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Neto auxiliary subunits regulate interneuron somatodendritic and presynaptic kainate receptors to control network inhibition

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Abstract

Although Netos are considered auxiliary subunits critical for kainate receptor (KAR) function, direct evidence for their regulation of native KARs is limited. As Neto KAR regulation is GluK subunit/Neto isoform specific, such regulation must be determined in cell-type specific contexts. We demonstrate Neto1/2 expression in somatostatin- (SOM), cholecystokinin/cannabinoid receptor 1- (CCK/CB1), and parvalbumin- (PV) containing interneurons. KAR-mediated excitation of these interneurons is contingent upon Neto1 as kainate yields comparable effects in Neto2 knockouts and wildtypes, but fails to excite interneurons or recruit inhibition in Neto1 knockouts. In contrast, presynaptic KARs in CCK/CB1 interneurons are dually regulated by both Neto1 and Neto2. Neto association promotes tonic presynaptic KAR activation dampening CCK/CB1 interneuron output and loss of this brake in Neto mutants profoundly increases CCK/CB1 interneuron-mediated inhibition. Our results confirm that Neto1 regulates endogenous somatodendritic KARs in diverse interneurons and demonstrate Neto regulation of presynaptic KARs in mature inhibitory presynaptic terminals.

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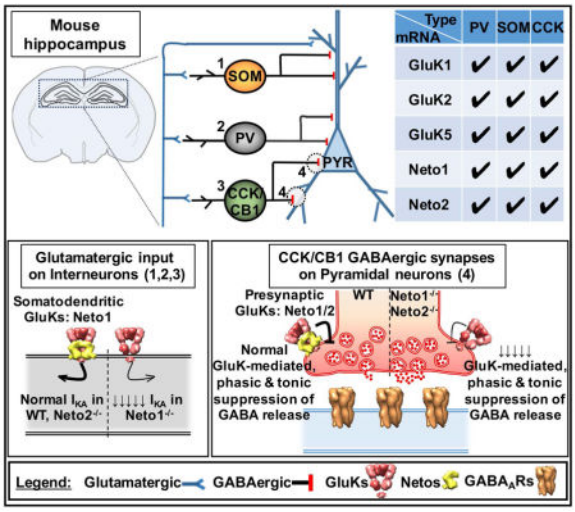
Graphical Abstract



INTRODUCTION

KARs typically serve as modulators of synaptic transmission and neuronal excitability in diverse central circuits, functionally distinguishing them from AMPA/NMDA receptors that dominate rapid excitatory transmission throughout the central nervous system (Contractor et al., 2011). Even in circuits with relatively abundant synaptic KARs, such as the hippocampal mossy fiber pathway, they typically contribute to use-dependent plasticity with ongoing phasic transmission primarily mediated by AMPA/NMDA receptors. This modulatory role makes KARs attractive therapeutic candidates as the receptors can be targeted for rapid and potent control of circuit excitability with minimal direct interference of ongoing synaptic communication and computation (Contractor et al., 2011; Jane et al., 2009).

KARs comprise tetrameric assemblies from combinations of five pore-forming subunits (GluK1-5) with the stipulation that GluK4-5 require co-assembly with GluK1-3 (Lerma and Marques, 2013). Though each subunit offers a potential therapeutic substrate, strategies focused on ligand-gated channels, particularly ones sharing an endogenous ligand such as glutamate receptors, may benefit by targeting auxiliary subunits. Recently, Neto1/Neto2 have emerged as auxiliary KAR subunits capable of regulating almost every parameter of receptor function (Copits and Swanson, 2012; Howe, 2015). Overexpression studies in heterologous cells or neurons have demonstrated that Netos regulate KAR desensitization and deactivation kinetics, channel open probability, ligand affinity, ion permeation, and subcellular localization (Brown et al., 2016; Copits et al., 2011; Fisher, 2015; Fisher and Mott, 2012, 2013; Griffith and Swanson, 2015; Orav et al., 2017; Palacios-Filardo et al., 2016; Zhang et al., 2014; Zhang et al., 2009). Consistent with these findings, studies at hippocampal mossy fiber to CA3 pyramidal cell (MF-CA3) synapses indicate that Neto1 regulates binding affinity, kinetics, and synaptic targeting of native GluK2/3-containing postsynaptic KARs (Straub et al., 2011a; Tang et al., 2011; Wyeth et al., 2014). However, direct evidence for Neto2 regulation of endogenous KAR function in central neurons remains lacking despite association with native cortical, hippocampal, and cerebellar KAR

complexes (Zhang et al., 2009; Straub et al., 2011a; Tang et al., 2011). Similarly, despite a wealth of overexpression data supporting Neto1/2 regulation of GluK1-containing KARs, direct evidence for endogenous Neto association with and regulation of native GluK1-containing KARs in neurons is limited. Recently, Neto1 was found to regulate tonic suppression of transmission at neonatal CA3 to CA1 pyramidal synapses by presynaptic GluK1 (Orav et al., 2017) while Neto2 was confirmed as an auxiliary subunit of native GluK1-containing KARs in peripheral sensory neurons (Vernon and Swanson, 2017) raising the possibility that cell type in combination with KAR subunit composition dictates Neto isoform affiliation.

Importantly, Neto-mediated regulation of recombinant KARs can exhibit GluK subunit and Neto isoform specificity (Copits et al., 2011; Fisher, 2015). Thus, as Neto1/2 and GluK1-5 display discrete expression profiles throughout the CNS it is critical to consider network and cell-type specificity in Neto regulation of native KARs. Despite prominent KAR expression within hippocampal pyramidal cells the dominant feature of network-wide KAR activation is a massive increase in inhibitory tone through recruitment of local circuit interneurons that are exquisitely sensitive to kainate (Christensen et al., 2004; Cossart et al., 1998; Cossart et al., 2001; Fisahn et al., 2004; Frerking et al., 1999; Jiang et al., 2001; Maingret et al., 2005; Mulle et al., 2000; Semyanov and Kullmann, 2001; Wondolowski and Frerking, 2009). In addition KARs on GABAergic terminals, particularly those of CCK/CB1 expressing interneurons, regulate presynaptic release (Christensen et al., 2004; Clarke et al., 1997; Daw et al., 2010; Lourenco et al., 2010; Mulle et al., 2000; Rodriguez-Moreno et al., 1997). Based on these observations interneuronal KARs have been proposed as key substrates to target for control of circuit excitability in disorders involving imbalanced excitation and inhibition (Christensen et al., 2004; Frerking and Nicoll, 2000; Khalilov et al., 2002).

Though Straub and colleagues (2011a) noted prominent Neto1 expression in hippocampal interneurons and observed reduced kainate-induced currents in unidentified interneurons of Neto1 knockouts, studies focused on Neto expression and KAR regulation in specific interneuron subpopulations are currently lacking. Using combined in situ hybridization (ISH), immunohistochemical (IHC), and genetic reporting strategies we localize Neto1/2 in combination with GluK1/2/5 in SOM, CCK/CB1, and PV-expressing subsets of hippocampal interneurons. Moreover, we demonstrate that Neto1, but not Neto2, regulates KAR currents in SOM, CCK/CB1, and PV interneurons as well as recruitment of inhibitory drive onto pyramidal cells.

Finally, we provide evidence that presynaptic KARs on CCK/CB1 interneurons are regulated by both Neto1 and Neto2, with presynaptic KAR function requiring Neto1 and Neto2 modulating KAR agonist efficacy/affinity.

RESULTS

Neto and KAR expression in interneurons

Inhibitory transmission in the hippocampus is mediated by a remarkably varied population of GABAergic interneurons. Though rigorous classification requires knowledge of developmental origins, neurochemical content, morphology and electrophysiological

properties, discrete subsets of interneurons can be broadly parsed by relatively specific molecular markers. In particular SOM, CCK/CB1, and PV expressing interneuron cohorts represent largely non-overlapping interneuron populations that have been extensively characterized and together encompass the majority of hippocampal interneurons (Klausberger and Somogyi, 2008; Tricoire et al., 2011). Importantly, each of these three classes has been demonstrated to express functional KARs (Cossart et al., 1998; Daw et al., 2010; Goldin et al., 2007). As antibodies capable of providing individual cellular resolution for Neto IHC are currently unavailable we first probed for Neto1/2 mRNA in SOM, CCK/CB1, and PV mRNA-labeled interneurons in sections from wildtype mice using multi probe fluorescent ISH. In addition, we probed for Grik1,2, and 5 mRNA to confirm prior reports of KAR subunits within diverse interneurons (Bahn et al., 1994; Bureau et al., 1999; Paternain et al., 2000). Signal for each of Neto1, Neto2, Grik1, Grik2, and Grik5 mRNA species was regularly observed in interneurons containing SOM, CB1, and PV mRNA signals, confirming robust transcription of both *Neto1* and *Neto2* genes in combination with those encoding KAR subunits in these interneuron populations (Fig. 1A–D and see Fig. S1). Interestingly, both Neto1 and Neto2 mRNA signals were frequently encountered within individual interneurons indicating potential redundant functionality, or more intriguingly, unique contributions from Neto1 and Neto2 to KAR regulation within the same interneuron (e.g. Fig. 1A,B).

