



Published in final edited form as:

Cell Rep. 2021 November 09; 37(6): 109960. doi:10.1016/j.celrep.2021.109960.

## Distinct regulation of tonic GABAergic inhibition by NMDA receptor subtypes

Kunwei Wu<sup>1</sup>, David Castellano<sup>1</sup>, Qingjun Tian<sup>1</sup>, Wei Lu<sup>1,2,\*</sup>

<sup>1</sup>Synapse and Neural Circuit Research Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892, USA

<sup>2</sup>Lead contact

### SUMMARY

Tonic inhibition mediated by extrasynaptic GABA<sub>A</sub>Rs regulates various brain functions. However, the mechanisms that regulate tonic inhibition remain largely unclear. Here, we report distinct actions of GluN2A- and GluN2B-NMDA receptors (NMDARs) on tonic inhibition in hippocampal neurons under basal and high activity conditions. Specifically, overexpression of GluN2B, but not GluN2A, reduces  $\alpha$ 5-GABA<sub>A</sub>R surface expression and tonic currents. Additionally, knockout of GluN2A and GluN2B decreases and increases tonic currents, respectively. Mechanistically, GluN2A-NMDARs inhibit and GluN2B-NMDARs promote  $\alpha$ 5-GABA<sub>A</sub>R internalization, resulting in increased and decreased surface  $\alpha$ 5-GABA<sub>A</sub>R expression, respectively. Furthermore, GluN2A-NMDARs, but not GluN2B-NMDARs, are required for homeostatic potentiation of tonic inhibition induced by prolonged increase of neuronal activity. Last, tonic inhibition decreases during acute seizures, whereas it increases 24 h later, involving GluN2-NMDAR-dependent signaling. Collectively, these data reveal an NMDAR subunit-specific regulation of tonic inhibition in physiological and pathological conditions and provide mechanistic insight into activity-dependent modulation of tonic inhibition.

### Graphical Abstract

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

\*Correspondence: luw4@mail.nih.gov.

#### AUTHOR CONTRIBUTIONS

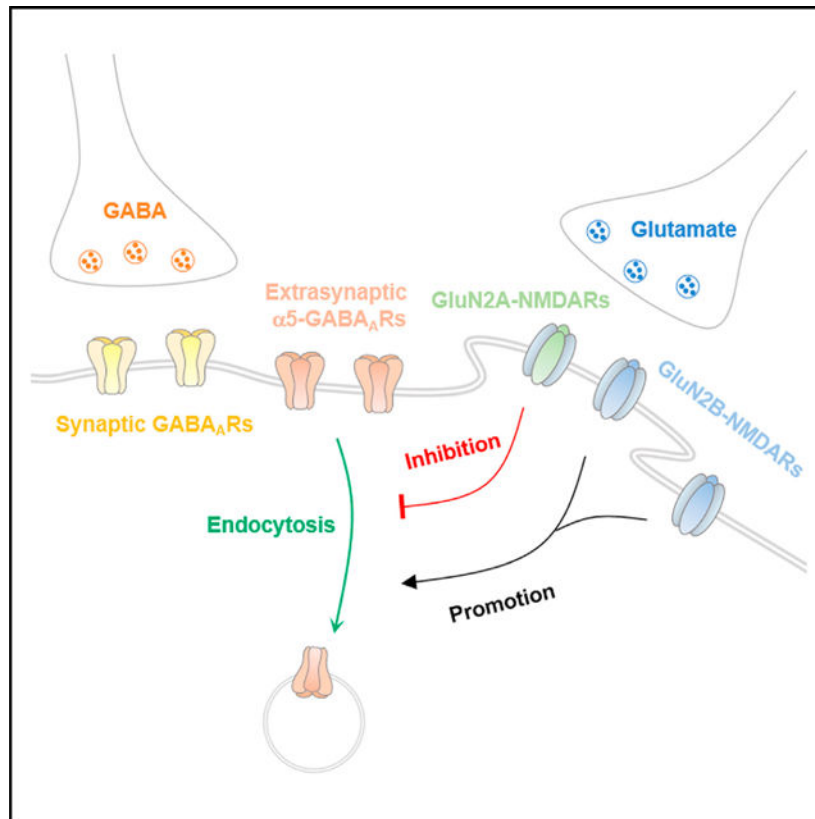
K.W. and W.L. designed the project. W.L. supervised the project. K.W. performed imaging, biochemical, behavioral, and electrophysiological experiments. K.W. and D.C. cloned sgRNA constructs. Q.T. performed neuronal cultures. W.L. and K.W. wrote the manuscript. All authors read and commented on the manuscript.

#### DECLARATION OF INTERESTS

The authors declare no competing interests.

#### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2021.109960>.



## In brief

Wu et al. find differential modulation of tonic inhibition by NMDA receptor subtypes and reveal distinct roles of GluN2A- and GluN2B-NMDA receptors in regulating α5-GABA<sub>A</sub> receptor trafficking, tonic inhibition, and its homeostatic plasticity. They also demonstrate the regulation of tonic inhibition by NMDA receptors in a kainate-induced seizure model.

## INTRODUCTION

γ-Aminobutyric acid (GABA), the major inhibitory neurotransmitter in the adult mammalian brain, exerts fast inhibitory effects on ubiquitously expressed γ-aminobutyric acid type A receptors (GABA<sub>A</sub>Rs). Generally, GABA<sub>A</sub>Rs can be classified as mediating either phasic or tonic inhibition (Belelli et al., 2009; Farrant and Nusser, 2005). Phasic inhibition is mediated by synaptically localized GABA<sub>A</sub>Rs that respond to presynaptic GABA release, whereas tonic inhibition is mediated by GABA<sub>A</sub>Rs localized either extrasynaptically or perisynaptically that respond to low ambient levels of GABA. With respect to tonic inhibition, accumulating evidence has revealed its involvement in a variety of neurophysiological processes such as regulating neuronal excitability, synaptic plasticity, and neuronal development (Belelli et al., 2009; Brickley and Mody, 2012; Farrant and Nusser, 2005; Holter et al., 2010; Lee and Maguire, 2014). Although the molecular and cellular mechanisms regulating phasic inhibition have been extensively studied (Han et al.,

2021; Jacob et al., 2008; Luscher et al., 2011; Vithlani et al., 2011), those involved in tonic inhibition remain less clear.

NMDA receptors (NMDARs) are heteromeric complexes assembled from the GluN1 and GluN2 or GluN3 subunit (Chatterton et al., 2002; McBain and Mayer, 1994). The GluN2 subunit has four isoforms (GluN2A to GluN2D), which are differently distributed across the brain (Monyer et al., 1992). Specifically, both GluN2A and GluN2B are the predominant GluN2 subunits expressed in the adult hippocampus (Monyer et al., 1994). Importantly, GluN2A- and GluN2B-NMDARs exhibit differences in kinetics, ligand sensitivity, ion permeability, and interactions with intracellular proteins (Vieira et al., 2020). They also have distinct roles in regulating neuronal development (Gambrill and Barria, 2011; Gonda et al., 2020; Sepulveda et al., 2010) and are implicated in pathological conditions such as epilepsy (Chen et al., 2007) and stroke (Chen et al., 2008).

Recent studies have documented a critical role for NMDAR-dependent regulation of GABAergic synapse development and function (Chiu et al., 2018; Gaïarsa, 2004; Gu and Lu, 2018; Gu et al., 2016; Henneberger et al., 2005; Horn and Nicoll, 2018; Marsden et al., 2007; Petrini et al., 2014; Rajgor et al., 2020). However, much less is known about how NMDARs regulate tonic inhibition. It has been shown that genetic deletion of GluN1, the obligatory subunit of the NMDAR, enhances tonic inhibition in immature hippocampal neurons (Gu et al., 2016). Tonic inhibition in hippocampal neurons could also be enhanced during NMDAR-dependent inhibitory long-term potentiation (Wyro lak et al., 2021). Additionally, pathological activation of NMDARs during stroke decreases expression of extrasynaptic  $\delta$ -GABA<sub>A</sub>Rs and reduces tonic currents in cortical neurons (Jaenisch et al., 2016). However, mechanistic understanding of the role of NMDAR-dependent regulation of tonic inhibition is lacking. Given that NMDAR subunit composition dictates its receptor properties, it is also unknown whether the subunit composition critically impacts tonic inhibition. Here, we employed genetic and pharmacological approaches to demonstrate the differential roles of GluN2A- and GluN2B-NMDARs in the regulation of extrasynaptic GABA<sub>A</sub>R trafficking and tonic inhibition in hippocampal neurons.

## RESULTS

### Overexpression of GluN2B inhibits tonic inhibition

To assess the role of GluN2 subunits in modulation of tonic inhibition, we first overexpressed GluN2A or GluN2B in cultured hippocampal neurons at days *in vitro* (DIV)11 and then measured miniature inhibitory postsynaptic currents (mIPSCs) and tonic inhibitory currents at DIV14 (Figure 1A). We observed that tonic currents were significantly reduced in neurons overexpressing GluN2B, whereas overexpression of GluN2A had no effect on tonic currents, compared to control neurons expressing GFP (Figure 1B). However, neither GluN2A nor GluN2B overexpression had a significant effect on mIPSCs (Figures S1A and S1B), indicating the existence of GluN2-dependent regulation of tonic, but not phasic, inhibition in neurons. Previous work has shown that extrasynaptic  $\alpha$ 5-GABA<sub>A</sub>Rs mediate a majority of tonic inhibition in hippocampal pyramidal neurons (Caraiscos et al., 2004; Glykys et al., 2008; Wu et al., 2021). To investigate whether overexpression of GluN2 subunits regulated  $\alpha$ 5-GABA<sub>A</sub>R surface abundance, we performed  $\alpha$ 5-GABA<sub>A</sub>R

immunostaining assays in cultured neurons. Consistent with the observed effect on tonic currents (Figure 1B), overexpression of GluN2B, but not GluN2A or the control, GFP, reduced the surface expression of  $\alpha 5$ -GABA<sub>A</sub>Rs (Figure 1C).

