/-} cultures (**Table 1** [↗](#)). For immunofluorescence results from the same Rab3A^{-/-} cultures, no trend towards higher values was apparent for GluA2 receptor cluster characteristics (**Table 1** [↗](#)). However, given the variability of the GluA2 response in cultures from Rab3A^{+/+} mice, it would require a much higher number of Rab3A^{+/+} and Rab3A^{-/-} cultures to make any firm conclusions about the role of Rab3A in the homeostatic response of GluA2 receptors.

Having used VGLUT1 immunofluorescence to mark synaptic sites, we could also ask whether there was a presynaptic effect of activity blockade on the size, intensity or integral of the VGLUT1 signal at sites apposed to the previously examined GluA2 postsynaptic sites. In the pooled data from cultures prepared from Rab3A^{+/+} mice, we found no change in the size of the VGLUT1-positive regions, and trends towards reduced intensity and integral (**Table 2** [↗](#)). These data do not support the idea that activity blockade caused an increase in the amount of VGLUT1 per vesicle, although we cannot rule out that a simultaneous reduction in the number of vesicles obscured the effect, since we did not have an independent label for synaptic vesicles. In cultures prepared from Rab3A^{-/-} mice, the size, intensity and integral of the VGLUT1-positive regions did not significantly change (**Table 2** [↗](#)).

We have demonstrated here for the first time that the synaptic vesicle protein Rab3A influences the homeostatic regulation of quantal amplitude. What are possible ways that Rab3A could exert its effect? It has previously been shown that exogenous addition of TNF α to hippocampal cultures causes an increase in surface expression of GluA1 receptors (although not GluA2 receptors), and that the homeostatic increase in mEPSC amplitudes is abolished in cultures prepared from the TNF α deletion mouse (Stellwagen et al., 2005 [↗](#); Stellwagen and Malenka, 2006 [↗](#)). Furthermore, neurons from TNF α ^{+/+} mice plated on glial feeder layers derived from TNF α ^{-/-} mice fail to show the increase in mEPSC amplitude after TTX treatment, indicating that the TNF α inducing the receptor increases following activity blockade comes from the glial cells (Stellwagen and Malenka, 2006 [↗](#)).

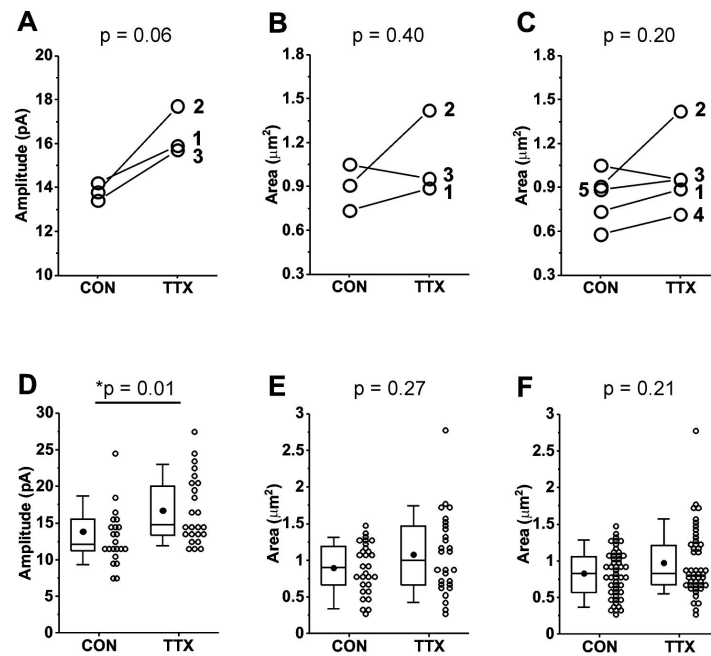


Figure 5.

Comparison of mEPSC amplitudes and GluA2 receptor cluster areas in matched and unmatched mouse cortical cultures prepared from Rab3A^{+/+} mice and treated with TTX for 48 hr.

(A) Culture averages of mEPSC amplitudes for untreated (CON) and TTX-treated coverslips (TTX) in each of 3 Rab3A^{+/+} mouse cortical co-cultures. Culture #1, CON, N = 6, 14.2 ± 2.2 pA; TTX, N = 6, 15.9 ± 1.9 pA; Culture #2, CON, N = 7, 13.8 ± 2.4 pA; TTX, N = 8, 17.7 ± 1.8 pA; Culture #3, CON, N = 10, 13.4 ± 0.8 pA; TTX, N = 9, 15.7 ± 1.0 pA. (B) Culture averages of GluA2 receptor cluster size in the same 3 cultures as shown in (A). Culture #1, CON, N = 10, 0.73 ± 0.09 μm²; TTX, N = 9, 0.89 ± 0.09 μm²; Culture #2, CON, N = 9, 0.91 ± 0.12 μm²; TTX, N = 9, 1.42 ± 0.24 μm²; Culture #3, CON, N = 10, 1.05 ± 0.11 μm²; TTX, N = 9, 0.95 ± 0.15 μm². (C) Two additional culture averages are included that did not have corresponding mEPSC recordings. Culture #4, CON, N = 10, 0.58 ± 0.07 μm²; TTX, N = 10, 0.71 ± 0.04 μm²; Culture #5, CON, N = 10, 0.89 ± 0.05 μm²; TTX, N = 10, 0.95 ± 0.08 μm². (D) Box plots of cell mean mEPSC amplitudes pooled from same 3 cultures as shown in (A). CON, N = 23 cells, 13.7 ± 0.9 pA; TTX, N = 24 cells, 16.4 ± 0.9 pA. (E) Box plots of dendrite mean GluA2 receptor cluster size pooled from same 3 cultures as shown in (A) and (B). CON, N = 29 dendrites, 0.90 ± 0.06 μm²; TTX, N = 28 dendrites, 1.08 ± 0.10 μm². (F) Box plots of dendrite mean GluA2 receptor cluster size pooled from the same 5 cultures shown in (C). CON, N = 49 dendrites, 0.83 ± 0.04 μm²; TTX, N = 48 dendrites, 0.98 ± 0.07 μm². Line series, Student's paired T test; box plots, Kruskal-Wallis test. Box plot parameters: box ends, 25th and 75th percentiles; whiskers, 10 and 90 percentiles; open circles indicate means of individual cells/dendrites; bin size 1 pA, 0.5 μm²; line, median; dot, mean. For all p-values, * with underline indicates significance with p < 0.05.

Table 1.

Comparison of mEPSC amplitude and GluA2 receptor cluster characteristics in mouse cortical cultures prepared from Rab3A^{+/+} mice, and Rab3A^{-/-} mice.

	Rab3A ^{+/+}				Rab3A ^{-/-}			
Quantity	CON Mean, SE	TTX Mean, SE (N, cultures)	% change	p value	CON Mean, SE (N, cultures)	TTX Mean, SE (N, cultures)	% change	p value
mEPSC amplitude	13.7 ± 0.9 pA (23 cells, 3 cultures)	16.4 ± 0.9 pA (24 cells, 3 cultures)	+19.7	*p = 0.02	14.9 ± 0.8 pA (21 cells, 3 cultures)	13.5 ± 0.9 pA (21 cells, 3 cultures)	-9.4	p = 0.18
GluA2 cluster size	0.83 ± 0.04 μm ² (49 cells, 5 cultures)	0.98 ± 0.07 μm ² (48 cells, 5 cultures)	+18.1	p = 0.21	0.93 ± 0.05 μm ² (30 cells, 3 cultures)	0.91 ± 0.05 μm ² (29 cells, 3 cultures)	-2.2	p = 0.75
GluA2 cluster intensity	733 ± 14 a.u. (49 cells, 5 cultures)	738 ± 13 a.u. (48 cells, 5 cultures)	+0.7	p = 0.69	766 ± 12 a.u. (30 cells, 3 cultures)	776 ± 15 a.u. (29 cells, 3 cultures)	+1.3	p = 0.46
GluA2 cluster integral	311021 ± 19282 a.u. (49 cells, 5 cultures)	365366 ± 27080 a.u. (48 cells, 5 cultures)	+17.4	p = 0.22	369436 ± 23439 a.u. (30 cells, 3 cultures)	364237 ± 25833 a.u. (29 cells, 3 cultures)	-0.8	p = 0.86

Table 2.

Comparison of mEPSC amplitude and VGLUT1 positive presynaptic site characteristics in mouse cortical cultures prepared from Rab3A^{+/+} and Rab3A^{-/-} mice. mEPSC data are identical to that in Table 1, reproduced here for comparison purposes.