In Neto1 knockout (Neto1KO) mice the *Neto1* gene is replaced with the *β-galactosidase (β-Gal)* gene (Ng et al., 2009). Thus, to confirm expression in hippocampal interneurons we examined endogenous *Neto1* promoter driven β-Gal expression in Neto1 homozygous knockout or heterozygous mice using IHC. Consistent with our ISH we observed localization of β-Gal within SOM, CCK/CB1, and PV-expressing interneurons (Fig. 2A–F). These results were verified by crossing Neto1KOs to interneuron reporter mouse lines and confirm β-Gal expression in the vast majority of medial ganglionic eminence-derived interneurons (*Nkx2.1Cre*:RCE/Neto1$^{+/-}$ mice) including SOM and PV cohorts as well as caudal ganglionic eminence-derived interneurons (*Htr3a*-GFP/Neto1$^{+/-}$ mice) including CCK/CB1 populations (Fig. S2). In addition we confirmed KAR expression in all three interneuron populations as GluK2/3 staining colocalized with SOM, CCK, and PV labeled hippocampal cells of wildtype rodents (Fig. 2G–I). Together our combined ISH an IHC findings reveal robust expression of both Neto isoforms in combination with KAR subunits in SOM, CCK/CB1, and PV interneurons.

Neto1 is required for kainate-induced excitation of interneurons and consequent recruitment of circuit inhibition

Previous work has demonstrated direct KAR-mediated excitation of hippocampal interneurons exhibiting features consistent with SOM oriens interneurons, PV fast-spiking cells, and CCK/CB1 radiatum interneurons (Cossart et al., 1998; Wondolowski and Frerking, 2009; Bureau et al., 1999; Frerking and Nicoll, 1999; Mulle et al., 2000). Having established the expression of Neto1 and 2 in each of these populations we investigated whether their KAR-mediated responses are altered in Neto knockout mice (Fig. 3). We performed whole-cell voltage-clamp recordings from putative SOM, CCK/CB1, and PV interneurons in acute hippocampal slices to record pharmacologically isolated KAR-mediated currents. Recorded

cells were grouped into SOM, CCK/CB1, and PV subsets based primarily on post-hoc morphological evaluation, but additionally using IHC (for SOM), and in some cases spiking properties (particularly for PV fast-spiking interneurons) (Fig. 3A–C). Consistent with previous reports, bath-applied kainate (200 nM) elicited currents across all three interneuron populations in wildtype mice (Fig. 3D,E). However, in slices from Neto1KO mice, as well as Neto nulls, kainate-induced currents were dramatically reduced in all categories (Fig. 3D,E). In Neto2 knockout (Neto2KO) mice kainate yielded currents similar to those in wildtype recordings (Fig. 3D,E). These observations extended to all anatomically distinct subsets of interneurons within each of the three main cohorts (eg. PV and CCK/CB1 perisomatic and dendrite targeting interneurons), and thus, interneurons were not further parsed. Together these findings illustrate a principal role for Neto1, but not Neto2, in regulating somatodendritic KARs expressed by SOM, CCK/CB1, and PV interneurons. Importantly, Neto null mice continued to express Grik1,2, and 5 mRNA transcripts in SOM, CCK/CB1, and PV interneurons at frequencies comparable to wildtypes (Fig. 1D and Fig. S1) suggesting that loss of Neto1 does not interfere with interneuronal KAR subunit transcription. Indeed loss of Netos did not alter total hippocampal mRNA levels of Grik1 which is interneuron specific in mature hippocampus (Fig. S3). Moreover, in Neto1KO mice GluK2/3 immunolabeling continued to decorate the soma and proximal dendrites of neurons outside of stratum pyramidale including SOM-expressing interneurons emphasizing a functional deficit in the absence of Neto1 rather than lack of KAR expression (Fig. 3F,G and Fig. S3).

Network-wide excitation of interneurons by kainate dramatically increases spontaneous inhibitory drive onto hippocampal principal cells (Christensen et al., 2004; Cossart et al., 1998; Cossart et al., 2001; Fisahn et al., 2004; Frerking et al., 1999; Jiang et al., 2001; Maingret et al., 2005; Mulle et al., 2000; Semyanov and Kullmann, 2001; Wondolowski and Frerking, 2009). However, the relative contributions of distinct interneuron cohorts to this phenomenon have not been examined. As presynaptic KARs can depress release, particularly in CCK/CB1 interneurons (Daw et al., 2010), bath-applied kainate may presynaptically suppress the contributions of some interneuron populations despite simultaneously exciting them through somatodendritic KARs. Indeed though CCK/CB1 interneurons were effectively excited by kainate (see above) they contributed little to kainate-induced enhancement of spontaneous inhibitory postsynaptic currents (sIPSCs) recorded in wildtype CA3 pyramidal cells since inhibitory drive remained elevated during selective inhibition of CCK/CB1 interneuron release with the CB1 agonist WIN-55,212-2 (Fig. 4A). In contrast blockade of PV (and perhaps SOM) interneuron release with the P/Q-type calcium channel antagonist agatoxin (Hefft and Jonas, 2005) completely reversed the kainate-induced enhancement of sIPSCs (Fig. 4B). Thus, in wildtype mice sIPSCs recruited by kainate are dominantly contributed by PV (and perhaps SOM) interneurons with little participation from CCK/CB1 interneurons.

We next examined recruitment of sIPSCs in CA3 pyramidal cells of Neto mutants to validate Neto-mediated regulation of interneuronal KARs. In Neto2KO as in wildtype mice kainate produced a robust increase in sIPSC amplitude and frequency (Fig. 4C–E). However, kainate recruitment of sIPSCs was dramatically attenuated in Neto1KO and Neto null mice (Fig 4C–E). Importantly, kainate-driven changes in sIPSCs were confirmed to rely on KARs rather

than indirect circuit effects as kainate failed to recruit inhibition in GluK1/2 double knockout mice (Fig. 4C–E). Our findings from individual interneuron recordings in conjunction with our evaluation of network-wide inhibition overwhelmingly implicate Neto1, but not Neto2, as critical for proper somatodendritic KAR function in diverse hippocampal interneurons.

Neto1 is required for kainate-induced gamma oscillations

In CA3 hippocampus low doses of kainate, acting selectively on KARs, drive network rhythmicity in the gamma frequency range (Fisahn et al., 2004; Hormuzdi et al., 2001). Generation and stability of such gamma oscillations critically depend upon the synaptic interplay of CA3 pyramidal cells and interneurons, particularly PV basket cells (Buzsaki and Wang, 2012). Given the requirement of Neto interactions for proper KAR function in both of these populations we examined kainate-induced gamma oscillations in Neto mutants. In acute hippocampal slices from wildtype and Neto2KO mice kainate consistently triggered robust oscillations of similar magnitude in the gamma frequency range (30–80 Hz, Fig. S4A,B). In contrast, kainate-induced gamma oscillations were severely compromised in Neto1KO mice (Fig. S4A,B). The deficit in Neto1KOs does not reflect developmental circuit alterations that generally prevent the CA3 network from supporting gamma oscillations as carbachol-induced gamma oscillations, which do not rely upon KARs, were comparable in all three genotypes (eg. Fig. S4A; Fisahn et al., 2004). These findings are consistent with the dominant role of Neto1 in regulating interneuron and CA3 pyramidal cell KARs and further highlight the importance of Neto association for KAR-mediated tuning of circuit activity.

CCK/CB1 interneuron output is profoundly influenced by Neto regulation of presynaptic KARs

Presynaptic KARs are well known regulators of transmitter release at diverse synapses throughout the nervous system (Contractor et al., 2011). Though not exhaustively studied, Neto1 and Neto2 knockout mice exhibited normal MF-CA3 short-term presynaptic plasticity (Straub et al., 2011a; Tang et al., 2011) despite considerable evidence for presynaptic KAR involvement in regulating release at this connection (Contractor et al., 2001; Contractor et al., 2000; Lauri et al., 2001; Schmitz et al., 2001). At immature (P4–P6) CA3-CA1 synapses Neto1 but not Neto2 is required for tonic suppression of glutamate release by GluK1-containing receptors (Orav et al., 2017). As outlined above, CCK/CB1 interneurons express presynaptic GluK1-containing KARs capable of depressing GABA release (Daw et al., 2010; Lourenço et al., 2010). Thus, to directly test for Neto regulation of presynaptic KARs we examined kainate modulation of GABAergic transmission in paired recordings between CCK/CB1 interneurons and CA1 pyramidal cells (CCK/CB1-PC). All pairs included for analysis were tested for both asynchronous release and CB1-mediated depolarization-induced suppression of inhibition (DSI) to confirm presynaptic cells as CCK/CB1 interneurons (e.g. Fig. 5A,B; Hefft and Jonas, 2005; Daw et al., 2010). Anatomical recovery of asynchronous releasing and typically DSI-sensitive interneurons yielded morphologies consistent with CCK/CB1 interneurons including perisomatic-targeting and Schaffer collateral-associated subtypes (e.g. Fig. 5A,B). Consistent with prior observations kainate significantly depressed unitary inhibitory postsynaptic currents (uIPSCs) in wildtype mice (Fig. 5C–G). Modest depression was observed in 100 nM kainate with maximal depression elicited at 800 nM (Fig. 5G). Importantly, this depression of CCK/CB1-PC unitary

transmission was associated with an increase in paired pulse ratio (PPR) confirming that kainate depressed presynaptic release rather than altering postsynaptic GABA receptor function (Fig, 5F). In the absence of Neto1, kainate-induced depression of CCK/CB1-PC uIPSCs was severely attenuated even at doses up to 1600 nM (Fig. 5C–G). Interestingly, CCK/CB1-PC pairs in Neto2KO mice were unaffected by kainate at lower doses (200 nM) but exhibited similar kainate sensitivity to wildtypes at higher doses (400nM) (Fig. 5E–G). Overall our data indicate that CCK/CB1 interneuron presynaptic KARs are regulated by both Neto1 and Neto2, with Neto1 being required for presynaptic KAR function and Neto2 modulating agonist efficacy/affinity.