### Knockout of GluN2A and GluN2B differentially regulates tonic inhibition

To complement overexpression experiments, we developed single-guide RNAs (sgRNAs) to perform single-cell genetic deletion of endogenous GluN2A or GluN2B in cultured hippocampal neurons and then measured tonic inhibitory currents (Figure 1D). The knockout (KO) efficacy was confirmed by western blot in co-transfected HEK293 cells (Figures S1C and S1D), as well as by NMDAR-mediated mEPSC and NMDA-evoked whole-cell current recordings in cultured neurons transfected with both sgRNAs (Figures S1E–S1G). Although KO of GluN2A significantly decreased tonic currents, we observed that GluN2B KO increased tonic currents, compared with control neurons expressing the empty vector (Figure 1E). Importantly, the changes in tonic currents induced by either GluN2A or GluN2B KO were restored by co-expression of the corresponding GluN2 sgRNA-resistant constructs (Figures 1E and S1D), suggesting that these effects were due to loss of endogenous GluN2A or GluN2B but not off-target effects.

### Pharmacological suppression of GluN2A- and GluN2B-NMDARs differentially modulates $\alpha 5$ -GABA<sub>A</sub>R surface expression and tonic inhibition

To corroborate our genetic findings, we next employed a pharmacological approach to further determine the role of GluN2A and GluN2B in the regulation of tonic inhibition. Specifically, we treated cultured neurons at DIV14 with NVP-AAM077 (NVP, GluN2A-preferring antagonist; 100 nM), ifenprodil (Ifen, GluN2B-preferring antagonist; 5  $\mu$ M) or APV (pan-NMDAR antagonist; 100  $\mu$ M) for 24 h, and then measured tonic inhibitory currents (Figure 2A). We found that Ifen increased  $\alpha 5$  surface expression and tonic inhibitory currents, whereas treatment with NVP induced opposite effects (Figures 2B and 2C). Interestingly, APV had little effect on  $\alpha 5$  surface expression and tonic currents (Figures 2B and 2C), indicating that antagonism of both GluN2A- and GluN2B-NMDARs exerts a combinatory effect leading to little change in tonic inhibition. Taken together, these results suggest differential regulation of  $\alpha 5$  surface expression and tonic inhibition by GluN2A- and GluN2B-NMDARs. Considering that  $\delta$ -GABA<sub>A</sub>Rs also generate a tonic inhibitory conductance in the hippocampus (Glykys et al., 2008; Penna et al., 2014), we recorded THIP (3  $\mu$ M, a GABA<sub>A</sub>R agonist with a preference for  $\delta$ -GABA<sub>A</sub>Rs)-evoked currents in hippocampal neurons at DIV15 after 24-h treatment with NVP or Ifen. We found that neither NVP nor Ifen treatment affected THIP-evoked currents (Figure S2), suggesting that NMDARs do not regulate  $\delta$ -GABA<sub>A</sub>R-mediated tonic inhibition in hippocampal neurons.

During development, NMDARs undergo a developmental switch from those incorporating the GluN2B subunit to the GluN2A subunit in neocortex and hippocampus (Dong et al., 2006; McKay et al., 2018; Sheng et al., 1994). Consistent with these previous studies, we confirmed this developmental switch from primarily GluN2B-NMDARs to primarily GluN2A-NMDARs in cultured hippocampal neurons (Figure S3A). In addition, both  $\alpha 5$ -GABA<sub>A</sub>R subunit expression and tonic currents increased during development within the first month in cultured neurons (Figures S3A and S3B), suggesting a possible link between

developmental switch of NMDARs subunits and increase of  $\alpha 5$ -GABA<sub>A</sub>R expression within the first month in neuronal cultures. Thus, we tested the effects of GluN2A and GluN2B antagonists on tonic inhibition in immature and mature neurons. In immature neurons at DIV7–8, Ifen or APV increased  $\alpha 5$  surface expression and tonic currents, whereas NVP had little effect (Figures S3C and S3D), consistent with higher expression of GluN2B in developing, immature neurons. By contrast, in mature neurons at DIV25–DIV-26, NVP and APV decreased  $\alpha 5$  surface expression and tonic currents, whereas Ifen had little effect (Figures S3C and S3D). These results suggest that regulation of tonic inhibition by GluN2A- and GluN2B-NMDARs in neuronal culture is developmentally dependent.

### Differential regulation of $\alpha 5$ -GABA<sub>A</sub>R internalization by GluN2A- and GluN2B-NMDARs

Considering that opposing actions of GluN2A- and GluN2B-NMDAR antagonism on  $\alpha 5$ -GABA<sub>A</sub>R surface expression and tonic currents were observed at 2–3 weeks neurons, we then investigated the mechanism underlying the distinct effects at this developmental stage. The abundance of  $\alpha 5$ -GABA<sub>A</sub>R surface expression is dynamically controlled by the balance of receptor endocytosis and exocytosis. Because  $\alpha 5$ -GABA<sub>A</sub>R surface expression is differentially regulated by NMDAR GluN2 subunits, we hypothesized that  $\alpha 5$ -GABA<sub>A</sub>R endocytosis and/or exocytosis might be critically regulated by GluN2A- or GluN2B-NMDARs. To test this hypothesis, we first performed antibody-feeding experiments to label surface and internalized endogenous  $\alpha 5$  in live hippocampal neurons at DIV15 and examined  $\alpha 5$  endocytosis. We found that 24-h NVP treatment increased  $\alpha 5$ -GABA<sub>A</sub>R internalization, whereas 24-h Ifen treatment decreased  $\alpha 5$ -GABA<sub>A</sub>R internalization (Figure 2D). In addition, 24-h APV treatment did not alter  $\alpha 5$ -GABA<sub>A</sub>R internalization (Figure 2D). These results suggest that GluN2A and GluN2B have distinct roles in regulating  $\alpha 5$ -GABA<sub>A</sub>R internalization at this developmental stage. Next, we combined fluorescence recovery after photobleaching (FRAP) with fluorescence loss in photobleaching (FLIP) to investigate exocytosis of superecliptic pHluorin-tagged  $\alpha 5$  (SEP- $\alpha 5$ ) as a measurement of the receptor exocytosis (Wu et al., 2021). In this experiment, repetitive photobleaching occurred at dendritic regions bilateral to the central FRAP area, thus excluding laterally diffusing SEP- $\alpha 5$  to the central area and allowing the measurement of newly exocytosed SEP- $\alpha 5$  (Figure S3E). We found that neurons treated with either selective NMDAR antagonists or pan-NMDAR antagonism exhibited similar fluorescence recovery after photobleaching (Figure S3F), indicating that neither GluN2A- nor GluN2B-NMDARs regulate  $\alpha 5$ -GABA<sub>A</sub>R exocytosis. The extrasynaptic abundance of  $\alpha 5$ -GABA<sub>A</sub>R is also affected by phosphorylation level of the actin-binding protein radixin, an ezrin/radixin/moesin (ERM)-family member (Hausrat et al., 2015). Specifically, phosphorylated radixin anchors  $\alpha 5$ -GABA<sub>A</sub>Rs at extrasynaptic sites (Hausrat et al., 2015). We found that antagonism of GluN2A- and GluN2B-NMDARs had little effect on ERM and phosphorylated ERM (p-ERM) levels (Figure S3G), suggesting that the regulation of  $\alpha 5$ -GABA<sub>A</sub>Rs by GluN2A- and GluN2B-NMDARs is not due to changes in the phosphorylation of radixin.

### GluN2A-NMDARs are required for homeostatic potentiation of tonic inhibition

Chronic pharmacological manipulation of neuronal activity can induce homeostatic adaptations in both excitatory and inhibitory transmission, which are powerful mechanisms

that control neuronal excitability and neural network function (Turrigiano, 2012). Our recent work demonstrated that tonic inhibition in hippocampal neurons exhibited homeostatic potentiation after prolonged elevation of neuronal activity (Wu et al., 2021). Consistent with this study, we found that surface  $\alpha 5$  expression and tonic inhibition increased following 48-h bicuculline treatment (Figure 3). To determine the role of GluN2A- and GluN2B-NMDARs in homeostatic plasticity of tonic inhibition, we treated hippocampal neurons at DIV16 with bicuculline (40  $\mu$ M) and 24 h later with NVP (100 nM), Ifen (5  $\mu$ M), or APV (100  $\mu$ M) (Figure 3A). 24 h later, we found that bicuculline-induced effects on tonic inhibitory currents (Figures 3B and 3C) and surface  $\alpha 5$  expression (Figures 3D and 3E) were significantly diminished by treatment with NVP and APV, but not by Ifen. These observations support a GluN2A-, but not GluN2B-NMDAR-dependent mechanism in homeostatic potentiation of tonic inhibition.

### GluN2A- and GluN2B-NMDARs regulate tonic inhibition in a KA-induced seizure model

Finally, we examined whether GluN2-dependent regulation of  $\alpha 5$ -GABA<sub>A</sub>Rs may occur during seizures based on evidence that deficits in tonic inhibition are potentially involved in epilepsy (Chen et al., 2007). To this end, we utilized an acute seizure model by systemically administering kainic acid (KA), an agonist for kainate- and AMPA-type ionotropic glutamate receptors. All mice injected with KA (20 mg/kg) showed epileptic behavior within 30 min and recovered 24 h after injection (Figure S4A). We then analyzed total and surface expression levels of GluN2A, GluN2B, and GABA<sub>A</sub>Rs in hippocampal slices prepared from mice at 1 h or 24 h after KA injection (Figure S4B). We found that KA administration had little effect on the total expression level of NMDAR and GABA<sub>A</sub>R subunits, comparing with saline-injected control (Figures 4A–4C). In contrast, 1-h post-KA injection significantly increased surface GluN2B, but not GluN2A expression (Figures 4A and 4B). Interestingly, 24 h after KA injection, surface GluN2A was increased, whereas surface GluN2B was unaffected (Figures 4A and 4B). As expected from the regulation of surface  $\alpha 5$  expression by GluN2A- and GluN2B-NMDARs (Figure 2C), surface expression of  $\alpha 5$ -GABA<sub>A</sub>R was decreased at 1 h and then increased at 24 h after KA injection (Figure 4C). In addition, surface  $\alpha 1$ -GABA<sub>A</sub>Rs were increased at 24 h after KA injection (Figure S4C). These results suggest the expression of surface GluN2 subunits, synaptic and extrasynaptic GABA<sub>A</sub>Rs are dynamically regulated by KA-induced seizures in a time-dependent manner.