	Rab3A ^{+/+}				Rab3A ^{-/-}			
Quantity	CON Mean, SE	TTX Mean, SE	% change	p value	CON Mean, SE	TTX Mean, SE	% change	p value
mEPSC amplitude	13.7 ± 0.9 pA (23 cells, 3 cultures)	16.4 ± 0.9 pA (24 cells, 3 cultures)	+19.7	*p = 0.02	14.9 ± 0.8 pA (21 cells, 3 cultures)	13.5 ± 0.9 pA (21 cells, 3 cultures)	-9.4	p = 0.18
VGLUT1 site size	1.17 ± 0.08 μm ² (29 cells, 3 cultures)	1.07 ± 0.06 μm ² (24 cells, 3 cultures)	-7.7	p = 0.97	0.89 ± 0.03 μm ² (30 cells, 3 cultures)	0.95 ± 0.04 μm ² (29 cells, 3 cultures)	+6.7	p = 0.55
VGLUT1 site intensity	699 ± 39 a.u. (29 cells, 3 cultures)	639 ± 31 a.u. (28 cells, 3 cultures)	-8.6	p = 0.28	554 ± 13 a.u. (30 cells, 3 cultures)	570 ± 19 a.u. (29 cells, 3 cultures)	+2.9	p = 0.18
VGLUT1 site integral	430787 ± 36818 a.u. (29 cells, 3 cultures)	351653 ± 21689 a.u. (28 cells, 3 cultures)	-18.4	p = 0.13	257159 ± 18265 a.u. (30 cells, 3 cultures)	289857 ± 18521 a.u. (29 cells, 3 cultures)	+12.7	p = 0.21

It has recently been shown that the source of TNF α is astrocytes rather than microglia (Heir et al., 2024 [\[1\]](#)). Rab3A has been detected in astrocytes (Maienschein et al., 1999 [\[2\]](#); Hong et al., 2016 [\[3\]](#)), so to determine whether Rab3A is acting via regulating TNF α release from astrocytes, we performed experiments similar to those of Stellwagen and Malenka (2006 [\[4\]](#)) (schema illustrated in **Figure 6** [\[5\]](#), Left). Under our culturing conditions, the glial feeder layers would be expected to be composed mainly of astrocytes (Heir et al., 2024 [\[1\]](#)). We compared the effect of activity blockade on mEPSC amplitudes recorded from cortical neurons from Rab3A^{+/+} mice plated on Rab3A^{+/+} glial feeder layers (**Figure 6A** [\[5\]](#)); neurons from Rab3A^{+/+} mice plated on Rab3A^{-/-} glia (**Figure 6B** [\[5\]](#)), and neurons from Rab3A^{-/-} mice plated on Rab3A^{+/+} glia (**Figure 6C** [\[5\]](#)). If Rab3A is required for TNF α release from glia, then Rab3A^{+/+} neurons plated on Rab3A^{-/-} glia should not show a homeostatic increase in mEPSC amplitude after treatment with TTX, and any cultures with Rab3A^{+/+} glia should have a normal homeostatic response. We found the opposite result: mEPSC amplitudes increased dramatically in cultures where Rab3A was present in neurons (WT on WT, CON, 13.3 $\pm$ 0.5 pA, TTX, 16.7 $\pm$ 1.2 pA; WT on KO, CON, 13.3 $\pm$ 1.0 pA, TTX, 18.8 $\pm$ 1.4 pA), but the increase was greatly diminished in cultures where Rab3A was present only in glia (KO on WT, CON, 15.2 $\pm$ 1.1 pA, TTX, 16.9 $\pm$ 0.7 pA). In the glial feeder layer culture, the loss of Rab3A in the neurons may have caused an increase in mEPSC amplitude at baseline, similar to the Earlybird mutant in dissociated cultures, but this difference was not significant (CON, WT on WT, 13.3 $\pm$ 0.5 pA; CON, KO on WT, 15.2 $\pm$ 1.1 pA, p = 0.23, Kruskal-Wallis test), so it is also possible this can be attributed to culture to culture variability. Regarding mEPSC frequency, in cultures with Rab3A^{+/+} neurons, frequency trended upward after activity blockade, whereas in the culture with Rab3A^{-/-} neurons, frequency was already increased (although not significantly, CON, WT on WT, 2.54 $\pm$ 0.57 sec⁻¹; CON, KO on WT, 4.46 $\pm$ 1.21 sec⁻¹, p = 0.23, Kruskal-Wallis test), and trended downward after activity blockade.

In summary, in the absence of Rab3A from glia, the homeostatic plasticity of mEPSC amplitude following activity blockade was normal, whereas in the absence of Rab3A in neurons, homeostatic plasticity was greatly diminished. This result makes it highly unlikely that Rab3A is required for the release of TNF α , or another factor from glia, that induces a homeostatic upregulation of postsynaptic receptors and thereby increases mEPSC amplitude following TTX treatment. Neuronal Rab3A appears to mediate the homeostatic increase in mEPSC amplitude following activity blockade.

Discussion

We found that homeostatic synaptic plasticity of mEPSC amplitude in dissociated mixed cultures of mouse cortical neurons and glia behaved remarkably similar to the mouse NMJ in response to loss of Rab3A function: in cultures from Rab3A^{-/-} mice, the increase in mEPSC amplitudes following prolonged network silencing by TTX was strongly diminished, and in cultures from Rab3A^{Ebd/Ebd} mice, basal mEPSC amplitude was increased compared to that of wild-type cultures and was not further modified by TTX treatment. These results suggest that normal function of the synaptic vesicle protein Rab3A is required for the homeostatic increase of mEPSC amplitude in cortical cultures. At the NMJ, the effect of Rab3A on homeostatic upregulation of mEPC amplitude was not mediated by upregulation of postsynaptic receptors. In neurons, the increase in mEPSC amplitude following block of activity was not due to increases in Ca²⁺-permeable GluA1 homomers because acute NASPM application was unable to reverse the TTX-induced increase in amplitude. Imaging of immunofluorescence of GluA2 receptors revealed the response was more variable than that of mEPSC amplitude. The simplest explanation for our findings at both the NMJ and in neurons is that Rab3A regulates a presynaptic contributor to alteration of quantal amplitude during homeostatic synaptic plasticity.

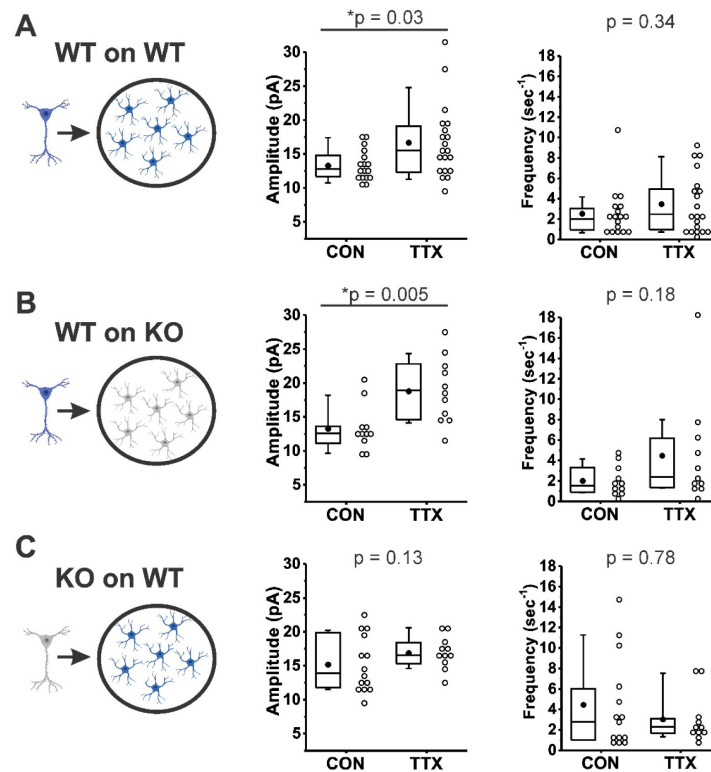


Figure 6.

Rab3A in neurons, not astrocytes, was required for full TTX-induced homeostatic plasticity.

(A)-(C), mEPSC amplitude (Middle) and frequency (Right) data from dissociated cortical neurons plated on an astrocyte feeder layer, each prepared separately from the type of mice depicted in the schema (Left): (A) Neurons from Rab3A^{+/+} mice plated on astrocytes from Rab3A^{+/+} mice. Box plots for mEPSC amplitudes (CON, N = 17 cells, 13.3 ± 0.5 pA; TTX, N = 20 cells, 16.7 ± 1.2 pA) and mEPSC frequency in the same recordings (mean, CON, 2.54 ± 0.57 sec⁻¹; TTX, 3.48 ± 0.64 sec⁻¹); from 4 cultures. (B) Neurons from Rab3A^{+/+} mice plated on astrocytes from Rab3A^{-/-} mice. Box plots for average mEPSC amplitude (CON, N = 11 cells, 13.3 ± 1.0 pA; TTX, N = 11 cells, 18.8 ± 1.4 pA) and average mEPSC frequency in the same recordings (CON, 2.01 ± 0.41 sec⁻¹; TTX, 4.47 ± 1.53 sec⁻¹); from 2 cultures. (C) Neurons from Rab3A^{-/-} neurons plated on astrocytes from Rab3A^{+/+} mice. Box plots for average mEPSC amplitude (CON, N = 14 cells, 15.2 ± 1.1 pA; TTX, N = 11 cells, 16.9 ± 0.7 pA) and mEPSC frequency in the same recordings (CON, 4.47 ± 1.21 sec⁻¹; TTX, 3.02 ± 0.70 sec⁻¹); from 3 cultures. Box plot parameters: box ends, 25th and 75th percentiles; whiskers, 10th and 90th percentiles; open circles represent means from individual cells; line, median; dot, mean. p values (shown on the graphs) are from Kruskal-Wallis test. p-values denoted with * and underline indicates significance of p < 0.05.