In examining kainate-induced recruitment of sIPSCs onto CA3 pyramidal cells we initially found no evidence for contributions from CCK/CB1 interneurons in wildtype mice despite effective excitation of this interneuron cohort (Figs. 3E and 4A–B). We reasoned this reflects simultaneously engaged presynaptic suppression of GABA release by the exogenous kainate. If this is correct the reduced affinity of CCK/CB1 interneuron presynaptic KARs in the absence of Neto2 predicts that CCK/CB1 interneuron-mediated inhibition will be recruited by kainate (200 nM) in Neto2KO mice. Indeed examination of inhibitory drive in CA3 pyramidal cells of Neto2KO mice revealed significant WIN-55,212-2 sensitivity of kainate-recruited sIPSCs (Fig. 5H).

We additionally noted that overall basal inhibitory tone was significantly greater in Neto2KO compared to wildtype mice (Fig. 5H). This could reflect increased basal circuit participation of CCK/CB1 interneurons due to reduced tonic presynaptic KAR activation. Prior evidence indicates that high affinity GluK1-containing presynaptic KARs can be tonically activated by endogenous glutamate in the basolateral amygdala and developing hippocampus (Braga et al., 2003; Orav et al., 2017). Thus, reduced affinity (Neto2KO) or functional absence (Neto1KO/Neto null) of presynaptic KARs could relieve tonic depression of CCK/CB1 interneuron release in mice lacking Neto1, 2 or both (collectively referred to as Neto mutant mice). To examine this possibility we further analyzed the basal uIPSC properties of wildtype and Neto mutant CCK/CB1-PC pairs. Analysis was limited to basket cell (CCK/CB1BC)-PC pairs to eliminate potential confounding influences of differential targeting by presynaptic terminals from dendrite-targeting CCK/CB1 interneurons and data from all Neto mutants were pooled. Consistent with a loss of basal presynaptic KAR tone, Neto mutants exhibited dramatically increased transmission with significantly larger uIPSC amplitudes, higher release probabilities (as indicated by lower failures, PPR, and CV) and reduced synaptic latencies with less jitter compared to wildtype (Fig 6A–C, and Fig. S5). Importantly, most of the alterations in unitary synaptic properties of CCK/CB1BCs observed in Neto mutants were mimicked by selectively antagonizing KARs (but not AMPARs) in wildtype mice confirming that the changes observed in mutant mice primarily relate to changes in KAR function (Fig. 6B–C and see Fig. S5). We additionally found that CCK/CB1BC-PC synapses in Neto mutants exhibit significantly more asynchronous release during trains of activity in comparison to those in wildtype mice (Fig. 6D–G). This is consistent with the increased release probability observed in Neto mutants as artificially increasing release probability at wildtype CCK/CB1-PC synapses by elevating extracellular Ca^{2+} similarly increases the asynchronous component of transmission (Daw et al., 2009).

Presynaptic KAR-mediated inhibition of CCK/CB1 interneuron release has previously been linked to gating of CB1R function (Lourenço et al., 2010). Thus, increased CCK/CB1 interneuron release observed in Neto mutants or after pharmacological KAR blockade could result from reduced CB1 receptor expression or function. However, we found that DSI of uIPSCs recorded from CCK/CB1BC-PC pairs was not significantly different between mutant and wildtype mice (Fig. 7A–B). Moreover, hippocampal Cnr1 mRNA levels and CB1R expression in CA1 stratum pyramidale were similar in Neto null and wildtype mice (Figs. S1, S3, and S6). Though DSI of CCK/CB1BC-PC unitary connections was similar between wildtype and mutant mice, Neto mutants consistently exhibited DSI of CA1 pyramidal cell sIPSCs while wildtype mice did not, further confirming greater basal CCK/CB1 interneuron inhibitory drive in Neto mutants (Fig. 7A–B). Finally, to probe whether the increased release probability of CCK/CB1 interneurons in Neto mutants translates to increased contribution of CCK/CB1 interneurons in afferent driven recruitment of inhibition we compared feedforward inhibition (FFI) in the Schaffer collateral to CA1 pyramidal cell pathway of wildtype and Neto2KO mice (Fig. 7C–E). The ratio of disynaptically recruited inhibition to monosynaptic excitation was significantly greater in Neto2KO mice resulting in a greater inhibition to excitation (I/E) ratio (Fig. 7C–D). Moreover, the afferent driven inhibition recruited by Schaffer collateral stimulation was significantly more DSI-sensitive in Neto2KOs than in wildtypes consistent with increased contribution of CCK/CB1 interneurons to FFI (Fig. 7C,E). Considered together our findings reveal that Neto proteins profoundly influence inhibitory drive from CCK/CB1 interneurons by regulating tonic activation of presynaptic KARs by endogenous glutamate.

DISCUSSION

As Neto regulation of KARs is GluK subunit and Neto isoform specific it is important to catalog Neto regulation of KARs in cell subtype and even subcellular compartment specific contexts. We investigated Neto expression and regulation of KARs in PV, SOM, and CCK/CB1 hippocampal interneurons. Both cellular and circuit level analyses demonstrated that Neto1 regulates endogenous somatodendritic KARs in all three interneuron populations. Moreover, Netos regulate presynaptic KARs with Neto1 serving an obligate function and Neto2 contributing to receptor affinity on CCK/CB1 interneuron terminals. Our findings further demonstrate that Neto association promotes presynaptic KAR activation by endogenous glutamate to tonically suppress CCK/CB1 interneuron output. Loss of this brake in Neto mutants profoundly increases CCK/CB1 interneuron-mediated inhibition which may have therapeutic relevance to neuropsychatric disorders associated with circuit I/E imbalances.

Our ISH profiling unambiguously localized signal for multiple KAR subunits (Grik1,2,5) and both Neto isoforms within the three interneuron populations. Moreover, combined IHC and genetic reporting strategies confirmed GluK2/3 and Neto1 expression in SOM, CCK/CB1, and PV interneurons. Previous studies using pharmacological and genetic knockout strategies indicate that kainate-induced currents in hippocampal interneurons (i.e. excitation and consequent recruitment of inhibitory drive) reflect activation of mixed populations of GluK1, 2 and 5 subunit-containing somatodendritic KARs (Cossart et al., 1998; Bureau et al., 1999; Mulle et al., 2000; Paternain et al., 2000; Christensen et al., 2004; Fisahn et al.,

2004; Wondolowski and Frerking, 2009). Regulation of interneuron somatodendritic GluK2-dominated KARs by Neto1 is entirely consistent with findings in CA3 pyramidal cells where Neto1 controls somatodendritic/synaptic GluK2/5 heteromeric KARs (Straub et al., 2011a; Tang et al., 2011; Wyeth et al., 2014). Neto1 regulation of native GluK1-containing receptors on interneurons is similarly consistent with clear evidence for Neto1 association with and regulation of recombinant GluK1-containing KARs and GluK1-containing receptors at immature glutamatergic synapses (Copits et al., 2011; Fisher, 2015; Fisher and Mott, 2013; Orav et al., 2017; Palacios-Filardo et al., 2016; Sheng et al., 2015). The KARs regulating CCK/CB1 presynaptic release are dominated by GluK1 (Daw et al., 2010; Bureau et al., 1998; Christensen et al., 2004). Thus, our current findings demonstrating Neto1 regulation of both somatodendritic and presynaptic KARs within diverse hippocampal interneurons provide confirmation that native GluK1-containing KARs are regulated by Neto1 auxiliary subunits.

Despite overwhelming evidence supporting the ability of Neto2 to regulate recombinant GluK1-, GluK2-, and GluK5-containing KARs (Copits et al., 2011; Fisher, 2015; Fisher and Mott, 2012, 2013; Griffith and Swanson, 2015; Palacios-Filardo et al., 2016; Sheng et al., 2015; Sheng et al., 2017; Straub et al., 2011b; Zhang et al., 2009) and native GluK1 containing KARs in developing peripheral neurons (Vernon and Swanson, 2017), physiological relevance for endogenous Neto2 functional regulation of native KARs in central neurons has remained elusive. Indeed prior work failed to detect any defect in CA3 pyramidal cell KAR function in Neto2KO mice and Neto2 was not able to compensate for the dramatic defects observed in Neto1KOs (Tang et al., 2011, Wyeth et al., 2014, Straub et al., 2011a). Here we similarly observed a dominant role for Neto1 in regulating both somatodendritic and presynaptic KARs in SOM, CCK/CB1, and PV interneurons. However, in the absence of Neto2 CCK/CB1 interneuron release was significantly less sensitive to agonist-evoked KAR-mediated inhibition. It is possible that Neto2 regulation of native KARs in central neurons is limited to presynaptic KARs. However, in cerebellum biochemical evidence indicates that Neto2 localizes GluK2 to postsynaptic sites (Tang et al., 2012). Alternatively, Neto2 regulation of somatodendritic, but not presynaptic, KARs may be fully compensated by Neto1 in neurons that express both isoforms.