It has been shown that the CA3 region of the hippocampus is substantially impacted in KA-induced seizures (Vincent and Mulle, 2009). We thus recorded tonic currents in CA3 pyramidal cells (Figure S4D). Consistent with KA-induced alterations in surface  $\alpha 5$  expression, we found that tonic currents in hippocampal CA3 neurons at 1 h and 24 h post-injection in KA-administrated mice were decreased and increased, respectively, compared to saline controls (Figures 4D and 4E). Interestingly, Ifen (10 mg/kg) or NVP (10 mg/kg) treatment 1 h prior to KA injection respectively restored the decreased or increased tonic currents at corresponding time point after KA injection (Figures 4D and 4E). These results suggest that KA-induced changes in tonic inhibition are GluN2-dependent in a temporal-specific manner. In contrast, although the mIPSC amplitude was increased 24 h after KA treatment compared to control, antagonism of GluN2A- or GluN2B- NMDARs had little

effect on this alteration (Figures S4E and S4F), indicating that KA-induced increases in phasic inhibition are independent of GluN2A- and GluN2B-NMDAR signaling.

## DISCUSSION

In this study, we investigate the regulatory role of GluN2A- and GluN2B-NMDARs in tonic inhibition. Our genetic and pharmacological analyses indicate opposing actions of GluN2A- and GluN2B-NMDARs on  $\alpha 5$ -GABA<sub>A</sub>R internalization and tonic inhibition in hippocampal neurons. In addition, GluN2A-NMDARs, but not GluN2B-NMDARs, are required for homeostatic potentiation of tonic inhibition. Last, in a KA-induced acute seizure model, we observe a decrease in tonic inhibition during acute seizures, which is then increased 24 h later, and these alterations are dependent on the activity of distinct GluN2-NMDARs. Collectively, these data extend previous work showing the importance of NMDAR-dependent regulation of inhibitory synapse development and transmission (Chiu et al., 2018; Gu and Lu, 2018; Gu et al., 2016; Horn and Nicoll, 2018) and reveal important crosstalk between glutamatergic signaling and extrasynaptic GABA<sub>A</sub>Rs.

### Differential regulation of tonic inhibition by NMDA receptor subtypes

It has been reported that extrasynaptic GABA<sub>A</sub>R-mediated tonic inhibition is regulated by various receptors such as GABA<sub>B</sub> (Connelly et al., 2013; Tao et al., 2013) and glycine receptors (Zou et al., 2019). In addition to these inhibitory receptors, tonic inhibition is modulated by glutamate receptors. For example, deletion of GluN1 augments tonic inhibition in immature hippocampal neurons in culture (Gu et al., 2016). In line with this study, here we found that pharmacological blockade of GluN2B-NMDARs, the predominant NMDAR subtype expressed in immature neuron or blockade of all NMDARs, enhanced tonic inhibition in immature neurons. Interestingly, we also found that the effect of blockade of GluN2B-NMDARs on tonic inhibition disappeared in mature neurons. The temporal-specific regulation of tonic inhibition is likely due to differential expression of NMDAR GluN2 subunits during development. Indeed, during the first 2 weeks after birth, NMDARs undergo a developmental switch from predominantly GluN2B- to GluN2A-NMDARs in the hippocampus (Dong et al., 2006). This may explain the lack of effects on tonic inhibition by antagonism of GluN2A-NMDARs in immature neurons. It is also important to mention that NMDAR subunits have been found to localize at GABAergic synapses in developing neurons (Cserép et al., 2012; Gundersen et al., 2004; Szabadits et al., 2011), providing anatomic proximity to regulate perisynaptic and extrasynaptic GABA<sub>A</sub>Rs. In contrast to a predominant effect of GluN2B-dependent regulation of tonic currents in immature neurons, we observed opposing actions of GluN2A- and GluN2B-NMDAR antagonism on tonic inhibition at 2–3 weeks neurons when both GluN2A and GluN2B are abundantly expressed. Interestingly, inhibition of all NMDARs by APV abolished the differential modulation of tonic inhibition by NMDAR subtypes, presumably due to normalizing the opposing effects of GluN2A- and GluN2B-NMDARs on  $\alpha 5$ -GABA<sub>A</sub>Rs. Of note, although both pharmacological inhibition and genetic deletion of GluN2A diminished tonic inhibition at 2–3 weeks neurons, overexpression of GluN2A had no significant effect on tonic inhibition, showing that further increase in GluN2A expression over endogenous levels at this developmental stage is not sufficient to alter tonic inhibitory currents.

Our data indicate that  $\alpha 5$ -GABA<sub>A</sub>R endocytosis, but not exocytosis, is differentially regulated by GluN2A- and GluN2B-NMDARs. Specifically, GluN2A-NMDARs inhibit, and GluN2B-NMDARs promote  $\alpha 5$ -GABA<sub>A</sub>R internalization. Currently, the molecular mechanisms underlying the distinct regulation of  $\alpha 5$ -GABA<sub>A</sub>R endocytosis by NMDARs remain unclear. One scenario is that GluN2A- and GluN2B-NMDARs are coupled to distinct downstream phosphatase and kinase pathways (Shipton and Paulsen, 2013; Sun et al., 2018; Wu and Tymianski, 2018), which in turn may differentially regulate  $\alpha 5$ -GABA<sub>A</sub>R trafficking and tonic inhibition. For instance, it has been reported that activation of GluN2A- and GluN2B-NMDARs differentially regulates ERK1/2 (Chen et al., 2007) and Akt signaling (Liu et al., 2007). However, it remains unknown whether these molecular pathways are involved in the regulation of  $\alpha 5$ -GABA<sub>A</sub>R trafficking and tonic inhibition. Interestingly, it has been shown that neuronal nitric oxide synthase (nNOS) is expressed at GABAergic synapses and can be activated by NMDAR activation (Szabadits et al., 2011). It is therefore plausible that NMDAR-dependent regulation of nNOS activity could modulate protein kinase activity, leading to the regulation of  $\alpha 5$ -GABA<sub>A</sub>R trafficking and tonic inhibition. Recently, a number of proteins such as gephyrin (Brady and Jacob, 2015), Shisa7 (Wu et al., 2021), and Clptm1 (Ge et al., 2018) have been identified to interact with the  $\alpha 5$ -GABA<sub>A</sub>R, which raise the possibility that GluN2A- and GluN2B-NMDARs might differentially regulate their interaction involved in  $\alpha 5$ -GABA<sub>A</sub>R trafficking. Radixin, an actin binding protein, also binds to  $\alpha 5$ -GABA<sub>A</sub>Rs, and phosphorylated radixin accumulates  $\alpha 5$ -GABA<sub>A</sub>R clustering at the extrasynaptic membrane (Hausrat et al., 2015; Loebrich et al., 2006). However, we found that antagonism of GluN2A- or GluN2B-NMDARs did not affect the phosphorylation of radixin, suggesting radixin is unlikely involved in NMDAR modulation of tonic inhibition. It is also worth noting that CaMKII activation can increase cell-surface  $\alpha 5\beta 3$ -containing receptors and tonic currents (Saliba et al., 2012). Conversely, blockade of CaMKII activity reduces tonic currents in hippocampal neurons (Wu et al., 2021). Interestingly, GluN2A- and GluN2B-NMDARs can differentially regulate CaMKII activity (Barria and Malinow, 2005; Strack et al., 2000), which in turn might contribute to distinct modulation of  $\alpha 5$ -GABA<sub>A</sub>R trafficking and tonic inhibition. In the future, it will be important to determine how GluN2A- and GluN2B-NMDARs can distinctly modulate tonic inhibition involving further downstream signaling components.

### Alterations of tonic inhibition in higher activity condition and epilepsy

It has been reported that GluN2B-NMDARs are involved in the pathophysiology of epilepsy (Morimoto et al., 2004; Waxman and Lynch, 2005). Indeed, selective GluN2B-NMDAR antagonists suppressed epileptogenesis in rodent seizure models (Gorlewicz et al., 2021; Mareš et al., 2021) and decreased neuronal excitability of neocortical pyramidal neurons from epileptic patients (Wang et al., 2017). Additionally, in the seizure model induced by pilocarpine, a cholinergic muscarinic agonist, surface accumulation of GluN2B-NMDARs is increased in the hippocampus (Müller et al., 2013; Naylor et al., 2013). Likewise, a reduction in  $\alpha 5$ -GABA<sub>A</sub>Rs in CA1 pyramidal cells of pilocarpine-treated rats has also been observed (Houser and Esclapez, 2003). In this study, we show that administration of KA *in vivo* induces upregulation of surface GluN2B and downregulation of surface  $\alpha 5$ -GABA<sub>A</sub>Rs 1 h after KA injection in the hippocampus. Together with the experiments demonstrating that GluN2B-NMDARs regulate  $\alpha 5$ -GABA<sub>A</sub>R internalization, our data indicate that an



increase of GluN2B-NMDARs contributes to reduced  $\alpha 5$ -GABA<sub>A</sub>R surface expression by enhancing receptor endocytosis, resulting in downregulation of tonic inhibition during acute seizures. Given that the important role of tonic inhibition in regulating neural excitability (Belelli et al., 2009; Farrant and Nusser, 2005; Lee and Maguire, 2014), GluN2B-NMDAR-mediated reduction of tonic inhibition may contribute to KA-induced neuronal hyperexcitability at the early stage in this seizure model.

Our data also show that in the KA-induced acute seizure model, epileptic phenotypes disappear 24 h after KA induction with a corresponding increase in tonic inhibition in hippocampal CA3 neurons. Consistently, a recent study using a similar seizure model identified that neuronal hyperexcitability in cortical neurons returns to the basal level and tonic inhibition is increased 24 h after KA injection (Pan et al., 2018). Additionally, in the pilocarpine-induced seizure model, tonic current is enhanced in dentate gyrus granule cells 3 weeks after induction (Naylor et al., 2005). Considering the importance of tonic GABAergic signaling in regulating neural network excitability (Belelli et al., 2009; Farrant and Nusser, 2005; Lee and Maguire, 2014), enhanced tonic inhibition that occurs after acute seizures may act as a homeostatic adaptive response to KA-induced neuronal hyperexcitability and thus might help lessen the associated neurodegeneration. It is worth noting that the increase of tonic inhibition in hippocampal CA3 neurons 24 h after KA administration is abolished by blockade of GluN2A-NMDARs, showing an important role of GluN2A-NMDARs in control of tonic inhibition in the late stage of this seizure model. Interestingly, we found that homeostatic potentiation of tonic inhibition induced by prolonged elevation of neuronal activity is also dependent on the GluN2A subunit. Although the mechanisms underlying the regulation of tonic inhibition by GluN2A-NMDARs under these conditions remain unclear, it could be due to a variety of possibilities such as different electrophysiological properties or discrete intracellular signaling cascades that can be engaged (Paoletti et al., 2013). Additionally, in line with a previous report (Peng et al., 2010), we found that phasic inhibition could undergo homeostatic potentiation after KA administration. However, homeostatic potentiation of synaptic inhibition is independent of GluN2A, suggesting distinct mechanisms governing homeostatic plasticity of tonic and phasic inhibition induced by KA.