Is the lack of homeostatic plasticity in the Earlybird mutant due to occlusion?

We found that mEPSC amplitude was increased in untreated cultures prepared from Rab3A^{Ebd/Ebd} mice, compared to mEPSC amplitude in cultures from wild type mice. If there is a limit to how large an mEPSC can become, it is possible that the mutant Rab3A does not affect homeostatic plasticity, only occludes it. Similar increases in mEPSC amplitude at baseline, combined with a failure to increase further after activity blockade, were noted in cultures prepared from mECP2, AKAP, Homer1a, and Arc/Arg3.1 deletion mice (Shepherd et al., 2006 [DOI](#); Hu et al., 2010 [DOI](#); Xu and Pozzo-Miller, 2017 [DOI](#); Sanderson et al., 2018 [DOI](#)) as well as in cultures treated with ubiquitin proteasome inhibitors (Jakawich et al., 2010 [DOI](#)) or microRNA 186-5p (Silva et al., 2019 [DOI](#)), suggesting the possibility of a generalized phenomenon in which increased mEPSC amplitude is induced when homeostatic regulation is disrupted. Since we did not observe such an increase in mEPSC amplitude at baseline in cultures from Rab3A^{-/-} mice, it remains a possibility that the point mutant may bind to novel partners, causing activities that would not be either facilitated or inhibited by Rab3A. Still, it is a strong coincidence that the novel activity of the mutant Earlybird Rab3A would affect mEPSC amplitude, the same characteristic that is modulated by activity blockade in a Rab3A dependent manner. Although we cannot rule out occlusion, an alternate interpretation is that the presence of the mutant protein mimics the condition of activity blockade.

Homeostatic plasticity of postsynaptic receptors was less consistent than that of mEPSC amplitude

While we found that the effect of TTX treatment on mEPSC amplitude and GluA2 receptor levels were of similar magnitude, the effect on GluA2 receptor levels was not statistically significant for the pooled data sets acquired in the same cultures, nor in a larger data set containing 5 cultures. In light of the extensive literature documenting increases in GluA1 or GluA2 receptors, or both, after activity blockade, the less than convincing increase in GluA2 receptor levels in the current work came as a surprise. The reason for the lack of significance was that the increase in receptors was more variable. We are aware of three possible explanations for greater variability. 1) The number of synaptic sites sampled was smaller for the imaging data set. This was because we limited our analysis to synaptic receptors on the primary dendrite, where the increase in sensitivity to exogenously applied glutamate was shown to be greatest (see **Figure 3** [DOI](#) in (Turrigiano et al., 1998 [DOI](#))). To address this issue, we limited our sample size of mEPSCs in the matched experiments to the same number per cell as the mean sample size per cell for imaging, but there was still a discrepancy in the effect of TTX on mEPSCs vs. receptors. 2) The small sample of only 3 cultures was not enough to reveal the increase in receptors. We attempted to address this by adding 2 more cultures. Although both additional cultures showed increases in GluA2 receptor cluster size, the magnitudes of the increases were not large, and when cells from these cultures were included in the pooled data set, the p value was not substantially improved. The only remaining possibility is: 3) The complexity involved in processing cultures for immunofluorescence introduces variability that obscures the true effect. One can get around technical issues related to immunofluorescence processing by performing Western blots, but then any changes detected could be due to upregulation of extrasynaptic receptors. What remains unclear is why our data were impacted by this but data of other studies were not. Some other studies have used the number of synaptic sites or puncta as the sample size (for example, (Hou et al., 2008 [DOI](#); Ibata et al., 2008 [DOI](#); Wang et al., 2019 [DOI](#))). When we analyzed our data after including as few as 6 randomly selected cluster size measurements per cell, a Kruskal-Wallis test gave highly significant p values for 3 cultures (p = 0.001, n = 174 sites (6 * 29 cells)) and 5 cultures (p = 0.005, n = 294 sites (6 * 49 cells)). We found only a single study that had comparable sample sizes for receptors and mEPSC amplitudes, and the p value was < 0.01 for mEPSC amplitudes (n = 9 and 10 cells for CON and TTX, respectively), but five times higher (< 0.05) for receptor data (n = 13 and 14 cells for CON and TTX) (Xu and Pozzo-Miller, 2017 [DOI](#)). This single example is consistent with our suggestion that measurements of changes in

receptors after activity blockade are more variable than measurements of mEPSC amplitudes. In sum, our data suggest that TTX treatment of mouse cortical cultures consistently leads to an increase in mEPSC amplitudes, and sometimes leads to an increase in synaptic GluA2 receptor cluster size. Increased receptors likely contribute to increases in mEPSC amplitudes, but our current data, coupled with our previous findings at the mouse neuromuscular junction, support the idea that there are additional sources contributing to the homeostatic increase in quantal size.

Neuronal, not glial, Rab3A is required for homeostatic regulation of quantal amplitude

Astrocytic release of TNF α was shown to mediate the increase in mEPSC amplitudes in prolonged TTX-treated cultures of dissociated hippocampal neurons and cultures of hippocampal slices (Stellwagen and Malenka, 2006 [\[1\]](#); Heir et al., 2024 [\[2\]](#)), but we found that loss of Rab3A in glia did not disrupt the increase in mEPSC amplitude after activity blockade. Further, we previously showed that the homeostatic increase in NMJ mEPSC amplitude was completely normal in the absence of TNF α (Wang et al., 2011 [\[3\]](#)). This leaves neuronal Rab3A as the potential controller of homeostatic regulation of quantal amplitude.

Is Rab3A acting in the presynaptic cell to cause homeostatic increase in mEPSC amplitude?

Our current results support the surprising possibility that the presynaptic vesicle protein Rab3A either regulates postsynaptic AMPARs or regulates a presynaptic contributor to homeostatic synaptic plasticity. To our knowledge, there is no evidence localizing Rab3A to postsynaptic dendritic sites. Other presynaptic molecules, such as SNARE proteins, synaptotagmins, and NSF, have been identified in the postsynaptic compartment (Lledo et al., 1998 [\[4\]](#); Nishimune et al., 1998 [\[5\]](#); Osten et al., 1998 [\[6\]](#); Song et al., 1998 [\[7\]](#); Araki et al., 2010 [\[8\]](#); Kennedy et al., 2010 [\[9\]](#); Suh et al., 2010 [\[10\]](#); Jurado et al., 2013 [\[11\]](#); Hussain and Davanger, 2015 [\[12\]](#); Gu et al., 2016 [\[13\]](#); Wu et al., 2017 [\[14\]](#)). A recent report implicates Rab3A in the localization of plasma membrane proteins, including the EGF receptor, in rafts in HEK cells and Jurkat-T cells, which indicates Rab3A can be found in other specialized locations besides the presynaptic nerve terminal (Diaz-Rohrer et al., 2023 [\[15\]](#)). Without additional experiments targeting Rab3A deletion to the pre- or post-synaptic compartment, we cannot rule out that Rab3A is regulating homeostatic plasticity in mouse cortical cultures by acting postsynaptically on receptor trafficking. However, given our previous finding of a lack of any increase in AChR after TTX treatment the NMJ (Wang et al., 2011 [\[3\]](#)), the simplest interpretation is that in these two situations, Rab3A is required for an increase in the presynaptic quantum rather than an increase in postsynaptic receptors.

The presynaptic quantum, or the amount of transmitter released during a single fusion event, is an important contributor to quantal size. The amount of transmitter released during vesicle fusion can be affected by the kinetics of the fusion pore opening (Chang et al., 2017 [\[16\]](#)). We previously used amperometric measurements in mouse adrenal chromaffin cells to show loss of Rab3A increased the occurrence of very small amplitude fusion pore feet (Wang et al., 2008 [\[17\]](#)). Paired with our finding that there is an increase in the occurrence of very slow-rising, abnormally shaped mEPSCs at the neuromuscular junction of Rab3A^{-/-} mice, our data suggest that small synaptic vesicles may have a fusion pore step under certain circumstances (see also (Chiang et al., 2018 [\[18\]](#))). If activity blockade causes an increase in mEPSC amplitude due to a more rapid opening of a fusion pore, or a larger fusion pore conductance, it is possible that Rab3A is required for this modulation.

Another way to increase the presynaptic quantum is to increase levels of the transmitter transporter (Daniels et al., 2004 [\[19\]](#); De Gois et al., 2005 [\[20\]](#); Wilson et al., 2005 [\[21\]](#); Hartman et al., 2006 [\[22\]](#)). However, we found that immunofluorescence for the glutamate transporter, VGLUT1, was not increased after activity blockade under our experimental conditions. A similar lack of

increase in VGLUT1 levels, relative to synapsin, was previously observed in hippocampal cultures treated with NBQX, an AMPA receptor blocker (see supplemental data, (Wilson et al., 2005)). Taken together with our findings, it appears that there is not strong evidence that the amount of VGLUT1 at synapses is a contributing factor to the increase in mEPSC amplitude in neuronal cultures after activity blockade with TTX.