In recombinant studies Neto association with GluK1 increases glutamate sensitivity by 10–30 fold, greatly reduces macroscopic desensitization in response to submaximal (both Neto1 and 2)/maximal (Neto2) glutamate concentrations, and speeds recovery from desensitization (Fisher, 2015; Palacios-Filardo et al., 2015; Copits and Swanson, 2011; Straub et al., 2011b). Combined these functional changes could promote tonic activation of GluK1 KARs by low ambient glutamate levels (Fisher, 2015). Indeed the glutamate EC_{50} of Neto-associated GluK1 KARs (as low as 4 μM vs 125 μM for GluK1 alone) approaches that of NMDARs (~2 μM) which are tonically activated by ambient glutamate (Herman and Jahr, 2007). Presently, the reduced agonist sensitivity (Neto2KO) or functional ablation (Neto1KO/Neto null) of presynaptic KARs in CCK/CB1 interneurons was accompanied by increased inhibitory output from these cells, consistent with relief from a tonic KAR-mediated brake on CCK/CB1 interneuron release. This interpretation is bolstered by our findings that antagonizing KARs in wildtype mice similarly increased CCK/CB1 interneuron output. However, the greater magnitude of uIPSC property changes observed in Neto mutants

compared to KAR antagonism leaves open the possibility that Netos may influence CCK/CB1 interneuron presynaptic function independent of KARs. Though Neto2 associates with and regulates surface expression of KCC2 (Ivakine et al., 2013; Mahadevan et al., 2015) any potential confounding influence by such regulation on sIPSC/uIPSC properties was avoided by directly controlling postsynaptic chloride gradients and providing large chloride driving forces to maximize signal to noise ratios for synaptic currents. Moreover, the normal gamma oscillations observed in Neto2KOs argue against a catastrophic breakdown in postsynaptic GABAergic signaling.

Our discovery that Netos promote tonic activation of CCK/CB1 interneuron presynaptic KARs by ambient glutamate reveals a previously unappreciated constraint on output from this interneuron population. Loss of this brake in Neto mutants dramatically increases CCK/CB1 output promoting their contribution to FFI with consequent changes in I/E balance. CCK/CB1 interneurons are particularly well-suited to integrate and impart emotional features of an animal's physiological state to corticolimbic networks (Freund, 2003; Freund and Katona, 2007; Lee and Soltesz, 2011). Indeed CCK/CB1 interneurons are highly susceptible to neuromodulation by local and subcortically-generated signals commonly associated with mood, anxiety, and fear such as cannabinoids, serotonin, and acetylcholine (Armstrong and Soltesz, 2012). Moreover, CCK itself has potent anxiogenic properties and CCK/CB1 interneurons represent a dominant pool of synaptically available CCK-8 peptide (Lee and Soltesz, 2011). Thus, manipulation of CCK/CB1 output by targeting presynaptic Neto/KAR complexes could offer a promising therapeutic avenue for the treatment of mood disorders.

METHODS

Detailed experimental procedures are provided in Supplemental Information.

Animals

Experiments involved male and female wildtype, Neto1, Neto2 and Neto1+2 knockout mice in addition to GluK1+2 knockout mice and the heterozygous offspring of Neto1 knockouts crossed with *Nkx2.1Cre*:RCE or *Htr3a*-GFP mice. Neto1 and 2 knockout, Neto null and GluK1+2 knockout mice were characterized in previous studies (Fisahn et al., 2004; Tang et al., 2011). *Nkx2.1Cre*:RCE and *Htr3a*-GFP mice were previously characterized as reporters for MGE and CGE derived interneurons respectively (Chittajallu et al., 2013; Tricoire et al., 2011). In some experiments rats were used for CCK labeling for better resolution of somatic labeling. All experiments were conducted in accordance with animal protocols approved by the NIH.

ISH and IHC

ISH was performed on fresh frozen tissue from P21–P35 mice using the RNAscope Fluorescent Multiplex kit (Advanced Cell Diagnostics). For IHC brains from P21–P103 rodents were fixed with 4% PFA, cryoprotected, cryosectioned, and labeling for the indicated proteins was visualized with fluorescent secondaries.

RT-PCR

RT-PCR was carried out on P30 hippocampi using QuantStudio 3 Real-Time PCR systems (ThermoFisher Scienfic). Target gene levels were normalized to the endogenous control gene *gadph*.

Slice Electrophysiology

Whole cell recordings were made from hippocampal interneurons and pyramidal cells from P14–P24 wildtype and knockout mice to evaluate the pharmacologically-isolated KAR response to bath-applied and endogenous agonists. Interneuron types were identified through a combination of morphology, immunolabeling, spiking properties, as well as the presence of DSI and asynchronous release in the case of CCK/CB1-CA1 PC pairs. Kainate and carbachol were used to induce gamma oscillations in hippocampal slices from P14–P21 mice. Disynaptic FFI onto CA1 PCs was stimulated in CA1 radiatum isolated from CA3 and normalized to monosynaptic excitation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Armstrong C, Soltesz I. Basket cell dichotomy in microcircuit function. J Physiol. 2012; 590:683–694. [PubMed: 22199164]

Bahn S, Volk B, Wisden W. Kainate receptor gene expression in the developing rat brain. J Neurosci. 1994; 14:5525–5547. [PubMed: 8083752]

Braga MF, Aroniadou-Anderjaska V, Xie J, Li H. Bidirectional modulation of GABA release by presynaptic glutamate receptor 5 kainate receptors in the basolateral amygdala. J Neurosci. 2003; 23:442–452. [PubMed: 12533604]

Brown PM, Aurousseau MR, Musgaard M, Biggin PC, Bowie D. Kainate receptor pore-forming and auxiliary subunits regulate channel block by a novel mechanism. J Physiol. 2016; 594:1821–1840. [PubMed: 26682513]

Bureau I, Bischoff S, Heinemann SF, Mulle C. Kainate receptor-mediated responses in the CA1 field of wild-type and GluR6-deficient mice. J Neurosci. 1999; 19:653–663. [PubMed: 9880586]

Buzsaki G, Wang XJ. Mechanisms of gamma oscillations. Annu Rev Neurosci. 2012; 35:203–225. [PubMed: 22443509]

Chittajallu R, Craig MT, McFarland A, Yuan X, Gerfen S, Tricoire L, Erkkila B, Barron SC, Lopez CM, Liang BJ, et al. Dual origins of functionally distinct O-LM interneurons revealed by differential 5-HT(3A)R expression. Nat Neurosci. 2013; 16:1598–1607. [PubMed: 24097043]

Christensen JK, Paternain AV, Selak S, Ahring PK, Lerma J. A mosaic of functional kainate receptors in hippocampal interneurons. J Neurosci. 2004; 24:8986–8993. [PubMed: 15483117]

Clarke VR, Ballyk BA, Hoo KH, Mandelzys A, Pellizzari A, Bath CP, Thomas J, Sharpe EF, Davies CH, Ornstein PL, et al. A hippocampal GluR5 kainate receptor regulating inhibitory synaptic transmission. Nature. 1997; 389:599–603. [PubMed: 9335499]

Contractor A, Mulle C, Swanson GT. Kainate receptors coming of age: milestones of two decades of research. Trends Neurosci. 2011; 34:154–163. [PubMed: 21256604]

Contractor A, Swanson G, Heinemann SF. Kainate receptors are involved in short- and long-term plasticity at mossy fiber synapses in the hippocampus. Neuron. 2001; 29:209–216. [PubMed: 11182092]

Contractor A, Swanson GT, Sailer A, O'Gorman S, Heinemann SF. Identification of the kainate receptor subunits underlying modulation of excitatory synaptic transmission in the CA3 region of the hippocampus. J Neurosci. 2000; 20:8269–8278. [PubMed: 11069933]

Copits BA, Robbins JS, Frausto S, Swanson GT. Synaptic targeting and functional modulation of GluK1 kainate receptors by the auxiliary neuropilin and tolloid-like (NETO) proteins. J Neurosci. 2011; 31:7334–7340. [PubMed: 21593317]

Copits BA, Swanson GT. Dancing partners at the synapse: auxiliary subunits that shape kainate receptor function. Nat Rev Neurosci. 2012; 13:675–686. [PubMed: 22948074]

Cossart R, Esclapez M, Hirsch JC, Bernard C, Ben-Ari Y. GluR5 kainate receptor activation in interneurons increases tonic inhibition of pyramidal cells. Nat Neurosci. 1998; 1:470–478. [PubMed: 10196544]

Cossart R, Tyzio R, Dinocourt C, Esclapez M, Hirsch JC, Ben-Ari Y, Bernard C. Presynaptic kainate receptors that enhance the release of GABA on CA1 hippocampal interneurons. Neuron. 2001; 29:497–508. [PubMed: 11239438]

Daw MI, Pelkey KA, Chittajallu R, McBain CJ. Presynaptic kainate receptor activation preserves asynchronous GABA release despite the reduction in synchronous release from hippocampal cholecystokinin interneurons. J Neurosci. 2010; 30:11202–11209. [PubMed: 20720128]

Fisahn A, Contractor A, Traub RD, Buhl EH, Heinemann SF, McBain CJ. Distinct roles for the kainate receptor subunits GluR5 and GluR6 in kainate-induced hippocampal gamma oscillations. J Neurosci. 2004; 24:9658–9668. [PubMed: 15509753]

Fisher JL. The auxiliary subunits Neto1 and Neto2 have distinct, subunit-dependent effects at recombinant GluK1- and GluK2-containing kainate receptors. Neuropharmacology. 2015; 99:471–480. [PubMed: 26277340]

Fisher JL, Mott DD. The auxiliary subunits Neto1 and Neto2 reduce voltage-dependent inhibition of recombinant kainate receptors. J Neurosci. 2012; 32:12928–12933. [PubMed: 22973017]

Fisher JL, Mott DD. Modulation of homomeric and heteromeric kainate receptors by the auxiliary subunit Neto1. J Physiol. 2013; 591:4711–4724. [PubMed: 23798491]