Taken together, we have uncovered a critical role for NMDAR-dependent regulation of  $\alpha 5$ -GABA<sub>A</sub>R trafficking and tonic inhibition during development, under higher activity, and in a KA seizure model. We have also revealed distinct roles for NMDAR subtypes in regulating tonic inhibition under a variety of conditions both *in vitro* and *in vivo*. As dysregulation of tonic inhibition has been shown to be a mechanism underlying a number of pathological brain states (Brickley and Mody, 2012; Hines et al., 2012), our findings also offer insight into crosstalk between glutamatergic and GABAergic systems, as well as how dysregulation of this crosstalk could be involved in epileptic conditions.

### Limitations of study

Given the importance of assessing sex as a biological variable, one limitation of our study is that we only utilized male mice for these experiments. Because of the rising significance of neurosteroids, endogenous positive allosteric modulators of GABA<sub>A</sub>Rs (although with

greater selectivity for  $\delta$ -GABA<sub>A</sub>Rs), it has been demonstrated that differences in seizure susceptibility could be dependent on their production in different genders (Samba Reddy, 2017). Additionally, distributions of extrasynaptic GABA<sub>A</sub>R subtypes differ in a cell- and brain region-specific manner. Because our recordings were from hippocampal cultures and CA3 neurons in acute brain slices, it cannot be ruled out that our observed NMDAR-dependent mechanisms regulating tonic inhibition might not be the same in neurons in other brain regions. Last, although we demonstrate distinct roles for NMDAR subtypes, we did not address the downstream mechanisms and how they might differ between NMDAR subtypes to regulate  $\alpha$ 5-GABA<sub>A</sub>R surface expression and tonic inhibition.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

### RESOURCE AVAILABILITY

**Lead contact**—Further information and requests for resources and reagents should be directed to the Lead Contact, Wei Lu (luw4@mail.nih.gov).

**Materials availability**—All unique reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

#### Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

**Animals**—All animal handling was performed in accordance with animal protocols approved by the Institutional Animal Care and Use Committee (IACUC) at NIH/NINDS. All mice were housed and bred in a conventional vivarium with *ad libitum* access to food and water under a 12-h circadian cycle. Time-pregnant mice at E17.5–18.5 were used for dissociated hippocampal neuronal culture. 6–8 weeks old male mice were used for biochemical, electrophysiological, and behavioral experiments.

**Cell Lines**—HEK293T cells (ATCC, Cat# CRL-11268) were maintained with culture media containing 1% penicillin-streptomycin (GIBCO), 10% FBS (GIBCO) in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO), in a humidified incubator at 37°C with 5% CO<sub>2</sub>.

**Dissociated Hippocampal Neuronal Culture**—Hippocampal neurons were prepared from E17.5–18.5 mice embryos of either sex as previously described (Wu et al., 2021). In brief, the hippocampi were dissected from embryonic brains and digested in the Hank's Balanced Salt Solution (HBSS, GIBCO) containing 20 U/ml papain (Worthington) and 100

U/ml DNase I (Worthington) at 37°C for 45 min. After centrifugation for 5 min at 800 rpm, the pellet was resuspended in HBSS containing 100 U/ml DNase I, and was fully dissociated by pipetting up and down. Cells were then transferred into HBSS containing trypsin inhibitor (10 mg/ml, Sigma-Aldrich) and BSA (10 mg/ml, Sigma-Aldrich). After centrifugation for 10 min at 800 rpm, cells were resuspended in Neurobasal media (GIBCO) supplemented with 2% B27 (GIBCO) and 2 mM GlutaMAX (GIBCO) and were plated on poly-D-lysine (Sigma-Aldrich)-coated glass coverslips or 6-well plates. Cultures were maintained in Neurobasal media supplemented with 2% B27 and 2 mM GlutaMAX in a humidified incubator at 37°C with 5% CO<sub>2</sub>. Culture media were changed by half volume once a week.

## METHOD DETAILS

**Plasmids**—pRK5-GFP-GluN2A and pRK5-GFP-GluN2B were gifts from Katherine Roche's lab at NINDS, NIH. pSpCas9(BB)-2A-Puro (PX459) V2.0 and pSpCas9(BB)-2A-GFP (PX458) were purchased from Addgene. Custom oligonucleotides were generated (GluN2A forward, 5' CACCGCGACGTGACAGAACGCGAAC 3'; and GluN2A reverse, 5' AAACGTTTCGCTTCTGTCACGTCGC 3'; and GluN2B forward, 5' CACCGTCTGACCGGAAGATCCAGG 3'; and GluN2B reverse, 5' AAACCCTGGATCTTCCGGTCAGAC 3'; IDT), and cloned into pSpCas9-BB-2A-GFP (PX458) or pSpCas9(BB)-2A-Puro (PX459) V2.0 vector at the BbsI cutting site. The coding sequence of GluN2A and GluN2B point mutations for sgRNA resistant plasmid (GluN2A: AGCCACGACGTGACAGAACGCGAACTT to AGTCACGACGTGACTGAGAGAGAACTT; GluN2B: ATGTCTGACCGGAAGATCCAGGGG to ATGTCTGATCGTAAGATTCAAGGA) were generated by Q5 Site-Directed Mutagenesis Kit (NEB).

**Cell Transfection**—HEK293T cells were transfected with GluN2A or GluN2B, together with sgRNA using CalPhos Mammalian Transfection Kit (Takara). Western blot was performed 48 h after transfection. Hippocampal neurons at DIV3–4 were transfected with GluN2A sgRNA or GluN2B sgRNA using NeuroMag reagent (Oz Biosciences), and were recorded at DIV16–17. Hippocampal neurons at DIV11 were co-transfected with pCAG-IRES-GFP and GluN2A or GluN2B using NeuroMag reagent. Electrophysiological recordings or immunostaining were performed 72 h after transfection. All transfection kits were used according to the manufacturer's instructions.

**Electrophysiology**—For recording in dissociated hippocampal cultures, neurons were continuously perfused with the extracellular solution containing (in mM): 140 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, and 10 Glucose (pH 7.3; osmolality 300–310 mOsm). The internal solution contained (in mM): 70 CsMeSO<sub>4</sub>, 70 CsCl, 8 NaCl, 10 HEPES, 0.3 Na-GTP, 4 Mg-ATP and 0.3 EGTA (pH 7.3; osmolality 285–290 mOsm). Miniature inhibitory postsynaptic currents (mIPSCs) and tonic currents were recorded at –70 mV in the presence of 0.5 μM TTX (Alomone Labs) and 20 μM DNQX (Alomone labs) without exogenous GABA. To test the effects of GluN2A- or GluN2B-preferring antagonist on tonic currents, neurons were treated with 100 nM NVP (Ding et al., 2021) or 5 μM Ifen (Sibarov et al., 2016) for 24 h prior to electrophysiological recording. For recording THIP-evoked currents,

THIP (3  $\mu\text{M}$ , Santa Cruz) was added to the extracellular solution via a computer-controlled multi-barrel perfusion system (Automate Scientific). For recording NMDA mEPSCs at +40 mV, 0.5  $\mu\text{M}$  TTX, 20  $\mu\text{M}$  DNQX, and 50  $\mu\text{M}$  picrotoxin (Sigma-Aldrich) were added into the extracellular solution. For recording NMDA-induced whole-cell currents, TTX (0.5  $\mu\text{M}$ ) were added into 0  $\text{Mg}^{2+}$  extracellular solution. NMDA-induced current was recorded at  $-70$  mV by rapid application/removal of NMDA (100  $\mu\text{M}$ ) using the perfusion system.

For recording in acute brain slices, transverse hippocampal slices (300  $\mu\text{m}$  thickness) were prepared from 6–8 weeks old male mice in chilled high sucrose cutting solution, containing (in mM): 2.5 KCl, 0.5  $\text{CaCl}_2$ , 7  $\text{MgCl}_2$ , 1.25  $\text{NaH}_2\text{PO}_4$ , 25  $\text{NaHCO}_3$ , 7 glucose, 210 sucrose and 1.3 ascorbic acid. The slices were recovered in artificial cerebrospinal fluid (ACSF) containing (in mM): 119 NaCl, 2.5 KCl, 26.2  $\text{NaHCO}_3$ , 1  $\text{NaH}_2\text{PO}_4$ , 11 glucose, 2.5  $\text{CaCl}_2$  and 1.3  $\text{MgSO}_4$  (pH 7.3; osmolality 300–310 mOsm) at  $32^\circ\text{C}$  for 30 min and then were maintained at room temperature prior to recording. To record tonic currents, slices were transferred to a submersion chamber, continuously perfused with ACSF with 0.5  $\mu\text{M}$  TTX, 20  $\mu\text{M}$  DNQX and 5  $\mu\text{M}$  GABA. The intracellular solution contained (in mM) 130 CsCl, 8.5 NaCl, 5 HEPES, 4  $\text{MgCl}_2$ , 4 Na-ATP, 0.3 Na-GTP and 1 QX-314 (pH 7.3; osmolality 285–290 mOsm).

To measure tonic inhibitory currents in neuronal cultures or in acute hippocampal slices, the  $\text{GABA}_A$  competitive antagonist bicuculline (20  $\mu\text{M}$ , Abcam) was bath applied after obtaining a stable baseline recording at  $-70$  mV. Igor Pro were used to determine the values of tonic currents. An all-points histogram was plotted for a 20 s period before and during bath-application of bicuculline, fitting the histogram with a Gaussian distribution gave the mean baseline holding currents, and the difference in baseline holding currents before and during bicuculline application was calculated to be the tonic currents. Tonic currents were normalized to membrane capacitance, to account for variability in cell size. Series resistance was monitored and not compensated, and cells in which series resistance was more than 25  $\text{M}\Omega$  or varied by 25% during a recording session were discarded. Whole-cell recordings were obtained from cells visualized with a fixed stage upright microscope (BX51WI, Olympus). Fluorescence-positive cells were identified by epifluorescence microscopy. Data were collected with a Multiclamp 700B amplifier (Axon Instruments), filtered at 2 kHz, and digitized at 10 kHz.