Finally, the amount of transmitter released by a vesicle could be increased due to larger vesicle size. Larger vesicle diameter has been observed following activity disruption in multiple paradigms, including ocular deprivation, cortical ablation, and immobilization (Kawana et al., 1971; Lloret and Saavedra, 1975; Cheresnarov et al., 1978). Loss of Rab3 family members is associated with increased vesicle size (Riedel et al., 2002; Schonn et al., 2010). Finally, an increase in volume has been shown to increase miniature endplate junctional current amplitude at the *Drosophila* NMJ (Karunanithi et al., 2002). Rab3A could be required for an activity-dependent increase in vesicle size, but the increase in diameter needed for a 25% increase in transmitter, and therefore mEPSC amplitude, would be difficult to detect.

The number of molecules shown to be required for homeostatic synaptic plasticity of quantal size in neuronal cultures may have surpassed ~20 (see (Koesters et al., 2024) for a recent summary), but fall into two main categories: molecules involved in glutamate receptor expression or trafficking, and signaling molecules. To our knowledge, this is the first evidence that a presynaptic protein is participating in this process. Homeostatic synaptic plasticity is becoming increasingly implicated in essential normal functions such as sleep (Tononi and Cirelli, 2014; Diering et al., 2017; Torrado Pacheco et al., 2021), and pathological conditions such as epilepsy, neuropsychiatric disorders, Huntington's, and alcohol use disorder (Trasande and Ramirez, 2007; Fernandes and Carvalho, 2016; Wang et al., 2017; Lovinger and Abrahao, 2018; Smith-Dijk et al., 2019; Lignani et al., 2020; Suzuki et al., 2021; Kavalali and Monteggia, 2023). Therefore, our identification of a novel Rab3A-dependent regulatory pathway in homeostatic synaptic plasticity may have important therapeutic implications. Our findings also further cement the importance of Rab3A in activity-dependent modulation of synaptic strength.

Materials and methods

Animals

To determine the role of Rab3A in homeostatic plasticity in mouse cortical cultures, we employed two distinct genetic mouse strains with altered Rab3A function. Rab3A deletion mice were generated as follows: Rab3A^{+/-} heterozygous mice were bred and genotyped as previously described (Kapfhamer et al., 2002; Wang et al., 2008), and maintained as a colony using multiple heterozygous breeding pairs. Homozygous Rab3A^{+/-} or Rab3A^{-/-} mouse pups were obtained by breeding Rab3A^{+/-} pairs and Rab3A^{-/-} pairs, respectively, so that the pups were the first-generation progeny of a homozygous mating. This protocol minimizes the tendency of multiple generations of Rab3A^{+/-} and Rab3A^{-/-} homozygous breedings to produce progeny that are more and more genetically distinct. Rab3A^{Ebd/Ebd} mice were identified in an EU-mutagenesis screen of C57BL/6J mice, and after a cross to C3H/HeJ, were backcrossed for 3 generations to C57BL/6J (Kapfhamer et al., 2002). Rab3A^{+Ebd} heterozygous mice were bred at Wright State University and genotyped in a two-step procedure: 1. a PCR reaction (forward primer: TGA CTC CTT CAC TCC AGC CT; reverse primer: TGC ACT GCA TTA AAT GAC TCC T) followed by 2. a restriction digest with enzyme Bsp1286I (New England Biolabs) that distinguishes the Earlybird mutant by its different base-pair products. Rab3A^{+/-} mice were backcrossed with Rab3A^{+/-} mice from the Earlybird heterozygous colony for 11 generations in an attempt to establish a single wild type strain, but differences in mEPSC amplitude and adrenal chromaffin cell calcium currents persisted, likely due to genes that are close to the Rab3A site, resulting in two wild type strains: 1. Rab3A^{+/-} from the Rab3A^{+/-} colony, and 2. Rab3A^{+/-} from the Rab3A^{+Ebd} colony.

Primary Culture of Mouse Cortical Neurons

Primary dissociated cultures of mixed neuronal and glia populations were prepared as previously described (Hanes et al., 2020 [DOI](#)). Briefly, postnatal day 0-2 (P0-P2) Rab3A^{+/+}, Rab3A^{-/-} or Rab3A^{Ebd/Ebd} neonates were euthanized by rapid decapitation, as approved by the Wright State University Institutional Animal Care and Use Committee, and brains were quickly removed. Each culture was prepared from the cortices harvested from two animals; neonates were not sexed. Cortices were collected in chilled Neurobasal-A media (Gibco) with osmolarity adjusted to 270 mOsm and supplemented with 40 U/ml DNase I (ThermoFisher Scientific). The tissues were digested with papain (Worthington Biochemical) at 20 U/ml at 37°C for 20 minutes followed by trituration with a sterile, fire-polished Pasteur pipette, then filtered through a 100 µm cell strainer, and centrifuged at 1100 rpm for 2 minutes. After discarding the supernatant, the pellet was resuspended in room temperature Neurobasal-A media (270 mOsm), supplemented with 5% fetal bovine serum for glia growth, and 2% B-27 supplement to promote neuronal growth (Gibco), L-glutamine, and gentamicin (ThermoFisher Scientific). Neurons were counted and plated at 0.15×10^6 cells/cover slip onto 12 mm coverslips pre-coated with poly-L-lysine (BioCoat, Corning). This plating density produces a “high density” culture characterized by a complex mesh of neuronal processes criss-crossing the field of view, completely filling the space between cell bodies (see **Figure 4** [DOI](#)). We found in preliminary experiments that an increase in mEPSC amplitude in mouse cortical cultures was inconsistent when cell density was low, likely due to lower levels of baseline activity, although it is a limitation of this study that we did not directly measure activity levels in cultures prepared from Rab3A^{+/+}, Rab3A^{-/-} or Rab3A^{Ebd/Ebd} mice, and therefore do not know if differences in results are due to differences in activity levels. While unlikely for Rab3A^{+/+} and Rab3A^{-/-} studies, given the mEPSC amplitude in untreated cultures was relatively small, it remains possible that in Rab3A^{Ebd/Ebd} studies, the larger mEPSC amplitude in untreated cultures was due to a loss of activity in these cultures. The culture media for the first day (0 DIV) was the same as the above Neurobasal-A media supplemented with FBS, B-27, L-glutamine, and gentamicin, and was switched after 24 hours (1 DIV) to media consisting of Neurobasal-A (270 mOsm), 2% B-27, and L-glutamine without FBS to avoid its toxic effects on neuronal viability and health (Stellwagen and Malenka, 2006 [DOI](#)). Half of the media was changed twice weekly and experiments were performed at 13-14 DIV. Two days prior to experiments, tetrodotoxin (TTX) (500 nM; Tocris), a potent Na⁺ channel blocker, was added to some cultures to chronically silence all network activity and induce homeostatic synaptic plasticity mechanisms, while untreated sister cultures served as controls. Cultures prepared from mutant mice were compared with cultures from wild-type mice from their respective colonies. Note that the cultures comprising the Rab3A^{+/+} data here are a subset of the data previously published in Hanes et al., 2020 [DOI](#). This smaller data set was restricted to the time period over which cultures were also prepared from Rab3A^{-/-} mice.

Preparation of Glial Feeder Layers

Glial feeder layers were prepared from the cortices of P0-P2 Rab3A^{+/+} or Rab3A^{-/-} mouse pups as described previously (Stellwagen and Malenka, 2006 [DOI](#)). Briefly, cortices were dissected and cells were dissociated as described above. Cell suspensions of mixed neuronal and glial populations were plated onto glass coverslips pre-coated with poly-L-lysine in Dulbecco's Modified Eagle Media (ThermoFisher Scientific) supplemented with 5% FBS (to promote glial proliferation and to kill neurons), L-glutamine, and gentamicin, and maintained in an incubator at 37°C, 5% CO₂; cultures were maintained in this manner for up to 1 month to generate purely glial cultures (all neurons typically died off by 7 DIV). Culture media was replaced after 24 hours, and subsequent media changes were made twice weekly, replacing half of the culture media with fresh media. Feeder layers were not used for neuronal seeding until all native neurons were gone and glia approached 100% confluency (visually inspected).

Plating of Neurons on Glial Feeder Layers

Cortical neurons were obtained as described above. The cell pellet obtained was resuspended in Neurobasal-A (osmolarity adjusted to 270 mOsm) containing B27 (2%, to promote neuronal growth), L-glutamine, and 5-fluorodeoxyuridine (FdU, a mitotic inhibitor; Sigma). Addition of FdU was used to prevent glial proliferation and contamination of the feeder layer with new glia, promoting only neuronal growth on the feeder layers (FdU-containing media was used for the maintenance of these cultures and all subsequent media changes). Glial culture media was removed from the feeder layer cultures, and the neuronal cell suspension was plated onto the glial feeder cultures. The culture strategy used to distinguish the relative roles of neuronal and glial Rab3A is outlined in Figure 8, Left. At 1 DIV, all of the culture media was removed and replaced with fresh Neurobasal-A media containing FdU described above, and half of the media was replaced twice per week for all subsequent media changes. Cultures were maintained in a 37°C, 5% CO₂ incubator for 13-14 DIV.