Frerking M, Nicoll RA. Synaptic kainate receptors. Curr Opin Neurobiol. 2000; 10:342–351. [PubMed: 10851174]

Frerking M, Petersen CC, Nicoll RA. Mechanisms underlying kainate receptor-mediated disinhibition in the hippocampus. Proc Natl Acad Sci U S A. 1999; 96:12917–12922. [PubMed: 10536023]

Freund TF. Interneuron Diversity series: Rhythm and mood in perisomatic inhibition. Trends Neurosci. 2003; 26:489–495. [PubMed: 12948660]

Freund TF, Katona I. Perisomatic inhibition. Neuron. 2007; 56:33–42. [PubMed: 17920013]

Goldin M, Epsztein J, Jorquera I, Represa A, Ben-Ari Y, Crepel V, Cossart R. Synaptic kainate receptors tune oriens-lacunosum moleculare interneurons to operate at theta frequency. J Neurosci. 2007; 27:9560–9572. [PubMed: 17804617]

Griffith TN, Swanson GT. Identification of critical functional determinants of kainate receptor modulation by auxiliary protein Neto2. J Physiol. 2015; 593:4815–4833. [PubMed: 26282342]

Hefft S, Jonas P. Asynchronous GABA release generates long-lasting inhibition at a hippocampal interneuron-principal neuron synapse. Nat Neurosci. 2005; 8:1319–1328. [PubMed: 16158066]

Herman MA, Jahr CE. Extracellular glutamate concentration in hippocampal slice. J Neurosci. 2007; 27:9736–9741. [PubMed: 17804634]

Hormuzdi SG, Pais I, LeBeau FE, Towers SK, Rozov A, Buhl EH, Whittington MA, Monyer H. Impaired electrical signaling disrupts gamma frequency oscillations in connexin 36-deficient mice. Neuron. 2001; 31:487–495. [PubMed: 11516404]

Howe JR. Modulation of non-NMDA receptor gating by auxiliary subunits. J Physiol. 2015; 593:61–72. [PubMed: 25556788]

Ivakine EA, Acton BA, Mahadevan V, Ormond J, Tang M, Pressey JC, Huang MY, Ng D, Delpire E, Salter MW, et al. Neto2 is a KCC2 interacting protein required for neuronal Cl- regulation in hippocampal neurons. Proc Natl Acad Sci U S A. 2013; 110:3561–3566. [PubMed: 23401525]

Jane DE, Lodge D, Collingridge GL. Kainate receptors: pharmacology, function and therapeutic potential. Neuropharmacology. 2009; 56:90–113. [PubMed: 18793656]

Jiang L, Xu J, Nedergaard M, Kang J. A kainate receptor increases the efficacy of GABAergic synapses. Neuron. 2001; 30:503–513. [PubMed: 11395010]

Khalilov I, Hirsch J, Cossart R, Ben-Ari Y. Paradoxical anti-epileptic effects of a GluR5 agonist of kainate receptors. J Neurophysiol. 2002; 88:523–527. [PubMed: 12091575]

Klausberger T, Somogyi P. Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. Science. 2008; 321:53–57. [PubMed: 18599766]

Lauri SE, Bortolotto ZA, Bleakman D, Ornstein PL, Lodge D, Isaac JT, Collingridge GL. A critical role of a facilitatory presynaptic kainate receptor in mossy fiber LTP. Neuron. 2001; 32:697–709. [PubMed: 11719209]

Lee SY, Soltesz I. Cholecystokinin: a multi-functional molecular switch of neuronal circuits. Dev Neurobiol. 2011; 71:83–91. [PubMed: 21154912]

Lerma J, Marques JM. Kainate receptors in health and disease. Neuron. 2013; 80:292–311. [PubMed: 24139035]

Lourenco J, Cannich A, Carta M, Coussen F, Mulle C, Marsicano G. Synaptic activation of kainate receptors gates presynaptic CB(1) signaling at GABAergic synapses. Nat Neurosci. 2010; 13:197–204. [PubMed: 20081851]

Mahadevan V, Dargaei Z, Ivakine EA, Hartmann AM, Ng D, Chevrier J, Ormond J, Nothwang HG, McInnes RR, Woodin MA. Neto2-null mice have impaired GABAergic inhibition and are susceptible to seizures. Front Cell Neurosci. 2015; 9:368. [PubMed: 26441539]

Maingret F, Lauri SE, Taira T, Isaac JT. Profound regulation of neonatal CA1 rat hippocampal GABAergic transmission by functionally distinct kainate receptor populations. J Physiol. 2005; 567:131–142. [PubMed: 15946969]

Mulle C, Sailer A, Swanson GT, Brana C, O'Gorman S, Bettler B, Heinemann SF. Subunit composition of kainate receptors in hippocampal interneurons. Neuron. 2000; 28:475–484. [PubMed: 11144357]

Orav E, Atanasova T, Shintyapina A, Kesaf S, Kokko M, Partanen J, Taira T, Lauri SE. NETO1 Guides Development of Glutamatergic Connectivity in the Hippocampus by Regulating Axonal Kainate Receptors. eNeuro. 2017:4.

Palacios-Filardo J, Aller MI, Lerma J. Synaptic Targeting of Kainate Receptors. Cereb Cortex. 2016; 26:1464–1472. [PubMed: 25316333]

Paternain AV, Herrera MT, Nieto MA, Lerma J. GluR5 and GluR6 kainate receptor subunits coexist in hippocampal neurons and coassemble to form functional receptors. J Neurosci. 2000; 20:196–205. [PubMed: 10627597]

Rodriguez-Moreno A, Herreras O, Lerma J. Kainate receptors presynaptically downregulate GABAergic inhibition in the rat hippocampus. Neuron. 1997; 19:893–901. [PubMed: 9354335]

Schmitz D, Mellor J, Nicoll RA. Presynaptic kainate receptor mediation of frequency facilitation at hippocampal mossy fiber synapses. Science. 2001; 291:1972–1976. [PubMed: 11239159]

Semyanov A, Kullmann DM. Kainate receptor-dependent axonal depolarization and action potential initiation in interneurons. Nat Neurosci. 2001; 4:718–723. [PubMed: 11426228]

Sheng N, Shi YS, Lomash RM, Roche KW, Nicoll RA. Neto auxiliary proteins control both the trafficking and biophysical properties of the kainate receptor GluK1. Elife. 2015:4.

Sheng N, Shi YS, Nicoll RA. Amino-terminal domains of kainate receptors determine the differential dependence on Neto auxiliary subunits for trafficking. Proc Natl Acad Sci U S A. 2017; 114:1159–1164. [PubMed: 28100490]

Straub C, Hunt DL, Yamasaki M, Kim KS, Watanabe M, Castillo PE, Tomita S. Distinct functions of kainate receptors in the brain are determined by the auxiliary subunit Neto1. Nat Neurosci. 2011a; 14:866–873. [PubMed: 21623363]

Straub C, Zhang W, Howe JR. Neto2 modulation of kainate receptors with different subunit compositions. J Neurosci. 2011b; 31:8078–8082. [PubMed: 21632929]

Tang M, Ivakine E, Mahadevan V, Salter MW, McInnes RR. Neto2 interacts with the scaffolding protein GRIP and regulates synaptic abundance of kainate receptors. PLoS One. 2012; 7:e51433. [PubMed: 23236500]

Tang M, Pelkey KA, Ng D, Ivakine E, McBain CJ, Salter MW, McInnes RR. Neto1 is an auxiliary subunit of native synaptic kainate receptors. J Neurosci. 2011; 31:10009–10018. [PubMed: 21734292]

Tricoire L, Pelkey KA, Erkkila BE, Jeffries BW, Yuan X, McBain CJ. A blueprint for the spatiotemporal origins of mouse hippocampal interneuron diversity. J Neurosci. 2011; 31:10948–10970. [PubMed: 21795545]

Wondolowski J, Frerking M. Subunit-dependent postsynaptic expression of kainate receptors on hippocampal interneurons in area CA1. J Neurosci. 2009; 29:563–574. [PubMed: 19144856]

Wyeth MS, Pelkey KA, Petralia RS, Salter MW, McInnes RR, McBain CJ. Neto auxiliary protein interactions regulate kainate and NMDA receptor subunit localization at mossy fiber-CA3 pyramidal cell synapses. J Neurosci. 2014; 34:622–628. [PubMed: 24403160]

Zhang W, Devi SP, Tomita S, Howe JR. Auxiliary proteins promote modal gating of AMPA- and kainate-type glutamate receptors. Eur J Neurosci. 2014; 39:1138–1147. [PubMed: 24712993]

Zhang W, St-Gelais F, Grabner CP, Trinidad JC, Sumioka A, Morimoto-Tomita M, Kim KS, Straub C, Burlingame AL, Howe JR, Tomita S. A transmembrane accessory subunit that modulates kainate-type glutamate receptors. Neuron. 2009; 61:385–396. [PubMed: 19217376]