**Immunostaining**—For surface  $\alpha 5$  receptor labeling, cultured hippocampal neurons at DIV15 on coverslips were incubated with anti- $\alpha 5$  antibody (1:500, Synaptic Systems) in culture medium for 15 min. Next, they were washed briefly with fresh culture medium and fixed with a solution containing 4% paraformaldehyde and 4% sucrose in PBS. Cultured neurons were subsequently incubated with Alexa 555-conjugated anti-rabbit secondary antibody (1:1000, Thermo Fisher Scientific) for the visualization of  $\alpha 5$ . For the endocytosis assay, cultured hippocampal neurons at DIV15 were incubated live with rabbit  $\alpha 5$  antibody (1:500, Synaptic Systems) at  $37^\circ\text{C}$  for 10 min in conditioned culture medium. After incubation, the neurons were washed with PBS and then incubated in antibody-free medium to allow antibody-bound receptors to undergo internalization at  $37^\circ\text{C}$  for 30 min, followed by fixation with 4% paraformaldehyde and 4% sucrose in PBS. After fixation, neurons were washed and then blocked with 10% NGS for 1 h, exposed to Alexa

488-conjugated anti-rabbit secondary antibody (1:200, Jackson ImmunoResearch Labs) for 1 h under the nonpermeabilized condition, and then internalized  $\alpha 5$  was labeled with Alexa 555-conjugated anti-rabbit secondary antibody (1:1000, Thermo Fisher Scientific) for 1 h after permeabilization in PBS containing 0.25% Triton X-100 and blocking in 10% NGS. Coverslips were washed for three times with PBS and mounted with Fluoromount-G.

Fluorescence images were acquired on a Zeiss LSM 880 laser scanning confocal microscope with a  $63 \times 1.4$  NA oil immersion objective. For quantification, sets of cells were prepared and stained simultaneously. Compared images were acquired at the same time using identical acquisition settings. The fluorescence intensity was analyzed using ImageJ. The results were based on the number of neurons from at least three independent experiments.

**Surface cell biotinylation of hippocampal slices**—Hippocampal slices were prepared from 6–8 weeks mice as described (Li et al., 2017). Surface expression of GluN2A, GluN2B,  $\alpha 1$ -GABA<sub>A</sub>R and  $\alpha 5$ -GABA<sub>A</sub>R was quantitated as described. Briefly, acute hippocampal slices were labeled for 30 min at 4°C with 1 mg/ml sulfo-NHS-SS biotin (ThermoFisher Scientific). Membranes were prepared and the biotinylated proteins were precipitated with streptavidin agarose resin (ThermoFisher Scientific) and detected by western blot.

**Kainic acid-induced seizure model**—Young adult male mice (6–8 weeks old) were administered an intraperitoneal (i.p.) injection of kainic acid (KA, Abcam) dissolved in 0.9% saline solution at 20 mg/kg body weight. Seizure score was evaluated at 0 h, 0.5 h, 1 h and 24 h after KA injection according to the modified Racine scale (Racine, 1972): stage 0, normal behavior; stage 1, immobility and rigidity; stage 2, repetitive behaviors, head nodding or bobbing; stage 3, Forelimb clonus with partial or intermittent rearing; stage 4, continuous rearing and falling; stage 5, severe clonic-tonic seizures; stage 6, death. The expression levels of GluN2A, GluN2B and GABA<sub>A</sub>Rs in hippocampi were examined at 1 h or 24 h after KA injection. To examine the effects of GluN2A- and GluN2B-containing receptors on the tonic inhibition in KA-induced seizure model, mice were injected with 10 mg/kg NVP (Gordillo-Salas et al., 2018), 10 mg/kg Ifen (Raybuck et al., 2017) or saline 1 h prior to KA injection and were sacrificed for electrophysiological recordings at 1 h or 24 h after KA injection.

## QUANTIFICATION AND STATISTICAL ANALYSIS

**Statistical Analysis**—Statistical analysis was performed in GraphPad Prism 8.0 software. Normality distribution was tested by the Shapiro-Wilk test before carrying out a subsequent statistical test. Direct comparisons between two groups were made using two-tailed Student's t test or Mann-Whitney U test. Multiple comparisons were performed using one-way ANOVA, Kruskal-Wallis test or two-way ANOVA with corrections for multiple comparisons test (see figure legends for specifics).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGMENTS

We are grateful to all members from Lu laboratory for critical comments on the manuscript. We thank Dr. Ryan Shepard for editing the manuscript. This work was supported by the NIH/NINDS Intramural Research Program (to W.L.).

## REFERENCES

- Barria A, and Malinow R (2005). NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. *Neuron* 48, 289–301. [PubMed: 16242409]
- Belelli D, Harrison NL, Maguire J, Macdonald RL, Walker MC, and Cope DW (2009). Extrasynaptic GABAA receptors: form, pharmacology, and function. *J. Neurosci.* 29, 12757–12763. [PubMed: 19828786]
- Brady ML, and Jacob TC (2015). Synaptic localization of  $\alpha 5$  GABA (A) receptors via gephyrin interaction regulates dendritic outgrowth and spine maturation. *Dev. Neurobiol.* 75, 1241–1251. [PubMed: 25663431]
- Brickley SG, and Mody I (2012). Extrasynaptic GABA(A) receptors: their function in the CNS and implications for disease. *Neuron* 73, 23–34. [PubMed: 22243744]
- Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, et al. (2004). Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by alpha5 subunit-containing gamma-aminobutyric acid type A receptors. *Proc. Natl. Acad. Sci. USA* 101, 3662–3667. [PubMed: 14993607]
- Chatterton JE, Awobuluyi M, Premkumar LS, Takahashi H, Talantova M, Shin Y, Cui J, Tu S, Sevarino KA, Nakanishi N, et al. (2002). Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. *Nature* 415, 793–798. [PubMed: 11823786]
- Chen Q, He S, Hu XL, Yu J, Zhou Y, Zheng J, Zhang S, Zhang C, Duan WH, and Xiong ZQ (2007). Differential roles of NR2A- and NR2B-containing NMDA receptors in activity-dependent brain-derived neurotrophic factor gene regulation and limbic epileptogenesis. *J. Neurosci.* 27, 542–552. [PubMed: 17234586]
- Chen M, Lu TJ, Chen XJ, Zhou Y, Chen Q, Feng XY, Xu L, Duan WH, and Xiong ZQ (2008). Differential roles of NMDA receptor subtypes in ischemic neuronal cell death and ischemic tolerance. *Stroke* 39, 3042–3048. [PubMed: 18688011]
- Chiu CQ, Martenson JS, Yamazaki M, Natsume R, Sakimura K, Tomita S, Tavalin SJ, and Higley MJ (2018). Input-Specific NMDAR-Dependent Potentiation of Dendritic GABAergic Inhibition. *Neuron* 97, 368–377.e3. [PubMed: 29346754]
- Connelly WM, Fyson SJ, Errington AC, McCafferty CP, Cope DW, Di Giovanni G, and Crunelli V (2013). GABAB Receptors Regulate Extrasynaptic GABAA Receptors. *J. Neurosci.* 33, 3780–3785. [PubMed: 23447590]
- Cserép C, Szabadits E, Szanyi A, Watanabe M, Freund TF, and Nyiri G (2012). NMDA receptors in GABAergic synapses during postnatal development. *PLoS ONE* 7, e37753. [PubMed: 22662211]
- Ding F, Bai Y, Cheng Q, Yu S, Cheng M, Wu Y, Zhang X, Liang X, and Gu X (2021). Bidentate, a Novel Plant Peptide Derived from *Achyranthes bidentata* Blume: Isolation, Characterization, and Neuroprotection through Inhibition of NR2B-Containing NMDA Receptors. *Int. J. Mol. Sci.* 22, 7977. [PubMed: 34360755]
- Dong YN, Wu HY, Hsu FC, Coulter DA, and Lynch DR (2006). Developmental and cell-selective variations in N-methyl-D-aspartate receptor degradation by calpain. *J. Neurochem.* 99, 206–217. [PubMed: 16899064]
- Farrant M, and Nusser Z (2005). Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. *Nat. Rev. Neurosci.* 6, 215–229. [PubMed: 15738957]
- Gaňarsa JL (2004). Plasticity of GABAergic synapses in the neonatal rat hippocampus. *J. Cell. Mol. Med.* 8, 31–37. [PubMed: 15090258]
- Gambrill AC, and Barria A (2011). NMDA receptor subunit composition controls synaptogenesis and synapse stabilization. *Proc. Natl. Acad. Sci. USA* 108, 5855–5860. [PubMed: 21427228]