Whole-Cell Voltage Clamp to Record mEPSCs

At 13-14 DIV, mEPSCs from TTX-treated and untreated sister cultures of Rab3A^{+/+} or Rab3A^{-/-} neurons from the Rab3A^{+/+} colony, or Rab3A^{+/+} or Rab3A^{Ebd/Ebd} neurons from the Rab3A^{+/Ebd} colony, were recorded via whole-cell voltage clamp to assess the role of Rab3A in homeostatic synaptic plasticity. Recordings were taken from pyramidal neurons, which were identified visually by a prominent apical dendrite; images were taken of all cells recorded from. Cells were continuously perfused with a solution consisting of (in mM): NaCl (115), KCl (5), CaCl₂ (2.5), MgCl₂ (1.3), dextrose (23), sucrose (26), HEPES (4.2), pH = 7.2 (Stellwagen and Malenka, 2006). On the day of recording, the osmolarity of the media from the cultures was measured (normally 285 – 295 mOsm) and the perfusate osmolarity was adjusted to match the culture osmolarity to protect against osmotic shock to the neurons. To isolate glutamatergic mEPSCs, TTX (500 nM) and picrotoxin (50 μM), were included in the perfusion solution to block action potentials and GABAergic currents, respectively. The NMDA receptor antagonist, APV, was not included in the perfusion solution because in pilot studies, all mEPSCs were blocked by 10 μM CNQX and 50 μM picrotoxin, demonstrating no APV-sensitive mEPSCs were present (data not shown). Previous studies have described NMDA mEPSCs in neuronal cultures, but recordings were performed in 0 Mg²⁺ (Watt et al., 2000; Sutton et al., 2006). In our recordings, the presence of extracellular Mg²⁺ (1.3 mM) and TTX would mean that the majority of NMDA receptors are blocked by extracellular Mg²⁺ at resting V_m. Patch electrodes (3.5 – 5 MΩ) were filled with an internal solution containing (in mM): K-gluconate (130), NaCl (10), EGTA (1), CaCl₂ (0.132), MgCl₂ (2), HEPES (10), pH = 7.2. Osmolarity was adjusted to 10 mOsm less than the perfusion solution osmolarity, which we have found improves success rate of achieving low access resistance recordings. Neurons were clamped at a voltage of -60 mV using an Axopatch 200B patch-clamp (Axon Instruments), recorded from for 2 – 5 minutes, and data were collected with Clampex 10.0/10.2 (Axon Instruments).

NASPM application

The antagonist of Ca²⁺-permeable AMPA receptors, N-naphthyl acetylspermine (NASPM, 20 μM; Tocris), was applied during recordings in a subset of experiments. NASPM is a synthetic analog of Joro Spider Toxin (JSTX) (Koike et al., 1997), and chemicals in this family block Ca²⁺ permeable glutamate receptors (Blaschke et al., 1993; Herlitze et al., 1993; Iino et al., 1996). The presence of the edited GluA2 subunit disrupts both the Ca²⁺ permeability and the sensitivity to JSTX and related substances (Blaschke et al., 1993; Bochet et al., 1994; Jonas and Burnashev, 1995). Therefore, 20 μM NASPM is expected to completely block AMPA receptors containing GluA1, GluA3 or GluA4 subunits (Hollmann et al., 1991; Burnashev et al., 1992), or the Kainate receptors containing GluA5 or GluA6 (Koike et al., 1997; Sun et al., 2009), but be ineffective against any heteromer or homomer containing GluA2 subunits. Because the effect of the spider toxins and their analogues are use-dependent (Herlitze et al., 1993; Iino et al., 1996; Koike et

al., 1997 [↗](#)), NASPM was applied with a depolarizing high K^+ solution (25 mM KCl, 95 mM NaCl) which we expected to trigger release of glutamate and opening of glutamate-activated receptors, allowing entry of NASPM into the pore. Baseline recordings were performed for 2 minutes in our standard perfusate, followed by perfusion of NASPM + High K^+ solution for 45 seconds, and then perfusion of a NASPM only solution for 5 minutes, after which recording was recommenced for 5 minutes (because we found in pilot experiments that frequency was reduced following NASPM application).

Analysis of mEPSCs

Miniature excitatory postsynaptic currents (mEPSCs) were manually selected using Mini Analysis software (Synaptosoft), a standard program used for mEPSC event detection and analysis. Records were filtered at 2 kHz using a low-pass Butterworth filter prior to selection. The program detection threshold was set at 3 pA but the smallest mEPSC selected was 4.01 pA. A fully manual detection process means that the same criterion (“does this *look* like an mEPSC?”) is applied to all events, not just the false positives or the false negatives, which prevents the bias from being primarily at one end or the other of the range of mEPSC amplitudes. It is important to note that when performing the MiniAnalysis process, the researcher did not know whether a record was from an untreated cell or a TTX-treated cell.

Immunofluorescence, microscopy, and data analysis

Primary cultures of mouse cortical neurons were grown for 13-14 DIV. Antibodies to GluA2 (mouse ab against N-terminal, EMD Millipore) were added directly to live cultures at 1:40 dilution, and incubated at 37 °C in a CO₂ incubator for 45 minutes. Cultures were rinsed 3 times with PBS/5% donkey serum before being fixed with 4% paraformaldehyde. After 3 rinses in PBS/5% donkey serum, cultures were incubated in CY3-labeled donkey-anti-mouse secondary antibodies for 1 hour at room temperature, rinsed in PBS/5% donkey serum, permeabilized with 0.2% saponin, and incubated in chick anti-MAP2 (1:2500, AbCAM) and rabbit anti-VGLUT1 (1:4000, Synaptic Systems) for 1 hour at room temperature in PBS/5% donkey serum. After rinsing with PBS/5% donkey serum, coverslips were incubated with 488-anti chick and CY5-anti rabbit secondary antibodies for 1 hour at room temperature, rinsed, blotted to remove excess liquid, and inverted on a drop of Vectashield (Vector Labs). Coverslips were sealed with nail polish and stored at 4 °C for < 1 week before imaging. All secondary antibodies were from Jackson Immunoresearch and were used at 1:225 dilution.

Coverslips were viewed on a Fluoview FV1000 laser scanning confocal microscope with a 60x oil immersion, 1.35 NA objective. Once a pyramidal neuron was identified and a single confocal section of the cell acquired (see Top, **Figure 4** [↗](#)), Fluoview 2.1 software was used to zoom in on the primary dendrite (5X) and a series of confocal sections were taken every 0.5 μm. Images were analyzed offline with ImagePro 6 (Cybernetics). The composite image was used to locate synaptic sites containing both VGLUT1 and GluA2 immunoreactivity in close apposition to each other and to the primary dendrite or a secondary branch identified with MAP2 immunoreactivity. An area of interest (AOI) was manually drawn around the GluA2 cluster in the confocal section in which it was the brightest. The AOIs for a dendrite were saved in a single file; the AOI number and the confocal section it was associated with were noted for later retrieval. For quantification, AOIs were loaded, an individual AOI was called up on the appropriate section, and the count/measurement tool used to apply a threshold. Multiple thresholds were used, 400-550, but we found that within a given experiment, the magnitude of the TTX effect was not altered by different thresholds, therefore, all data presented here use a threshold of 450. Pixels within the cluster that were above the threshold were automatically outlined, and size, average intensity, and integral of the outlined region saved to Origin2020 (OriginLab) file for calculating mean values. For analyzing VGLUT1 sites, previous AOIs were examined, and if not directly over the VGLUT1 site, moved to encompass it (this happened when GluA2 and VGLUT1 were side by side rather than overlapping). The threshold was 200 for all VGLUT1 data except Rab3A^{+/+} Culture #1, where threshold was 400.

There was no threshold that worked for both that culture and the other cultures, with too much of the background regions being included for Culture #1 with a threshold of 200, and too little of the synaptic site area being included for the other Cultures with a threshold of 400.