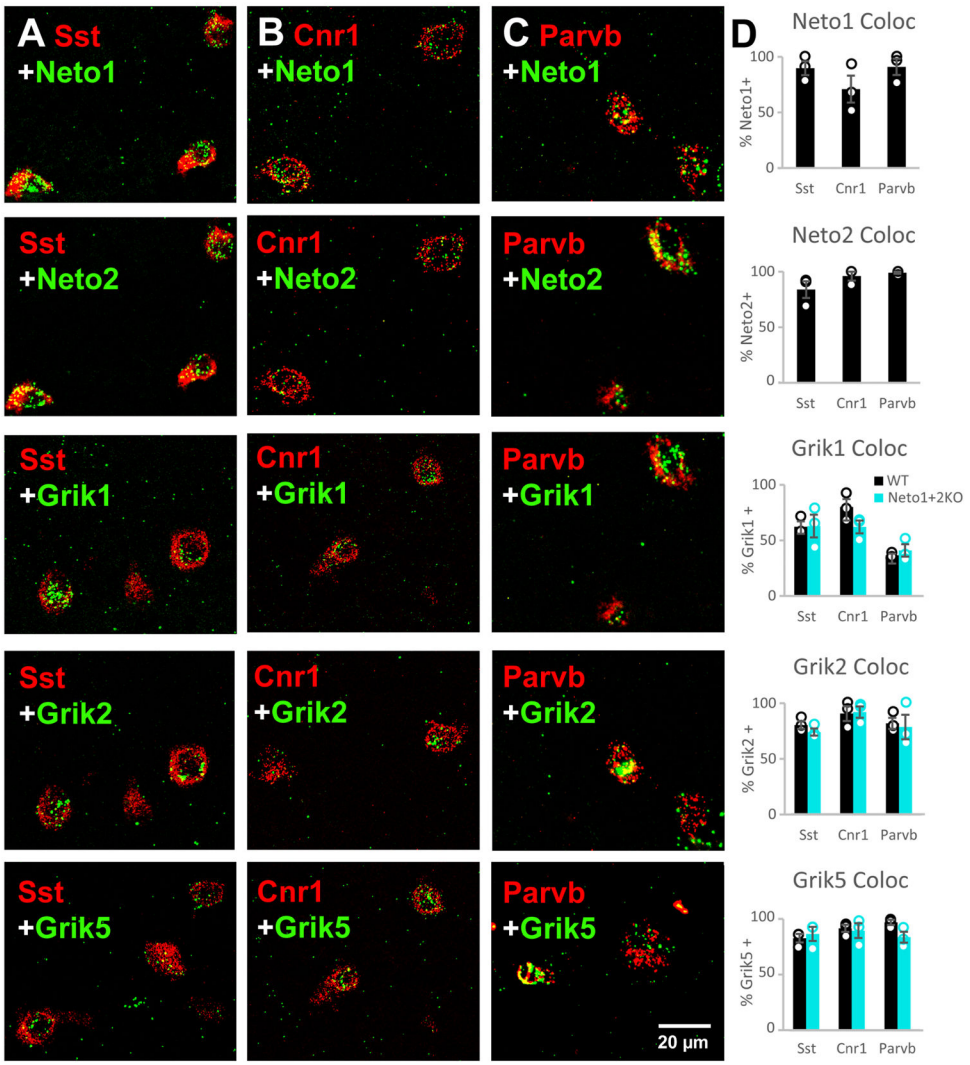

Figure 1. SOM (*Sst*), CCK/CB1 (*Cnr1*), and PV (*Parvb*) mRNA expressing interneurons co-express mRNA for Neto and kainate receptor subunits

A–C Representative images illustrating mRNA expression of *Neto1*, *Neto2* (green), and KAR subunits *Grik1, Grik2, and Grik5* (green) in SOM (A), CCK/CB1 (B), and PV (C) mRNA-expressing interneurons (red). Note that both Neto1 and Neto2 mRNA could be observed within the same interneuron (A–B, upper two panels).

D Bar charts summarizing the percentage of SOM, CCK/CB1, and PV mRNA expressing interneurons coexpressing mRNA for Neto1, Neto2, Grik1, Grik2, and Grik5 in wildtype (WT, black bars) and Neto null (Neto1+2 KO, cyan bars) mice. Individual points reflect observations from three different mice. For each colocalization measurement plotted, 72–339 individual SOM, CCK/CB1, and PV mRNA labeled interneurons (in sum 2956 cells) across 3 different mice per genotype (4–7 sections per mouse) were assessed for co-expression of the indicated Neto and Grik mRNA species. See also Fig. S1.

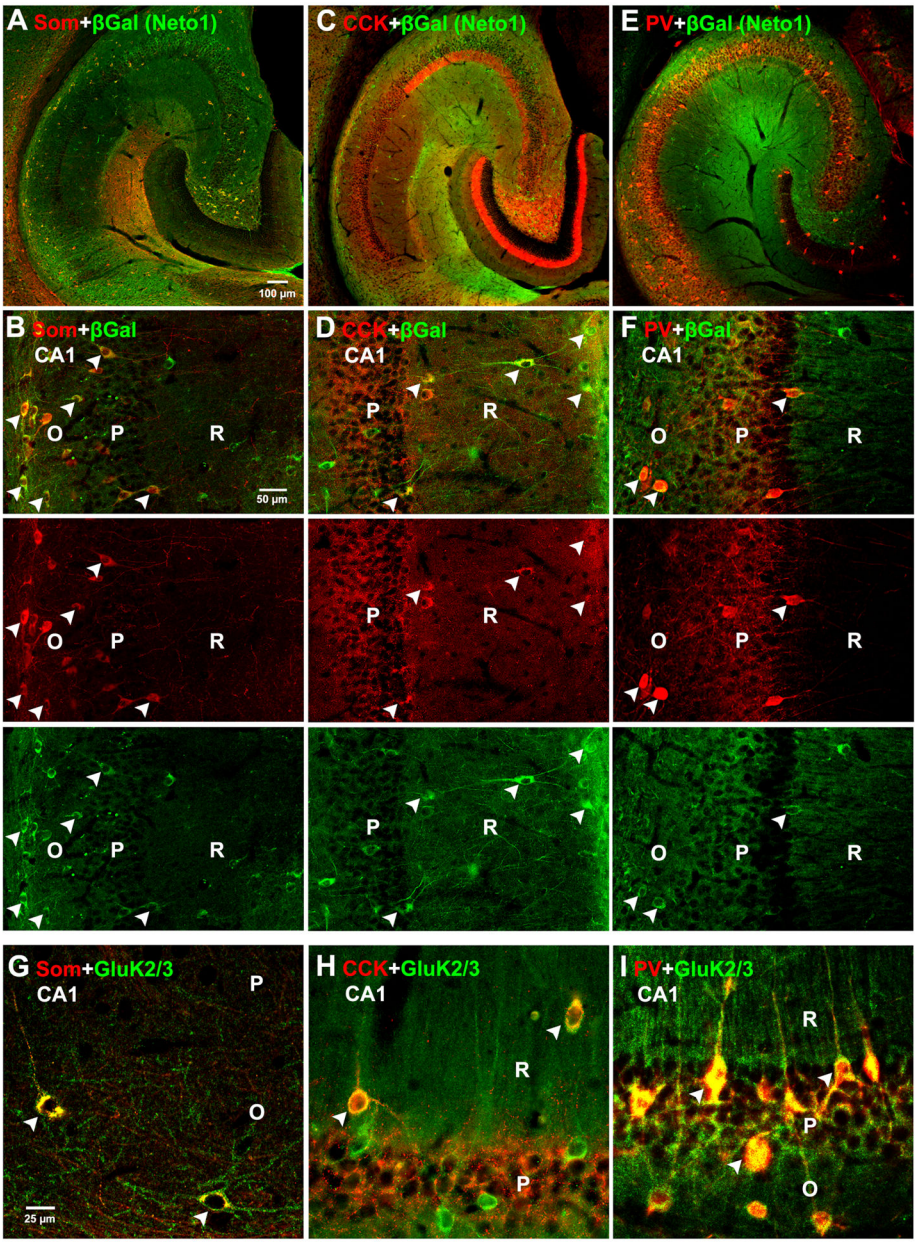

Figure 2. ***Neto1*** **promoter driven β-galactosidase (βGal) expression in SOM, CCK/CB1, and PV-containing interneurons of Neto1 mutant mice**

A–F Representative images from IHC studies illustrating *Neto1* promotor driven βGal (green) expression in SOM (A,B, red), CCK/CB1 (C,D, red), and PV (E,F, red) interneurons from Neto1KO (A–D) or Neto1 heterozygous (E,F) mice. Panels B, D, and F are higher magnification images with interneuron and βGal signals overlayed (upper) and separated (lower panels) for clarity.