- Ge Y, Kang Y, Cassidy RM, Moon KM, Lewis R, Wong ROL, Foster LJ, and Craig AM (2018). C1ptm1 Limits Forward Trafficking of GABA<sub>A</sub> Receptors to Scale Inhibitory Synaptic Strength. *Neuron* 97, 596–610.e8. [PubMed: 29395912]
- Glykys J, Mann EO, and Mody I (2008). Which GABA(A) receptor subunits are necessary for tonic inhibition in the hippocampus? *J. Neurosci.* 28, 1421–1426. [PubMed: 18256262]
- Gonda S, Giesen J, Sieberath A, West F, Buchholz R, Klatt O, Ziebarth T, Räk A, Kleinhubbert S, Riedel C, et al. (2020). GluN2B but Not GluN2A for Basal Dendritic Growth of Cortical Pyramidal Neurons. *Front. Neuroanat.* 14, 571351. [PubMed: 33281565]
- Gordillo-Salas M, Pilar-Cuéllar F, Auberson YP, and Adell A (2018). Signaling pathways responsible for the rapid antidepressant-like effects of a GluN2A-preferring NMDA receptor antagonist. *Transl. Psychiatry* 8, 84. [PubMed: 29666360]
- GORLEWICZ A, PIJET B, ORLOVA K, KACZMAREK L, and KNAPSKA E (2021). Epileptiform GluN2B-driven excitation in hippocampus as a therapeutic target against temporal lobe epilepsy. *bioRxiv*. 10.1101/2021.06.30.450508.
- Gu X, and Lu W (2018). Genetic deletion of NMDA receptors suppresses GABAergic synaptic transmission in two distinct types of central neurons. *Neurosci. Lett.* 668, 147–153. [PubMed: 29355693]
- Gu X, Zhou L, and Lu W (2016). An NMDA Receptor-Dependent Mechanism Underlies Inhibitory Synapse Development. *Cell Rep.* 14, 471–478. [PubMed: 26774487]
- Gundersen V, Holten AT, and Storm-Mathisen J (2004). GABAergic synapses in hippocampus exocytose aspartate on to NMDA receptors: quantitative immunogold evidence for co-transmission. *Mol. Cell. Neurosci.* 26, 156–165. [PubMed: 15121187]
- Han W, Shepard RD, and Lu W (2021). Regulation of GABA<sub>A</sub>Rs by Transmembrane Accessory Proteins. *Trends Neurosci.* 44, 152–165. [PubMed: 33234346]
- Hausrat TJ, Muhia M, Gerrow K, Thomas P, Hirdes W, Tsukita S, Heisler FF, Herich L, Dubroqua S, Breiden P, et al. (2015). Radixin regulates synaptic GABA<sub>A</sub> receptor density and is essential for reversal learning and short-term memory. *Nat. Commun.* 6, 6872. [PubMed: 25891999]
- Henneberger C, Jüttner R, Schmidt SA, Walter J, Meier JC, Rothe T, and Grantyn R (2005). GluR- and TrkB-mediated maturation of GABA receptor function during the period of eye opening. *Eur. J. Neurosci.* 21, 431–440. [PubMed: 15673442]
- Hines RM, Davies PA, Moss SJ, and Maguire J (2012). Functional regulation of GABA<sub>A</sub> receptors in nervous system pathologies. *Curr. Opin. Neurobiol.* 22, 552–558. [PubMed: 22036769]
- Holter NI, Zylla MM, Zuber N, Bruehl C, and Draguhn A (2010). Tonic GABAergic control of mouse dentate granule cells during postnatal development. *Eur. J. Neurosci.* 32, 1300–1309. [PubMed: 20846322]
- Horn ME, and Nicoll RA (2018). Somatostatin and parvalbumin inhibitory synapses onto hippocampal pyramidal neurons are regulated by distinct mechanisms. *Proc. Natl. Acad. Sci. USA* 115, 589–594. [PubMed: 29295931]
- Houser CR, and Esclapez M (2003). Downregulation of the alpha5 subunit of the GABA(A) receptor in the pilocarpine model of temporal lobe epilepsy. *Hippocampus* 13, 633–645. [PubMed: 12921352]
- Jacob TC, Moss SJ, and Jurd R (2008). GABA(A) receptor trafficking and its role in the dynamic modulation of neuronal inhibition. *Nat. Rev. Neurosci.* 9, 331–343. [PubMed: 18382465]
- Jaenisch N, Liebmann L, Guenther M, Hübner CA, Frahm C, and Witte OW (2016). Reduced tonic inhibition after stroke promotes motor performance and epileptic seizures. *Sci. Rep.* 6, 26173. [PubMed: 27188341]
- Lee V, and Maguire J (2014). The impact of tonic GABA<sub>A</sub> receptor-mediated inhibition on neuronal excitability varies across brain region and cell type. *Front. Neural Circuits* 8, 3. [PubMed: 24550784]
- Li J, Han W, Pelkey KA, Duan J, Mao X, Wang YX, Craig MT, Dong L, Petralia RS, McBain CJ, and Lu W (2017). Molecular Dissection of Neuroigin 2 and Slitrk3 Reveals an Essential Framework for GABAergic Synapse Development. *Neuron* 96, 808–826.e8. [PubMed: 29107521]

- Liu Y, Wong TP, Aarts M, Rooyakkers A, Liu L, Lai TW, Wu DC, Lu J, Tymianski M, Craig AM, and Wang YT (2007). NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. *J. Neurosci.* 27, 2846–2857. [PubMed: 17360906]
- Loebrich S, Bähring R, Katsuno T, Tsukita S, and Kneussel M (2006). Activated radixin is essential for GABAA receptor alpha5 subunit anchoring at the actin cytoskeleton. *EMBO J.* 25, 987–999. [PubMed: 16467845]
- Luscher B, Fuchs T, and Kilpatrick CL (2011). GABAA receptor trafficking-mediated plasticity of inhibitory synapses. *Neuron* 70, 385–409. [PubMed: 21555068]
- Mareš P, Kozlová L, Mikulecká A, and Kubová H (2021). The GluN2B-Selective Antagonist Ro 25–6981 Is Effective against PTZ-Induced Seizures and Safe for Further Development in Infantile Rats. *Pharmaceutics* 13, 1482. [PubMed: 34575558]
- Marsden KC, Beattie JB, Friedenthal J, and Carroll RC (2007). NMDA receptor activation potentiates inhibitory transmission through GABA receptor-associated protein-dependent exocytosis of GABA(A) receptors. *J. Neurosci.* 27, 14326–14337. [PubMed: 18160640]
- McBain CJ, and Mayer ML (1994). N-methyl-D-aspartic acid receptor structure and function. *Physiol. Rev.* 74, 723–760. [PubMed: 8036251]
- McKay S, Ryan TJ, McQueen J, Indersmitten T, Marwick KFM, Hasel P, Kopanitsa MV, Baxter PS, Martel MA, Kind PC, et al. (2018). The Developmental Shift of NMDA Receptor Composition Proceeds Independently of GluN2 Subunit-Specific GluN2 C-Terminal Sequences. *Cell Rep.* 25, 841–851.e4. [PubMed: 30355491]
- Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, and Seeburg PH (1992). Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science* 256, 1217–1221. [PubMed: 1350383]
- Monyer H, Burnashev N, Laurie DJ, Sakmann B, and Seeburg PH (1994). Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12, 529–540. [PubMed: 7512349]
- Morimoto K, Fahnestock M, and Racine RJ (2004). Kindling and status epilepticus models of epilepsy: rewiring the brain. *Prog. Neurobiol.* 73, 1–60. [PubMed: 15193778]
- Müller L, Tokay T, Porath K, Köhling R, and Kirschstein T (2013). Enhanced NMDA receptor-dependent LTP in the epileptic CA1 area via upregulation of NR2B. *Neurobiol. Dis.* 54, 183–193. [PubMed: 23313317]
- Naylor DE, Liu H, and Wasterlain CG (2005). Trafficking of GABA(A) receptors, loss of inhibition, and a mechanism for pharmacoresistance in status epilepticus. *J. Neurosci.* 25, 7724–7733. [PubMed: 16120773]
- Naylor DE, Liu H, Niquet J, and Wasterlain CG (2013). Rapid surface accumulation of NMDA receptors increases glutamatergic excitation during status epilepticus. *Neurobiol. Dis.* 54, 225–238. [PubMed: 23313318]
- Pan G, Chen Z, Zheng H, Zhang Y, Xu H, Bu G, Zheng H, and Li Y (2018). Compensatory Mechanisms Modulate the Neuronal Excitability in a Kainic Acid-Induced Epilepsy Mouse Model. *Front. Neural Circuits* 12, 48. [PubMed: 30008664]
- Paoletti P, Bellone C, and Zhou Q (2013). NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nat. Rev. Neurosci.* 14, 383–400. [PubMed: 23686171]
- Peng YR, Zeng SY, Song HL, Li MY, Yamada MK, and Yu X (2010). Postsynaptic spiking homeostatically induces cell-autonomous regulation of inhibitory inputs via retrograde signaling. *J. Neurosci.* 30, 16220–16231. [PubMed: 21123568]
- Penna A, Wang DS, Yu J, Lecker I, Brown PMGE, Bowie D, and Orser BA (2014). Hydrogen peroxide increases GABAA receptor-mediated tonic current in hippocampal neurons. *J. Neurosci.* 34, 10624–10634. [PubMed: 25100596]
- Petrini EM, Ravasenga T, Hausrat TJ, Iurilli G, Olcese U, Racine V, Sibarita JB, Jacob TC, Moss SJ, Benfenati F, et al. (2014). Synaptic recruitment of gephyrin regulates surface GABAA receptor dynamics for the expression of inhibitory LTP. *Nat. Commun.* 5, 3921. [PubMed: 24894704]
- Racine RJ (1972). Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr. Clin. Neurophysiol.* 32, 281–294. [PubMed: 4110397]



- Rajgor D, Purkey AM, Sanderson JL, Welle TM, Garcia JD, Dell'Acqua ML, and Smith KR (2020). Local miRNA-Dependent Translational Control of GABA<sub>A</sub>R Synthesis during Inhibitory Long-Term Potentiation. *Cell Rep.* 31, 107785. [PubMed: 32579917]
- Raybuck JD, Hargus NJ, and Thayer SA (2017). A GluN2B-Selective NMDAR Antagonist Reverses Synapse Loss and Cognitive Impairment Produced by the HIV-1 Protein Tat. *J. Neurosci.* 37, 7837–7847. [PubMed: 28716964]
- Saliba RS, Kretschmannova K, and Moss SJ (2012). Activity-dependent phosphorylation of GABA<sub>A</sub> receptors regulates receptor insertion and tonic current. *EMBO J.* 31, 2937–2951. [PubMed: 22531784]
- Samba Reddy D (2017). Sex differences in the anticonvulsant activity of neurosteroids. *J. Neurosci. Res.* 95, 661–670. [PubMed: 27870400]
- Sepulveda FJ, Bustos FJ, Inostroza E, Zúñiga FA, Neve RL, Montecino M, and van Zundert B (2010). Differential roles of NMDA Receptor Subtypes NR2A and NR2B in dendritic branch development and requirement of RasGRF1. *J. Neurophysiol.* 103, 1758–1770. [PubMed: 20107120]
- Sheng M, Cummings J, Roldan LA, Jan YN, and Jan LY (1994). Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature* 368, 144–147. [PubMed: 8139656]
- Shipton OA, and Paulsen O (2013). GluN2A and GluN2B subunit-containing NMDA receptors in hippocampal plasticity. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 369, 20130163. [PubMed: 24298164]
- Sibarov DA, Abushik PA, Giniatullin R, and Antonov SM (2016). GluN2A Subunit-Containing NMDA Receptors Are the Preferential Neuronal Targets of Homocysteine. *Front. Cell. Neurosci.* 10, 246. [PubMed: 27847466]
- Strack S, McNeill RB, and Colbran RJ (2000). Mechanism and regulation of calcium/calmodulin-dependent protein kinase II targeting to the NR2B subunit of the N-methyl-D-aspartate receptor. *J. Biol. Chem.* 275, 23798–23806. [PubMed: 10764765]
- Sun Y, Xu Y, Cheng X, Chen X, Xie Y, Zhang L, Wang L, Hu J, and Gao Z (2018). The differences between GluN2A and GluN2B signaling in the brain. *J. Neurosci. Res.* 96, 1430–1443. [PubMed: 29682799]
- Szabadits E, Cserép C, Szonyi A, Fukazawa Y, Shigemoto R, Watanabe M, Itoharu S, Freund TF, and Nyiri G (2011). NMDA receptors in hippocampal GABAergic synapses and their role in nitric oxide signaling. *J. Neurosci.* 31, 5893–5904. [PubMed: 21508214]
- Tao W, Higgs MH, Spain WJ, and Ransom CB (2013). Postsynaptic GABAB receptors enhance extrasynaptic GABA<sub>A</sub> receptor function in dentate gyrus granule cells. *J. Neurosci.* 33, 3738–3743. [PubMed: 23447585]
- Turrigiano G (2012). Homeostatic synaptic plasticity: local and global mechanisms for stabilizing neuronal function. *Cold Spring Harb. Perspect. Biol.* 4, a005736. [PubMed: 22086977]
- Vieira M, Yong XLH, Roche KW, and Anggono V (2020). Regulation of NMDA glutamate receptor functions by the GluN2 subunits. *J. Neurochem.* 154, 121–143. [PubMed: 31978252]
- Vincent P, and Mulle C (2009). Kainate receptors in epilepsy and excitotoxicity. *Neuroscience* 158, 309–323. [PubMed: 18400404]
- Vithlani M, Terunuma M, and Moss SJ (2011). The dynamic modulation of GABA(A) receptor trafficking and its role in regulating the plasticity of inhibitory synapses. *Physiol. Rev.* 91, 1009–1022. [PubMed: 21742794]
- Wang X, He X, Li T, Shu Y, Qi S, and Luan G (2017). Anti-epileptic effect of ifenprodil on neocortical pyramidal neurons in patients with malformations of cortical development. *Exp. Ther. Med.* 14, 5757–5766. [PubMed: 29285118]
- Waxman EA, and Lynch DR (2005). N-methyl-D-aspartate receptor subtypes: multiple roles in excitotoxicity and neurological disease. *Neuroscientist* 11, 37–49. [PubMed: 15632277]
- Wu QJ, and Tymianski M (2018). Targeting NMDA receptors in stroke: new hope in neuroprotection. *Mol. Brain* 11, 15. [PubMed: 29534733]
- Wu K, Han W, Tian Q, Li Y, and Lu W (2021). Activity- and sleep-dependent regulation of tonic inhibition by Shisa7. *Cell Rep.* 34, 108899. [PubMed: 33761345]