Statistics

In our previous publication (Hanes et al., 2020 [DOI](#)), we described the characteristics of homeostatic plasticity of mEPSC amplitudes in mouse cortical cultures using multiple mEPSC amplitude quantiles from each cell to create data sets with thousands of samples. We compared the cumulative distribution functions for mEPSC amplitudes in untreated vs. TTX-treated cultures, as well as sorting the values from smallest to largest, and plotted TTX values vs. control values in the rank order plot first described in (Turrigiano et al., 1998 [DOI](#)) to demonstrate synaptic scaling. Finally, we plotted the ratio of TTX values/control values vs. control values to show that the ratio, which represents the effect of TTX, increased with increasing control values, which we termed “divergent scaling.” Here, we wanted to avoid the inflation of sample size caused by pooling multiple mEPSCs per cell in order to statistically compare the effects of TTX in cultures of wild type mice, Rab3A^{-/-} mice and Rab3A^{Ebd/Ebd} mutant mice. Therefore, we computed the mean mEPSC amplitude for each cell and compared means from untreated and TTX-treated cultures with the non-parametric Kruskal-Wallis test, with N = the number of cells. The overall means are presented ± SEM in the text, and p values are displayed above each plot. The same process was carried out for the characteristics of synaptic GluA2 receptor clusters and VGLUT1 sites in imaging experiments, with N = the number of dendrites (one dendrite was sampled for each cell). Although we quantified three characteristics of the GluA2 clusters and VGLUT1 sites, size, average intensity, and integral, we have not corrected p values for multiple comparisons. Means are presented as box plots + data, with the box at 25 and 75 percentiles, whiskers at 10 and 90 percentiles, a dot indicating the mean, and a line indicating the median. In **Figure 3** [DOI](#), mEPSC amplitudes were measured within the same cell before and after NASPM application, so a paired t test was used to compare the pre- and post-NASPM mEPSC amplitudes. In **Figure 5A-C** [DOI](#) the mean mEPSC amplitude for untreated and TTX-treated coverslips in the same culture are compared with a paired t test. Statistics were computed and plots were created using Origin2020 (OriginLab).

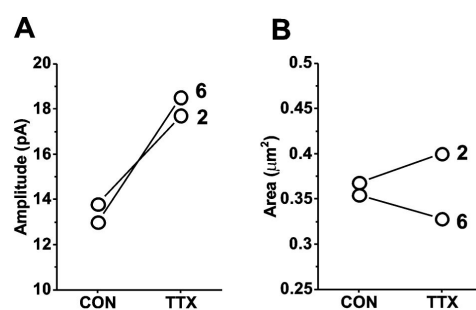
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Additional information

Author contributions

A.G.K., K.L.E. and M.M.R. designed research; A.G.K. performed research; A.G.K. and K.L.E. analyzed data; A.G.K., K.L.E., and M.M.R. wrote the paper.



Supplemental Figure 1.

Comparison of mEPSC amplitudes and GluA1 receptor cluster areas in matched mouse cortical cultures prepared from Rab3A^{+/+} mice and treated with TTX for 48 hr.

(A) Culture averages of mEPSC amplitudes for untreated (CON) and TTX-treated coverslips (TTX) in each of 2 Rab3A^{+/+} mouse cortical co-cultures. Culture #2, CON, N = 7, 13.8 ± 2.4 pA; TTX, N = 8, 17.7 ± 1.8 pA (+28.3%); Culture #6, CON, N = 6, 13.0 ± 1.5 pA; TTX, N = 6, 18.5 ± 2.0 pA (+42.3%). (B) Culture averages of GluA1 receptor cluster size in the same 2 cultures as shown in (A). Culture #2, CON, N = 10, 0.37 ± 0.04 μm²; TTX, N = 10, 0.40 ± 0.06 μm² (+8.1%) Culture #6, CON, N = 10, 0.35 ± 0.05 μm²; TTX, N = 10, 0.33 ± 0.04 μm² (-5.7%) "Culture #2" is the same Culture #2 depicted in [Figure 5](#), different coverslips were processed for GluA1 immunofluorescence labeling. GluA1 receptor cluster intensity did not increase in Culture #2 (CON, 364 vs. TTX, 357) or Culture #6 (CON, 357 vs. TTX, 363). Live cultures were exposed to GluA1 antibody against the extracellular domain (ABN241, purchased from EMD Millipore, now available from Millipore Sigma) before being fixed and processed with secondary antibodies.

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Reviewer #1 (Public review):

Koesters and colleagues investigated the role of the small GTPase Rab3A in homeostatic scaling of miniature synaptic transmission in primary mouse cortical cultures using electrophysiology and immunohistochemistry. The major finding is that TTX incubation for 48 hours does not induce an increase in the amplitude of excitatory synaptic miniature events in neuronal cortical cultures derived from Rab3A KO and Rab3A Earlybird mutant mice. NASPM application had comparable effects on mEPSC amplitude in control and after TTX, implying that Ca²⁺-permeable glutamate receptors are unlikely modulated during synaptic scaling. Immunohistochemical analysis revealed no significant changes in GluA2 puncta size, intensity, and integral after TTX treatment in control and Rab3A KO cultures. Finally, they provide evidence that loss of Rab3A in neurons, but not astrocytes, blocks homeostatic scaling. Based on these data, the authors propose a model in which neuronal Rab3A is required for homeostatic scaling of synaptic transmission, potentially through GluA2-independent mechanisms.

The major finding - impaired homeostatic up-scaling after TTX treatment in Rab3A KO and Rab3 earlybird mutant neurons - is supported by data of high quality. However, the paper falls short of providing any evidence or direction regarding potential mechanisms. The data on GluA2 modulation after TTX incubation are likely statistically underpowered, and do not allow drawing solid conclusions, such as GluA2-independent mechanisms of up-scaling.

The study should be of interest to the field because it implicates a presynaptic molecule in homeostatic scaling, which is generally thought to involve postsynaptic neurotransmitter receptor modulation. However, it remains unclear how Rab3A participates in homeostatic plasticity.

Major (remaining) point:

(1) Direct quantitative comparison between electrophysiology and GluA2 imaging data is complicated by many factors, such as different signal-to-noise ratios. Hence, comparing the variability of the increase in mini amplitude vs. GluA2 fluorescence area is not valid. Thus, I recommend removing the sentence "We found that the increase in postsynaptic AMPAR levels was more variable than that of mEPSC amplitudes, suggesting other factors may contribute to the homeostatic increase in synaptic strength." from the abstract.

Similarly, the data do not directly support the conclusion of GluA2-independent mechanisms of homeostatic scaling. Statements like "We conclude that these data support the idea that there is another contributor to the TTX- induced increase in quantal size." should be thus revised or removed.

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Reviewer #2 (Public review):

I thank the authors for their efforts in the revision. In general, I believe the main conclusion that Rab3A is required for TTX-induced homeostatic synaptic plasticity is well-supported by the data presented, and this is an important addition to the repertoire of molecular players involved in homeostatic compensations. I also acknowledge that the authors are more cautious in making conclusions based on the current evidence, and the structure and logic have been much improved.

The only major concern I have still falls on the interpretation of the mismatch between GluA2 cluster size and mEPSC amplitude. The authors argue that they are only trying to say that changes in the cluster size are more variable than those in the mEPSC amplitude, and they provide multiple explanations for this mismatch. It seems incongruous to state that the simplest explanation is a presynaptic factor when you have all these alternative factors that very likely have contributed to the results. Further, the authors speculate in the discussion that Rab3A does not regulate postsynaptic GluA2 but instead regulates a presynaptic contributor. Do the authors mean that, in their model, the mEPSC amplitude increases can be attributed to two factors- postsynaptic GluA2 regulation and a presynaptic contribution (which is regulated by Rab3A)? If so, and Rab3A does not affect GluA2 whatsoever, shouldn't we see GluA2 increase even in the absence of Rab3A? The data in Table 1 seems to indicate otherwise.

I also question the way the data are presented in Figure 5. The authors first compare 3 cultures and then 5 cultures altogether, if these experiments are all aimed to answer the same research question, then they should be pooled together. Interestingly, the additional two cultures both show increases in GluA2 clusters, which makes the decrease in culture #3 even more perplexing, for which the authors comment in line 261 that this is due to other factors. Shouldn't this be an indicator that something unusual has happened in this culture? Data in this figure is sufficient to support that GluA2 increases are variable across cultures, which hardly adds anything new to the paper or to the field. The authors further cite a study with comparable sample sizes, which shows a similar mismatch based on p values (Xu and Pozzo-Miller 2007), yet the effect sizes in this study actually match quite well (both ~160%). P values cannot be used to show whether two effects match, but effect sizes can. Therefore, the statement in lines 411-413 "... consistently leads to an increase in mEPSC amplitudes, and sometimes leads to an increase in synaptic GluA2 receptor cluster size" is not very convincing, and can hardly be used to support "the idea that there are additional sources contributing to the homeostatic increase in quantal size".

I would suggest simply showing mEPSC and immunostaining data from all cultures in this experiment as additional evidence for homeostatic synaptic plasticity in WT cultures, and leave out the argument for "mismatch". The presynaptic location of Rab3A is sufficient to speculate a presynaptic regulation of this form of homeostatic compensation.

Minor concerns:

(1) Line 214, I see the authors cite literature to argue that GluA2 can form homomers and can conduct currents. While GluA2 subunits edited at the Q/R site (they are in nature) can form homomers with very low efficiency in exogenous systems such as HEK293 cells (as done in the cited studies), it's unlikely for this to happen in neurons (they can hardly traffick to synapses if possible at all).

(2) Lines 221-222, the authors may have misinterpreted the results in Turrigiano 1998. This study does not show that the increase in receptors is most dramatic in the apical dendrite, in fact, this is the only region they have tested. The results in Figures 3b-c show that the effect size is independent of the distance from soma.