G–I Sample overlay images illustrating GluK2/3 immunolabeling (green) in mouse SOM (G, red) and PV (I, red) labeled interneurons as well as rat CCK/CB1-expressing cells (H, red). See also Fig. S2. (arrows indicate interneurons; O, oriens; P pyramidal layer; R, radiatum)

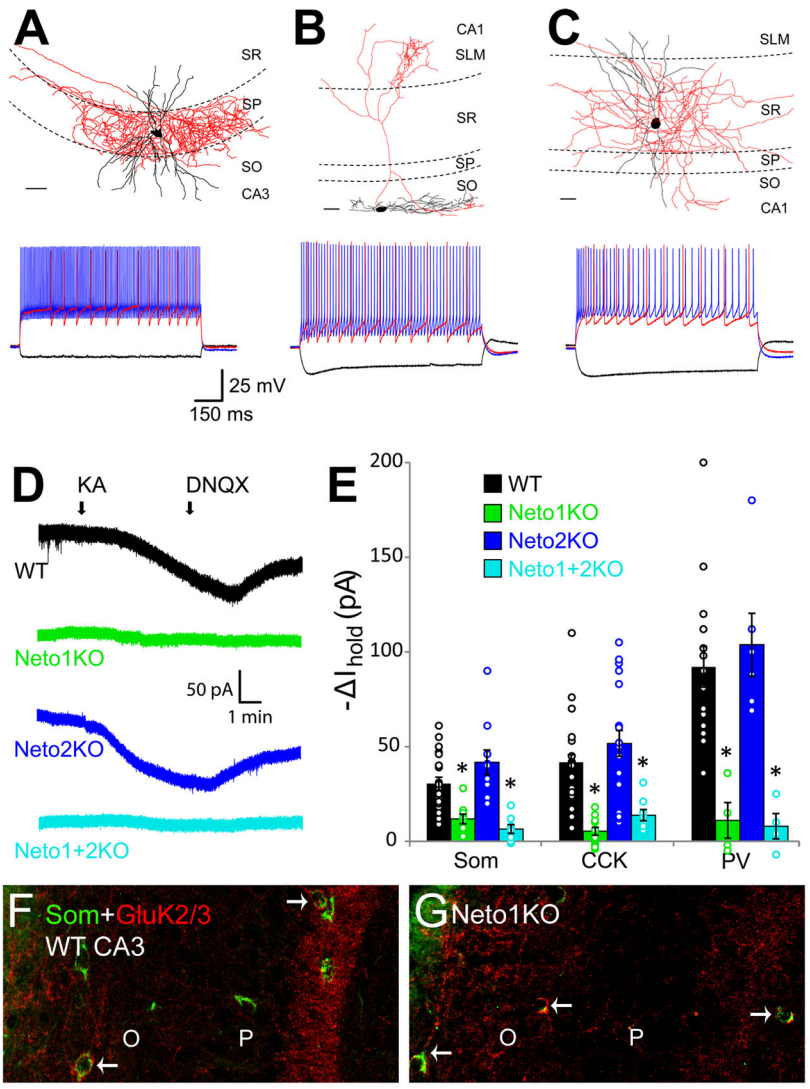

Figure 3. Impaired interneuron KAR-mediated currents in the absence of Neto1

A–C Anatomical recoveries of representative PV (A, fast-spiking basket cell), SOM (B, oriens-lacunosum molecular projecting, O-LM cell), and CCK/CB1 (C, Schaffer collateral-associated, SCA cell) interneurons, illustrating the 3 cohorts probed for kainate (KA)-induced currents (bars, 50 μm; SO, stratum oriens; SP; stratum pyramidale; SR, stratum radiatum; SLM, stratum lacunosum-moleculare). Below are voltage responses of each interneuron to hyperpolarizing current injections (−200 pA, black) as well as depolarizing current injections to threshold and twice threshold for action potential generation (red and blue respectively).

D Representative KA- (200 nM) induced currents observed in interneurons in wildtype (WT, black), Neto1KO (green), Neto2KO (blue), and Neto null (cyan) mice.

E Group data plot (individual observations overlayed) summarizing the maximal amplitude of KA-induced currents in each interneuron class across all genotypes. KA was applied in the presence of GYKI-53655 (50 μM), APV (100 μM), bicuculline (10 μM) and TTX (1 μM) and finished in DNQX (25 μM). KAR-mediated currents were significantly reduced in

Neto1 and Neto null mice in SOM (3 mice 9 cells, 3 mice 8 cells, respectively, *p<0.05 each vs. WT), CCK/CB1 (4 mice 12 cells, 4 mice 7 cells, respectively, *p<0.05 each vs. WT) and PV (2 mice 4 cells each, *p<0.05 each vs. WT) interneurons relative to WT (10 mice 21 SOM cells, 11 mice 22 CCK cells, 7 mice 14 PV cells) and Neto2KO mice (6 mice 10 SOM cells, 7 mice 21 CCK cells, 3 mouse 6 PV cells, p>0.05 each vs. WT).

F,G Representative images illustrating comparable GluK2/3 immunolabeling (red) in SOM-labeled interneurons (green) in CA3 oriens (O) and pyramidale (P) of a wildtype (F) and Neto1KO (G) (additional examples provided in Fig. S3).

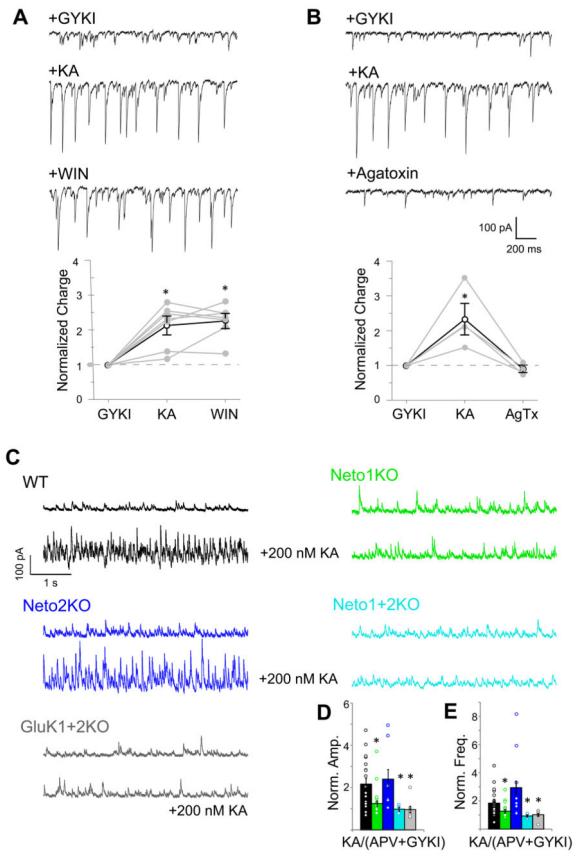

Figure 4. Impaired KAR-mediated recruitment of inhibitory drive in the absence of Neto1

A Representative recording (upper traces) and group data (lower plot) from WT CA3 pyramidal cells (PCs) illustrating that CB1 agonist WIN-55,212-2 (WIN, 5 μM) fails to alter kainate (KA)-recruited sIPSCs (7 cells, 4 mice, p=0.01 in WIN vs baseline).

B In contrast, agatoxin (AgTx, 250 nM) reverses KA (200 nM in 50 μM GYKI) recruitment of sIPSCs (4 cells, 3 mice, p=0.37 in agatoxin vs. baseline). For group data plots the combined effect of KA on sIPSC frequency and amplitude was accounted for by analyzing the overall change in charge associated with sIPSC recruitment by KA and data were normalized to charge transfer during the baseline period. sIPSCs were recorded at a holding potential of −70 mV with the chloride reversal potential set at 0 mV, yielding inward sIPSCs.

C Representative recordings illustrating KA-recruitment (in GYKI/APV) of sIPSCs in CA3 PCs of WT, Neto1KO, Neto2KO, Neto null and GluK1/2 double knockout mice.

D,E Group data bar charts (individual observations overlayed) summarizing the effect of KA on sIPSC amplitude (D) and frequency (E) in each genotype. KA-related changes are plotted normalized to events obtained during the baseline period prior to KA. Note, for these recordings CA3 PCs were voltage-clamped at 0 mV and chloride reversal was approximately −60 mV, yielding outward sIPSCs. KA increased the amplitude and frequency in WT (5 mice, 19 cells) and Neto2KO mice (2 mice, 9 cells, p=0.5 for amplitude, p=0.5 for frequency), but recordings from Neto1KO (4 mice, 24 cells, p=0.006 for amplitude and frequency) and Neto1+2KO mice (2 mice, 4 cells, p=0.02 for amplitude, p=0.004 for

frequency) responded similarly to GluK1+2KO mice (2 mice, 8 cells, $p<0.0004$ for amplitude, $p=0.002$ for frequency).

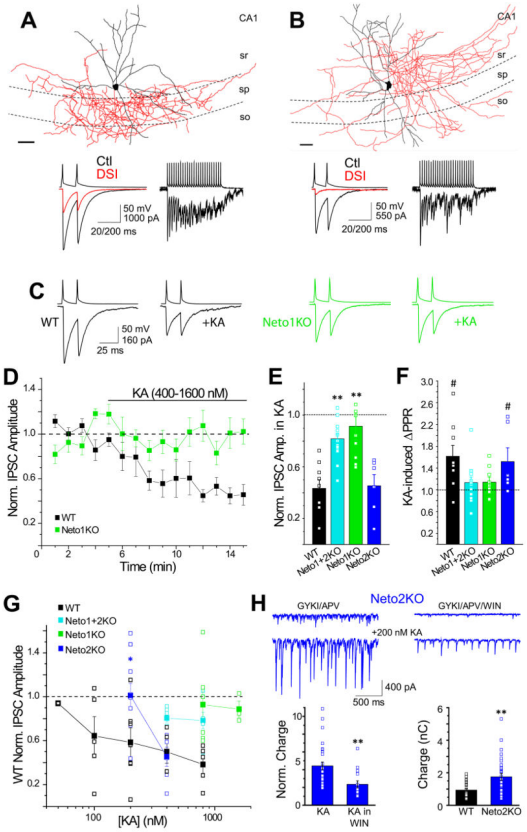

Figure 5. Neto1 and Neto2 regulation of CCK/CB1 interneuron presynaptic KARs

A,B Anatomical reconstructions of representative perisomatic targeting basket (A) and dendrite-targeting SCA (B) interneurons targeted for CCK/CB1-CA1 PC paired recordings (postsynaptic PCs not shown, bar 50 μm). Lower panels of (A) and (B) show traces of presynaptic action potentials and corresponding postsynaptic uIPSCs for pairs or trains of presynaptic stimuli to illustrate that recordings display prominent DSI (left, Ctl vs DSI after 5 s postsynaptic depolarization to 0 mV) and asynchronous release (right) to confirm presynaptic cells as CCK/CB1 interneurons.