- Wyroślak M, Lebida K, and Mozrzymas JW (2021). Induction of Inhibitory Synaptic Plasticity Enhances Tonic Current by Increasing the Content of  $\alpha 5$ -Subunit Containing GABA<sub>A</sub> Receptors in Hippocampal Pyramidal Neurons. *Neuroscience* 467, 39–46. [PubMed: 34033868]
- Zou G, Chen Q, Chen K, Zuo X, Ge Y, Hou Y, Pan T, Pan H, Liu D, Zhang L, and Xiong W (2019). Human Hyperekplexic Mutations in Glycine Receptors Disinhibit the Brainstem by Hijacking GABA<sub>A</sub> Receptors. *iScience* 19, 634–646. [PubMed: 31450193]

Author Manuscript

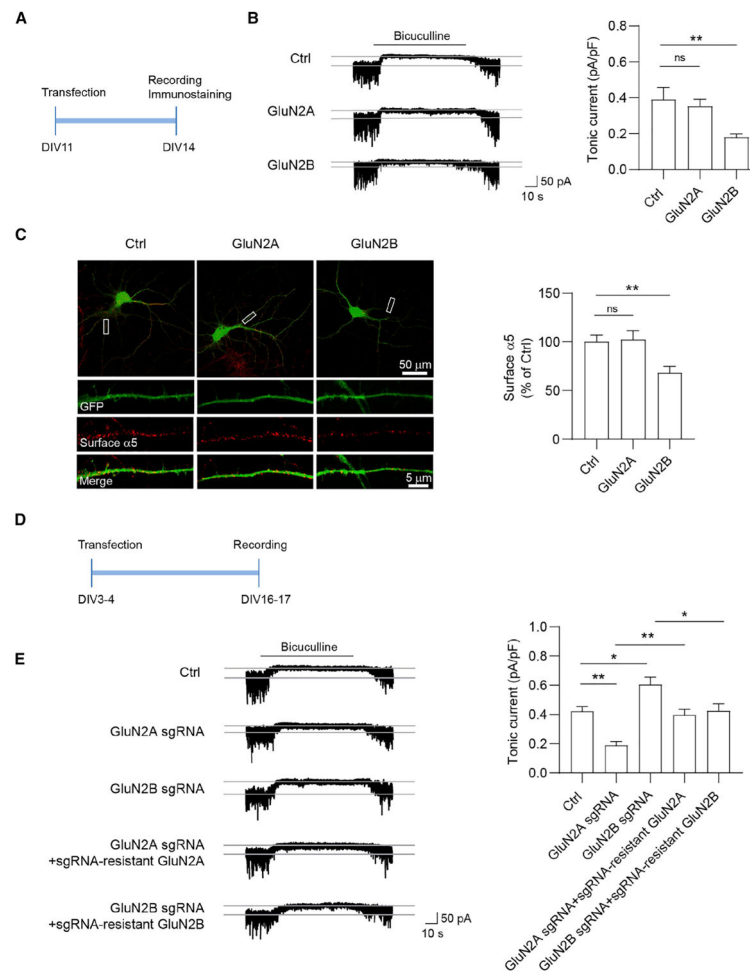
Author Manuscript

Author Manuscript

Author Manuscript

**Highlights**

- GluN2A- and GluN2B-NMDA receptors differentially regulate tonic inhibition
- NMDA receptor subunit-specific modulation of  $\alpha 5$ -GABA<sub>A</sub>R trafficking
- GluN2A-NMDARs are critical for homeostatic plasticity of tonic inhibition
- Regulation of tonic inhibition by NMDA receptors in a kainate-induced seizure model



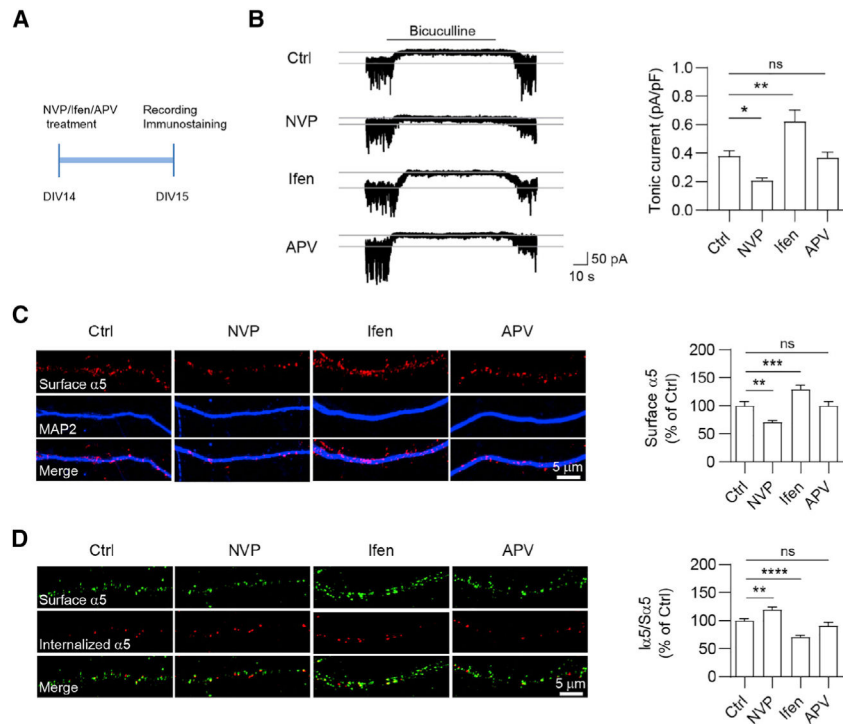
\*p < 0.05 and \*\*p < 0.01. All data are presented as mean ± SEM.  
See also Figure S1.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



**Figure 2. Pharmacological suppression of GluN2A- and GluN2B-NMDARs regulates tonic inhibition and  $\alpha 5$ -GABA<sub>A</sub>R internalization**

(A) Experimental design. Hippocampal neurons at DIV14 were treated with NVP (GluN2A-preferring antagonist NVP-AAM077, 100 nM), Ifen (GluN2B-preferring antagonist ifenprodil, 5  $\mu$ M), or APV (broad-spectrum NMDAR antagonist, 100  $\mu$ M) for 24 h and then recorded for tonic currents at DIV15.

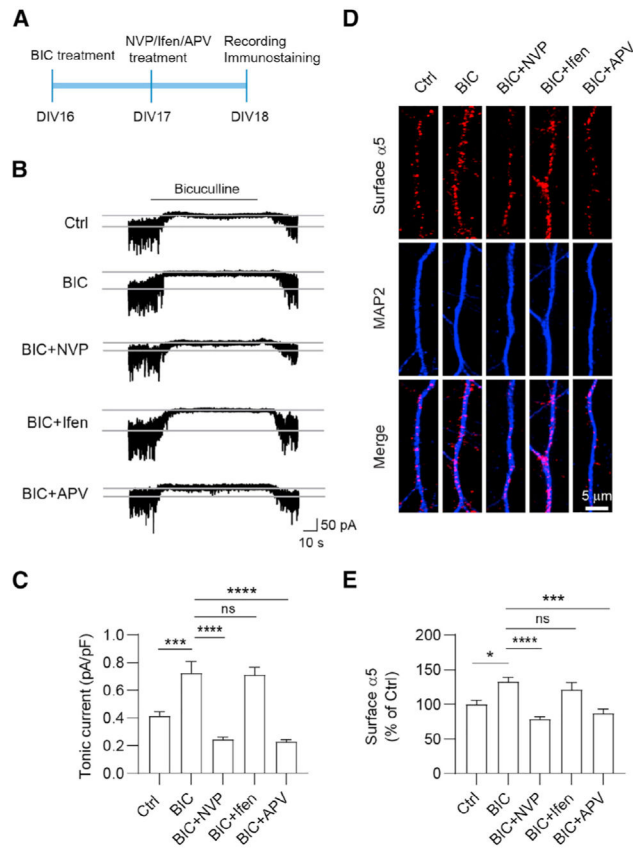
(B) Ifen treatment increased tonic currents, whereas NVP treatment decreased tonic currents.  $n = 10$ – $11$  for each group, one-way ANOVA test,  $F_{(3,38)} = 13.14$ ,  $p < 0.0001$  with Dunnett's multiple comparisons test, Ctrl versus NVP,  $p = 0.031$ ; Ctrl versus Ifen,  $p = 0.0023$ .