(3) Lines 309-310 (and other places mentioning TNFa), the addition of TNFa to this experiment seems out of place. The authors have not performed any experiment to validate the presence/absence of TNFa in their system (citing only 1 study from another lab is insufficient). Although it's convincing that glia Rab3A is not required for homeostatic plasticity here, the data does not suggest Rab3A's role (or the lack of) for TNFa in this process.

<https://doi.org/10.7554/eLife.90261.3.sa2>

Reviewer #3 (Public review):

This manuscript presents a number of interesting findings that have the potential to increase our understanding of the mechanism underlying homeostatic synaptic plasticity (HSP). The data broadly support that Rab3A plays a role in HSP, although the site and mechanism of action remain uncertain.

The authors clearly demonstrate that Rab3A plays a role in HSP at excitatory synapses, with substantially less plasticity occurring in the Rab3A KO neurons. There is also no apparent HSP in the Earlybird Rab3A mutation, although baseline synaptic strength is already elevated. In this context, it is unclear if the plasticity is absent, already induced by this mutation, or just occluded by a ceiling effect due to the synapses already being strengthened. Occlusion may also occur in the mixed cultures when Rab3A is missing from neurons but not astrocytes. The authors do appropriately discuss these options. The authors have solid data showing that Rab3A is unlikely to be active in astrocytes. Finally, they attempt to study the linkage between changes in synaptic strength and AMPA receptor trafficking during HSP, and conclude that trafficking may not be solely responsible for the changes in synaptic strength during HSP.

Strengths:

This work adds another player into the mechanisms underlying an important form of synaptic plasticity. The plasticity is likely only reduced, suggesting Rab3A is only partially required and perhaps multiple mechanisms contribute. The authors speculate about some possible novel mechanisms, including whether Rab3A is active pre-synaptically to regulate quantal amplitude.

As Rab3A is primarily known as a pre-synaptic molecule, this possibility is intriguing. However, it is based on the partial dissociation of AMPAR trafficking and synaptic response and lacks strong support. On average, they saw a similar magnitude of change in mEPSC amplitude and GluA2 cluster area and integral, but the GluA2 data was not significant due to higher variability. It is difficult to determine if this is due to biology or methodology - the imaging method involves assessing puncta pairs (GluA2/VGluT1) clearly associated with a MAP2 labeled dendrite. This is a small subset of synapses, with usually less than 20 synapses per neuron analyzed, which would be expected to be more variable than mEPSC recordings averaged across several hundred events. However, when they reduce the mEPSC number of events to similar numbers as the imaging, the mEPSC amplitudes are still less variable than the imaging data. The reason for this remains unclear. The pool of sampled synapses is still different between the methods and recent data has shown that synapses have variable responses during HSP. Further, there could be variability in the subunit composition of newly inserted AMPARs, and only assessing GluA2 could mask this (see below). It is intriguing that pre-synaptic changes might contribute to HSP, especially given the likely localization of Rab3A. But it remains difficult to distinguish if the apparent difference in imaging and electrophysiology is a methodological issue rather than a biological one. Stronger data, especially positive data on changes in release, will be necessary to conclude that pre-synaptic

factors are required for HSP, beyond the established changes in post-synaptic receptor trafficking.

Other questions arise from the NASPM experiments, used to justify looking at GluA2 (and not GluA1) in the immunostaining. First, there is a strong frequency effect that is unclear in origin. One would expect NASPM to merely block some fraction of the post-synaptic current, and not affect pre-synaptic release or block whole synapses. But the change in frequency seems to argue (as the authors do) that some synapses only have CP-AMPA receptors, while the rest of the synapses have few or none. Another possibility is that there are pre-synaptic NASPM-sensitive receptors that influence release probability. Further, the amplitude data show a strong trend towards smaller amplitude following NASPM treatment (Fig 3B). The p value for both control and TTX neurons was 0.08 - it is very difficult to argue that there is no effect. The decrease on average is larger in the TTX neurons, and some cells show a strong effect. It is possible there is some heterogeneity between neurons on whether GluA1/A2 heteromers or GluA1 homomers are added during HSP. This would impact the conclusions about the GluA2 imaging as compared to the mEPSC amplitude data.

To understand the role of Rab3A in HSP will require addressing two main issues:

(1) Is Rab3A acting pre-synaptically, post-synaptically or both? The authors provide good evidence that Rab3A is acting within neurons and not astrocytes. But where it is acting (pre or post) would aid substantially in understanding its role. The general view in the field has been that HSP is regulated post-synaptically via regulation of AMPAR trafficking, and considerable evidence supports this view. More concrete support for the authors' suggestion of a pre-synaptic site of control would be helpful.

(2) Rab3A is also found at inhibitory synapses. It would be very informative to know if HSP at inhibitory synapses is similarly affected. This is particularly relevant as at inhibitory synapses, one expects a removal of GABA receptors or a decrease in GABA release (ie the opposite of whatever is happening at excitatory synapses). If both processes are regulated by Rab3A, this might suggest a role for this protein more upstream in the signaling; an effect only at excitatory synapses would argue for a more specific role just at those synapses.

<https://doi.org/10.7554/eLife.90261.3.sa1>

Author response:

The following is the authors' response to the previous reviews.

Since multiple Reviewers requested that the results describing effects of TTX treatment on GluA2 receptor levels detected by immunofluorescence and confocal imaging be revised, we have made substantial changes, which are described below. We believe the changes have greatly improved the manuscript and thank the reviewers for their comments.

Lack of significant increase in GluA2 receptor data is due to too few cultures sampled; anything could have happened [in one] particular dissociation. A concern that the TTX effect might vary greatly from culture to culture was why we felt it was important to match the receptor measurements on the same cultures that we recorded mEPSCs. We now present the culture means in Figure 5A (mEPSCs) and 5B (GluA2 receptor cluster size). These plots make it clear that the variability in the GluA2 receptor cluster size effect is not attributable to a failure of that culture to show a homeostatic effect. That is, the variability in GluA2 receptor effect is independent of the variability in mEPSC effect. To increase sample size, we examined 2 additional cultures for synaptic GluA2 receptor levels in control vs. TTX treatment. These cultures showed very modest increases (Figure 5C). When cell means from these experiments

were pooled with those from the 3 matched cultures, the TTX effect was still not statistically significant (Figure 5G).

Lack of significant increase in GluA2 receptor data is due to the choice to restrict our analysis to the primary dendrite, close to the cell body. We restricted our analysis to the primary dendrite because Figure 3 in Turrigiano et al, 1998, shows the increased response to exogenously applied glutamate after TTX treatment is greatest close to the cell body and wanes as the glutamate is applied further away (added to Results, new lines 388-389).

Variability in GluA2 receptor data is due to the much smaller number of synapses sampled, compared to mEPSCs. We matched the sampling for mEPSC amplitude data to that of imaging data by taking only 20 samples from each electrophysiological recording. Each mEPSC represents one synapse; in a set of 20 mEPSCs some might come from the same synapse, so that we are sampling from ≤ 20 synapses. The effect of TTX on mEPSC amplitudes remained significant despite the reduced samples per cell (Figure 5A).

Why do we fail to show a significant increase in receptors when this has been shown in many studies?

We have added to our discussion the point that several studies, including Wang et al. 2019, use the number of puncta, rather than the number of cells, as the sample number. We ran an analysis of GluA2 receptor cluster size where we sampled multiple synapses per cell, and used the number of clusters as the sample n . We found that even with as few as 6 synapses randomly selected from each cell, the effect of TTX on GluA2 receptor cluster size became highly significant ($p = 0.001$ for data from 3 cultures and $p = 0.005$ for data from 5 cultures) (see new lines 400-406 in Discussion). In sum, our data are not very different from that of some previous studies. We are not arguing that receptors do not increase. Instead our point is that the increase is more variable than the increase in mEPSC amplitude and thus takes a much bigger sample size to detect. In sum, the difference between the mEPSC data and the receptor data is that the mEPSC data consistently show a ~20-25% increase, whereas the receptor data do not always show an increase and sometimes the increase is only ~10%. Finally, we added two matched culture experiments examining synaptic GluA1 receptor cluster characteristics. GluA1 receptor cluster size decreased in one culture, and increased very modestly in the other (Supplemental Figure 1B), whereas mEPSC amplitude robustly increased (Supplemental Figure 1A; Results, new lines 265-268).

We conclude that these data support the idea that there is another contributor to the TTX-induced increase in quantal size.

Other changes in presentation of GluA2 receptor results: Since the effects on intensity and integral are of lesser magnitude than that on cluster size, we have removed these results from the graphs, although they are presented in Table 1. We have removed Figure 6, the presentation of individual culture results, since these results are now conveyed in Figure 5A-C. We have removed graphs depicting GluA2 receptor cluster size in response to TTX in Rab3A^{-/-} cultures, but these data are still presented in Table 1.

We address other detailed comments below.

Public Reviews:

Reviewer #1 (Public review):

(2) The effects of Rab3A on TTX-induced mini frequency modulation remains unclear, because TTX does not induce a change in mini frequency in the Rab3A^{+/+}Ebd control (Fig. 2). The respective conclusions should be revised accordingly (l. 427).