C Averaged traces from representative recordings of CCK/CB1-PC uIPSCs before and after kainate (KA) application (800 nM, in GYKI 50 μM) in wildtype and Neto1KO mice.

D Group data time course plot illustrating the effect of KA (400–1600 nM) on CCK/CB1-PC uIPSC amplitude in WT and Neto1KO mice. IPSC amplitudes were binned for 1 minute intervals and normalized to the average amplitude observed prior to KA application.

E,F Bar charts summarizing the effect of KA (400–1600 nM) on uIPSC amplitude (E, ** $p<0.01$ vs. WT) and paired pulse ratio (F, # $p<0.05$ KA vs. baseline) in CCK/CB1-PC paired recordings from wildtypes (9 pairs, 3mice), Neto1KOs (9 pairs, 3 mice), Neto2KOs (6 pairs, 3 mice), and Neto nulls (7 pairs, 2 mice).

G, Group data summary plot illustrating uIPSC inhibition observed in CCK/CB1-PC pairs for various KA doses in each genotype (* $p<0.05$ vs. WT; for doses ranging from 400–1600 nM, recordings were grouped by genotype and statistical results are illustrated in panel E).

For 50 nM KA (WT, 3 pairs, 2 mice), 100 nM KA (WT, 6 pairs, 3 mice), 200 nM KA (WT, 7 pairs, 6 mice; Neto2 KO, 10 pairs, 4 mice).

H Upper traces are representative recordings of baseline and KA-recruited sIPSCs in Neto2KO CA3 PCs with (right) or without (left) WIN (5 μM) incubation. Lower bar chart at left summarizes degree of sIPSC recruitment by KA in the absence (29 cells, 7 mice) or presence of WIN (15 cells, 4 mice) quantified as total sIPSC charge recruited by KA normalized to the pre-KA baseline (**p<0.01 vs. KA alone). Lower right bar chart summarizes baseline sIPSC charge observed in WT (27 cells, 4 mice) and Neto2 KO (31 cells, 6 mice) CA3 PCs (**p<0.01 vs. WT).

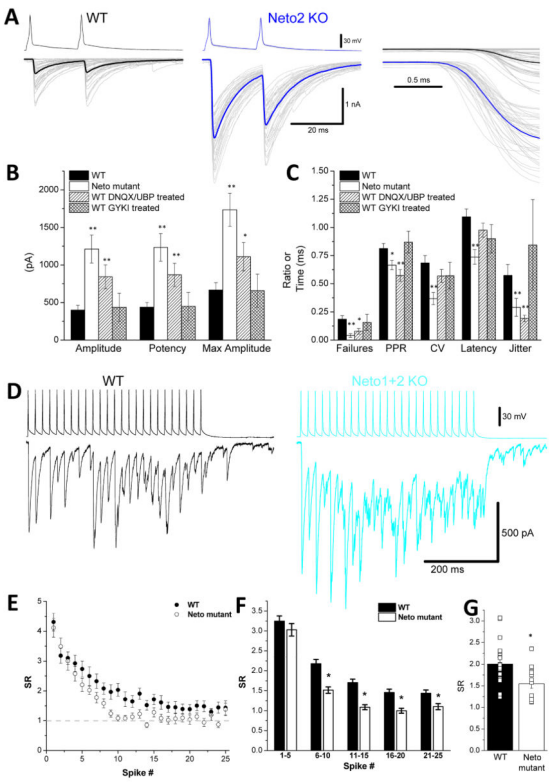

Figure 6. Loss of tonic presynaptic KAR activation in Neto mutants increases CCK/CB1 interneuron output

A CCK/CB1BC-CA1 PC uIPSCs from representative recordings in WT and Neto mutant (2 KO) mice. Thick black and blue traces are the averages of consecutive individual trials (thin grey sweeps). At right is a time expanded view of the first uIPSCs aligned from the peak of the first presynaptic action potential.

B–C Group data summary plots of CCK/CB1BC-PC uIPSC properties for WTs (37 pairs, 21 mice) and Neto mutants (18 pairs, 11 mice). Also summarized are uIPSC properties for CCK/CB1BC-PC pairs recorded in slices from WTs treated with DNQX (10 μM, 8 pairs, 3 mice) or UBP (5 μM, 11 pairs, 4 mice) to antagonize KARs (data combined) or GYKI 53655 (50 μM, 12 pairs, 4 mice) to control for DNQX-mediated block of AMPARs (*$p<0.05$ and **$p<0.01$ vs. WT). For clarity individual observations are not plotted but are provided in Fig. S4.

D Representative CCK/CB1BC-PC recordings from WT and Neto mutant (nulls) mice illustrating asynchronous release during train stimulation.

E–G Group data plots summarizing uIPSC synchronicity ratios (SRs) measured during trains for CCK/CB1BC-PC paired recordings in WT (22 pairs, 7 mice) and Neto mutants (15 pairs, 9 mice). SRs are plotted for release associated with each individual presynaptic action potential (E), grouped into bins of 5 successive action potentials (F) or for the entire train (G). Despite similar initial starting SRs Neto mutants exhibit reduced synchrony after the first 5 release events of the train (*$p<0.05$ vs. WT).

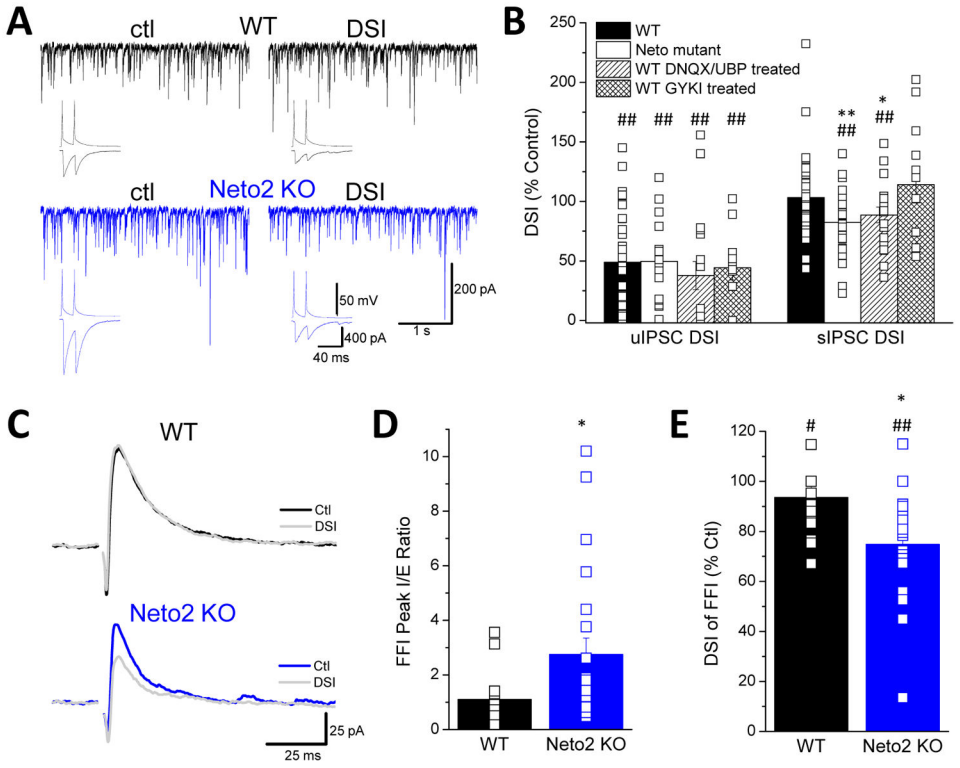

Figure 7. Increased contribution of CCK/CB1 interneurons to circuit inhibition in Neto mutant mice

A Representative recordings illustrating the effect of PC depolarization to evoke DSI on uIPSCs (lower insets) and simultaneously monitored sIPSCs (upper traces) during CCK/CB1BC-PC paired recordings in WT and Neto mutant (2 KO) mice.

B Bar chart summary of uIPSC and sIPSC DSI for the conditions indicated. DSI is expressed as percent of control pre-DSI levels using amplitude for uIPSCs and charge transfer for sIPSCs. For uIPSCs n=37 pairs from 21 WT mice, n=18 pairs from 11 Neto mutants, n=19 pairs from 7 WTs treated with DNQX/UBP, and n=12 pairs from 4 WTs treated with GYKI 53655. For sIPSCs n=42 cells from 22 WT mice, n=42 cells from 14 Neto mutant mice, n=19 cells from 7 DNQX/UBP-treated WT mice, and n=12 cells from 4 GYKI-treated WT mice (*p<0.05 and **p<0.01 vs. WT; ##p<0.01 vs. control pre-DSI baseline).

C Averaged waveforms of Schaffer collateral stimulation-evoked dual component synaptic events observed in representative recordings from CA1 PCs of WT and Neto2KO mice under control and DSI conditions. The initial monosynaptically driven excitatory postsynaptic current (inward/downward deflection) is rapidly followed by a disynaptically recruited inhibitory postsynaptic current (outward/upward deflection).

D Group data summary of FFI peak current I/E ratios obtained in WT and Neto2 KO CA1 PCs.

E Group data summary of the DSI sensitivity of disynaptically recruited IPSCs (FFI) in WT (17 cells, 4 mice) and Neto2KO (22 cells, 7 mice) CA1 PCs (*p<0.05 vs. WT; #p<0.05 and

##p<0.01 vs. ctl pre-DSI baseline). DSI is expressed as percent of control pre-DSI IPSC amplitude.