(C) Ifen treatment increased surface  $\alpha 5$  expression, whereas NVP treatment decreased surface  $\alpha 5$  expression.  $n = 33$ – $43$  for each group, Kruskal-Wallis test with Dunnett's multiple comparisons test, Ctrl versus NVP,  $p = 0.0041$ ; Ctrl versus Ifen,  $p = 0.0003$ .

(D) Endocytosis assay of  $\alpha 5$ -GABA<sub>A</sub>Rs in cultures. Surface  $\alpha 5$ -GABA<sub>A</sub>Rs (S $\alpha 5$ ) were labeled in green, and internalized  $\alpha 5$ -GABA<sub>A</sub>Rs (I $\alpha 5$ ) were in red. Bar graphs in the right showing that 24-h NVP treatment increased  $\alpha 5$  internalization, whereas 24-h Ifen treatment decreased  $\alpha 5$  internalization.  $n = 15$ – $16$  for each group, one-way ANOVA test,  $F_{(3,56)} = 22.96$ ,  $p < 0.0001$  with Dunnett's multiple comparisons test, Ctrl versus NVP:  $p = 0.0044$ ; Ctrl versus Ifen:  $p < 0.0001$ .

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ . All data are presented as mean  $\pm$  SEM.

See also Figures S2 and S3.



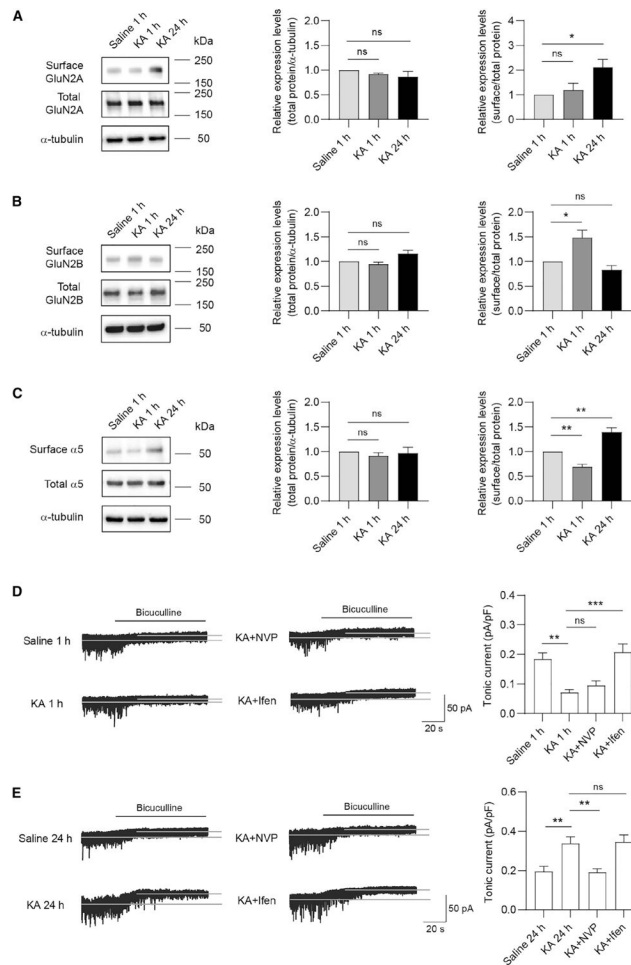
**Figure 3. GluN2A-NMDARs are required for homeostatic potentiation of tonic inhibition**

(A) Experimental design. Hippocampal neurons at DIV16 were treated with bicuculline (BIC, 40  $\mu$ M), and at DIV17 they were treated with NVP (100 nM), Ifen (5  $\mu$ M), or APV (100  $\mu$ M) for 24 h before recording.

(B and C) NVP and APV treatment abolished BIC-induced potentiation of tonic currents.  $n = 10$  for each group, one-way ANOVA test,  $F_{(4,45)} = 25.32$ ,  $p < 0.0001$  with Tukey's multiple comparison test, Ctrl versus BIC:  $p = 0.0004$ ; BIC versus BIC+NVP:  $p < 0.0001$ ; BIC versus BIC+APV:  $p < 0.0001$ .

(D and E) NVP and APV treatment abolished BIC-induced potentiation of surface  $\alpha 5$  expression.  $n = 20$ – $26$  for each group, Kruskal-Wallis test with Dunn's multiple comparisons test, Ctrl versus BIC:  $p = 0.0189$ ; BIC versus BIC+NVP:  $p < 0.0001$ ; BIC versus BIC+APV:  $p = 0.0002$ .

\* $p < 0.05$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ . All data are presented as mean  $\pm$  SEM.



**Figure 4. Pharmacological suppression of GluN2A- and GluN2B-NMDARs regulates tonic inhibition in the KA-induced seizure model**

(A–C) Representative western blots and summary graphs from cell-surface biotinylation assays showing that surface and total GluN2A (A,  $n = 3$  independent experiments, one-way ANOVA test,  $F_{(2,6)} = 5.928$ ,  $p = 0.0379$  with Dunnett's multiple comparisons test,  $p = 0.0321$ ), GluN2B (B,  $n = 3$  independent experiments, one-way ANOVA test,  $F_{(2,6)} = 10.80$ ,  $p = 0.0103$  with Dunnett's multiple comparisons test,  $p = 0.0283$ ), and  $\alpha 5$ -GABA<sub>A</sub>R (C,  $n = 4$  independent experiments, one-way ANOVA test,  $F_{(2,9)} = 34.75$ ,  $p < 0.0001$  with Dunnett's multiple comparisons test, saline 1 h versus KA 1 h:  $p = 0.0098$ ; saline 1 h versus KA 24 h:  $p = 0.0022$ ) expression in the KA-induced seizure model.

(D and E) Tonic currents in hippocampal CA3 neurons were decreased 1 h after KA injection, whereas increased 24 h after KA injection. Ifen or NVP treatment 1 h prior to KA injection, respectively, restored the decreased or increased tonic currents at corresponding time point after KA injection. (D)  $n = 10$  for each group, one-way ANOVA test,  $F_{(3,36)} = 11.42$ ,  $p < 0.0001$  with Dunnett's multiple comparisons test, saline 1 h versus KA 1 h:  $p = 0.0014$ ; KA 1 h versus KA + Ifen:  $p = 0.0001$ . (E)  $n = 10$  for each group, one-way ANOVA test,  $F_{(3,36)} = 8.925$ ,  $p = 0.0001$  with Dunnett's multiple comparisons test, saline 24 h versus KA 24 h:  $p = 0.0065$ ; KA 24 h versus KA+NVP:  $p = 0.0047$ .

\* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ . All data are presented as mean  $\pm$  SEM.



See also Figure S4.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Chicken Polyclonal Anti-GFP	Abcam	Cat# ab13970, RRID: AB_300798
Rabbit Polyclonal Anti-Glutamate Receptor NMDAR2A (GluN2A)	Sigma-Aldrich	Cat# M264, RRID: AB_260485
Rabbit Polyclonal Anti-Glutamate Receptor NMDAR2B (GluN2B)	Sigma-Aldrich	Cat# M265, RRID: AB_260487
Rabbit Polyclonal Anti-Phospho-Ezrin (Thr567)/Radixin(Thr564)/Moesin (Thr558) (p-ERM)	Cell Signaling Technology	Cat# 3141, RRID: AB_330232
Rabbit Polyclonal Anti-Ezrin/Radixin/Moesin (ERM)	Cell Signaling Technology	Cat# 3142, RRID: AB_2100313
Rabbit Polyclonal Anti-GABA(A) $\alpha$ 5 Receptor	Synaptic Systems	Cat# 224503, RRID: AB_2619944
Rabbit Polyclonal Anti-GABA(A) $\alpha$ 1 Receptor	Millipore	Cat# 06-868, RRID: AB_310272
Chemicals, peptides, and recombinant proteins		
NeuroMag reagent	Oz Biosciences	Cat# NM51000
CalPho Mammalian Transfection Kit	Takara	Cat# 631312
Bicuculline	Abcam	Cat# ab120110
D-APV	Abcam	Cat# ab120003
DNQX	Alomone labs	Cat# D-131
Tetrodotoxin (TTX)	Alomone Labs	Cat# T-550
Picrotoxin	Sigma-Aldrich	Cat# P1675
NVP-AAM077 Tetrasodium Hydrate	Sigma-Aldrich	Cat# 5.04528
Ifenprodil (+)-tartrate salt	Sigma-Aldrich	Cat# I2892
Kainic acid	Abcam	Cat# ab120100
4,5,6,7-tetrahydroisoxazolo(5,4-c) pyridin-3-ol (THIP)	Santa Cruz	Cat# SC204342
Q5 Site-Directed Mutagenesis Kit	NEB	Cat# E0554S
EZ-Link Sulfo-NHS-SS-Biotin	Thermo Fisher Scientific	Cat# 21331
Pierce Glutathione Agarose	Thermo Fisher Scientific	Cat# 16101
Experimental models: cell lines		
Primary cultures of hippocampal neurons	This paper	N/A
HEK293T	ATCC	Cat# CRL-1126
Experimental models: organisms/strains		
C57BL/6N mice	Charles River	Cat# 027
Oligonucleotides		
sgRNA targeting sequence: mouse GluN2A: CGACGTGACAGAACGCGAAC	This paper	N/A
sgRNA targeting sequence: mouse GluN2B: GTCTGACCGAAGATCCAGG	This paper	N/A
Recombinant DNA		
pRK5-GFP-GluN2A	Dr. Katherine Roche (NIH)	N/A

<b>REAGENT or RESOURCE</b>	<b>SOURCE</b>	<b>IDENTIFIER</b>
pRK5-GFP-GluN2B	Dr. Katherine Roche (NIH)	N/A
pSpCas9(BB)-2A-Puro (PX459) V2.0	Addgene	Cat# 62988
pSpCas9(BB)-2A-GFP (PX458)	Addgene	Cat# 48138
GluN2A sgRNA	This paper	N/A
GluN2B sgRNA	This paper	N/A
sgRNA-resistant GluN2A	This paper	N/A
sgRNA-resistant GluN2B	This paper	N/A
Software and algorithms		
ImageJ	NIH	<a href="https://imagej.nih.gov/ij/">https://imagej.nih.gov/ij/</a>
GraphPad Prism 8.0	GraphPad	<a href="https://www.graphpad.com">https://www.graphpad.com</a>
Igor Pro	Wavemetrics	<a href="https://www.wavemetrics.com">https://www.wavemetrics.com</a>