The effects on mini frequency were added for completeness, but given the lack of consistently significant changes with TTX treatment or changes in the KO or Rab3A^{Ebd/Ebd} cultures, we have removed comment on these results from the Discussion.

(3) The model is still not supported by the data. In particular, data supporting a negative regulation of Rab3A by APs, Rab3A-dependent release of a tropic factor, or a Rab3A-dependent increase in GluA2 abundance are not presented.

We have removed the model from the manuscript.

(4) Data points are not overlapping and appear "quantal" in most box plots. How were the data rounded?

The appearance of quantal variation in cell amplitude means is due to the binning that is part of the creation of the box plot. We have not remade the figures without binning, because the binning provides a visual depiction of the distribution of the data points. We have added the bin sizes to the appropriate figure legends.

Reviewer #2 (Public review):

However, the authors still have not provided further investigation of the mechanisms behind the role of Rab3A in this form of plasticity, and the revision therefore has added little to the significance of the study. Moreover, the experimental design for the investigation of the mismatch between mEPSC amplitude and GluA2 cluster fluorescence remains questionable, making it difficult to draw any credible conclusions from groups of data that not only look similar to the eye but also show no significance statistically.

To our knowledge, no other study has matched measurements of mEPSC amplitude in the same cultures where synaptic receptor levels were assessed. As stated above, we have revised the presentation of GluA2 receptor results, concluding from the lack of significant effects on receptor levels that the mEPSC amplitude increase cannot be fully explained by the receptor data (which is strengthened by addition of two more cultures analyzed for GluA2 immunofluorescence). This is an important addition to the significance of the study.

In summary, this study establishes that neuronal Rab3A plays a role in homeostatic synaptic plasticity, but so do a number of other molecules that have been implicated in homeostatic synaptic plasticity in the past two decades (only will grow with the new techniques such as RNAseq). Without going beyond this finding and demonstrating how exactly Rab3A participates in the induction and/or expression of this form of plasticity, or maybe the potential Rab3A-mediated functional and behavioral defects in vivo, the contribution of the current study to the field is limited. However, given the presynaptic location of Rab3A, this finding could serve as a starting point for researchers interested in pre-postsynaptic cross-talk during homeostatic plasticity in general.

We previously published a review in which we list 19 molecules known at that time to be important for homeostatic synaptic plasticity (see Table 2, Koesters et al., 2024), and they fall into two categories: molecules involved in glutamate receptor expression or trafficking, and signaling molecules. Rab3A is the first synaptic vesicle protein to be implicated in homeostatic plasticity of quantal size. We have added this point to the Discussion, new lines 473-476. By demonstrating that Rab3A is not acting in glia (which release TNF α , which regulates receptor expression), and that GluA2 receptor levels do not explain the homeostatic mEPSC increase in our experimental conditions, we have ruled out two major mechanisms.

Reviewer #3 (Public review):

Other questions arise from the NASPM experiments, used to justify looking at GluA2 (and not GluA1) in the immunostaining. First, there is a frequency effect that is unclear in origin. One would expect NASPM to merely block some fraction of the post-synaptic current, and not affect pre-synaptic release or block whole synapses. However the change in frequency seems to argue (as the authors do) that some synapses only have CP-AMPA, while the rest of the synapses have few or none. Another possibility is that there are pre-synaptic NASPM-sensitive receptors that influence release probability. Further, the amplitude data show a strong trend towards smaller amplitude following NASPM treatment (Fig 3B). The p value for both control and TTX neurons was 0.08 - it is very difficult to argue that there is no effect. The decrease on average is larger in the TTX neurons, and some cells show a strong effect. It is possible there is some heterogeneity between neurons on whether GluA1/A2 heteromers or GluA1 homomers are added during HSP. This would impact the weakly supported conclusions about the GluA2 imaging vs mEPSC amplitude data.

We cannot rule out that the NASPM-induced decrease in mEPSC frequency is due to a loss of presynaptic glutamate receptor enhancement of release probability, and have added this statement to the Results, new lines 202-204. Regarding the p value of 0.08—we are not arguing that NASPM has no effect on mEPSC amplitude, only that it has no effect on the homeostatic increase in amplitude after TTX treatment. An increase in GluA1/A2 heteromers should have been detected in our imaging studies.

Unaddressed issues that would greatly increase the impact of the paper:

(1) Is Rab3A acting pre-synaptically, post-synaptically or both? The authors provide good evidence that Rab3A is acting within neurons and not astrocytes. But where it is acting (pre or post) would aid substantially in understanding its role. They could use sparse knockdown of Rab3A, or simply mix cultures from KO and WT mice (with appropriate tags/labels). The general view in the field has been that HSP is regulated post-synaptically via regulation of AMPAR trafficking, and considerable evidence supports this view. The more support for their suggestion of a pre-synaptic site of control, the better.

We agree that doing co-cultures of Rab3A^{-/-} and Rab3A^{+/+} neurons is the definitive experiment to determine the locus of action of Rab3A in homeostatic synaptic plasticity. We hope to examine this question in a future manuscript.

(2) Rab3A is also found at inhibitory synapses. It would be very informative to know if HSP at inhibitory synapses is similarly affected. This is particularly relevant as at inhibitory synapses, one expects a removal of GABARs (ie the opposite of whatever is happening at excitatory synapses). If both processes are regulated by Rab3A, this might suggest a role for this protein more upstream in the signaling; an effect only at excitatory synapses would argue for a more specific role just at these synapses.

We agree that it would be very interesting to determine if the homeostatic decrease in mIPSCs after activity blockade depends on Rab3A. We hope to address this question in the future.

Recommendations for the authors:

Reviewer #3 (Recommendations for the authors):

Minor points:

The abstract is a bit repetitive in places. Some editing would be advised.

We did not identify anything repetitive in the abstract except the parallel construction referring to the previous findings at the NMJ and current findings in cortical neurons. However, we have eliminated a section in the introduction which went into detail about the receptor imaging results (previous lines 103-110).

Line 77: 'shift toward early awakening' is unclear; do you mean shorter sleep/wake cycle? Other circadian issues? A more complete description is needed.

We have moved the additional detail about the Earlybird mutation's effect on circadian period from the Results to the Introduction, new lines 77 to 79.

The results section has many passages that seem more like discussion, offering various interpretation and alternatives for the data. While some commentary is appropriate, to justify the next series of experiments and maintain a logical flow, this manuscript has rather a high amount of this. Some editing and shifting material to the discussion might be warranted.

We have reduced the commentary in the Results section.

Line 245: GluA2 homomers are really unlikely, as they won't pass current (unless unedited) and don't often if ever form. But GluA2/A3 heteromers are likely (and detected by their methods).

GluA2 homomers do conduct current, albeit less than heteromers (Swanson et al., 1997; Oh and Derkach, 2005; Coombs et al., 2019). [The Oh and Derkach paper shows a GluA2 homomer current in Supplementary Figure 3]. We have modified the text to acknowledge that the GluA2 receptor imaging will detect heteromers and homomers (Results, new lines 214 to 215).

Line 258: If the number of synaptic pairs analyzed was usually <20, what was the average and range of pairs? This gets into the sampling issue.

We have added the average number of synaptic sites (20.4 ± 6.5) and range (11-38) to the text, Results, new line 229.

Are the stats of the baseline mEPSC amplitude and frequency shifts (WT vs KO on WT feeder layer) given somewhere (lines 398-402)? If not, please add them.

These stats have been added to the text, mEPSC amplitude, (CON, WT on WT, 13.3 ± 0.5 pA; CON, KO on WT, 15.2 ± 1.1 pA, $p = 0.23$, Kruskal-Wallis test), new lines 325-326 and frequency, (CON, WT on WT, 2.54 ± 0.57 sec⁻¹; CON, KO on WT, 4.46 ± 1.21 sec⁻¹, $p = 0.23$, Kruskal-Wallis test), new lines, 329-330.

25mM K⁺ is going to be much more than 'mildly' depolarizing (line 697). Should just skip that word.

'mildly' has been removed.

The section on MiniAnalysis seems overly argumentative, and there is no need to discuss flaws in the Wu paper. The important thing (a bit buried at the end of this section) is that the manual mini selection was done blind to condition, which is the normal way of

dealing with potential bias. It would be better to limit the methods to describing what was done.

The bulk of the justification of manual analysis has been removed from the text.

The discussion of potential conductance changes (lines 534-6) seems somewhat unwarranted.

Modification of GluA1 phosphorylation in the GluA1/A2 heteromer would not be detected by NASPM (and the NASPM data being a bit inconclusive anyway). Further, auxiliary subunits (like TARPs) can alter conductance of any of the AMPARs. So I don't think they have enough data to exclude such a possibility.

The discussion of contributions of conductance have been removed from the text.

Coombs ID, Soto D, McGee TP, Gold MG, Farrant M, Cull-Candy SG (2019) Homomeric GluA2(R) AMPA receptors can conduct when desensitized. *Nat Commun* 10:4312.

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