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A Shared Vesicular Carrier Allows Synaptic Corelease of GABA and Glycine

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Summary

The type of vesicular transporter expressed by a neuron is thought to determine its neurotransmitter phenotype. We show that inactivation of the vesicular inhibitory amino acid transporter (Viaat, VGAT) leads to embryonic lethality, an abdominal defect known as omphalocele, and a cleft palate. Loss of Viaat causes a drastic reduction of neurotransmitter release in both GABAergic and glycinergic neurons, indicating that glycinergic neurons do not express a separate vesicular glycine transporter. This loss of GABAergic and glycinergic synaptic transmission does not impair the development of inhibitory synapses or the expression of KCC2, the K^+-Cl^- cotransporter known to be essential for the establishment of inhibitory neurotransmission. In the absence of Viaat, GABA-synthesizing enzymes are partially lost from presynaptic terminals. Since GABA and glycine compete for vesicular uptake, these data point to a close association of Viaat with GABA-synthesizing enzymes as a key factor in specifying GABAergic neuronal phenotypes.

Introduction

Inhibitory neurotransmission in the adult nervous system is chiefly mediated by release of γ-aminobutyric acid (GABA) and glycine from synaptic vesicles. GABAergic inhibition predominates in the brain, whereas both glycine and GABA act as the primary inhibitory neurotransmitters in spinal cord and brainstem. To date, only a single transporter for the filling of vesicles at both GABAergic and glycinergic synapses has been identified. It is referred to as either vesicular GABA transporter (VGAT) (McIntire et al., 1997) or vesicular inhibitory amino acid transporter (Viaat) (Sagne et al., 1997). In contrast, the glutamatergic, excitatory transmitter system employs three separate vesicular glutamate transporters with distinct expression patterns (Fremeau et al., 2004b).

The designation of Viaat as a GABA/glycine transporter is based on evidence from morphological studies showing the presence of Viaat in both GABAergic and glycinergic synaptic terminals (Chaudhry et al., 1998), from biochemical studies on neurotransmitter uptake characteristics of synaptic vesicle preparations (Burger et al., 1991; Christensen and Fonnum, 1991), and from electrophysiological studies demonstrating corelease of GABA and glycine from single vesicles in spinal cord neurons (Jonas et al., 1998). However, despite the fact that multiple lines of evidence indicate that Viaat is a vesicular carrier for both GABA and glycine, direct evidence is lacking, and the question as to whether Viaat is the sole vesicular carrier responsible for all GABAergic and glycinergic neurotransmission or whether glycinergic neurons possess an additional glycine transporter has remained unresolved. In fact, the affinity of heterologously expressed Viaat for glycine was shown to be considerably lower than its affinity for GABA (McIntire et al., 1997; Sagne et al., 1997). This finding is in line with some studies on the endogenous GABA/glycine uptake system (Bedet et al., 2000) but is contradicted by other studies reporting lower but comparable uptake of glycine versus GABA by the endogenous system (Burger et al., 1991) or effective inhibition of GABA uptake at low concentrations of glycine (Christensen et al., 1991).

GABA and glycine exert their well established roles as inhibitory neurotransmitters in the mature nervous system, where intraneuronal Cl^- concentrations are low, and the opening of $GABA_A$ receptor ($GABA_AR$) and glycine receptor (GlyR) channels causes Cl^- influx and membrane hyperpolarization. In immature neurons, GABA and glycine act instead as depolarizing and even excitatory neurotransmitters (Ben-Ari, 2002), because intraneuronal Cl^- concentrations are high and receptor Cl^- channel opening is followed by Cl^- efflux and membrane depolarization. This depolarization caused by $GABA_AR$ and GlyR activation is sufficient to activate voltage-dependent Ca^{2+} channels and even elicit action potentials (Chen et al., 1996; Leinekugel et al., 1995; Owens et al., 1996; Reichling et al., 1994; Yuste and Katz, 1991). The relatively high intracellular Cl^- concentration of immature neurons is due to the developmentally early appearance of inwardly directed Cl^- transporters, in particular the Na^+-K^+-$2Cl^-$ cotransporter NKCC1 (Clayton et al., 1998). The shift from the depolarizing to the hyperpolarizing and thereby inhibitory action of GABA and glycine depends on the expression of KCC2, a K^+-Cl^- cotransporter that is responsible for the Cl^- extrusion capacity of mature neurons (Rivera et al., 1999). KCC2 transcript levels increase throughout development in parallel with neuronal maturation (Stein et al., 2004). Although significant KCC2 upregulation occurs postnatally, its critical function in the transition to the adult pattern of GABA/glycine hyperpolarizing action is already apparent at birth, when KCC2 deletion mutant mice fail to establish normal breathing patterns and die (Hubner et al., 2001). The developmental upregulation

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of KCC2 expression has been suggested to be an activity-dependent event, produced by the depolarizing action of GABA and glycine in immature neurons (Ganguly et al., 2001), a finding that was contradicted by later studies (Ludwig et al., 2003; Titz et al., 2003). Moreover, the maturation of postsynaptic receptor clusters at glycinergic contacts is also thought to be an activity-dependent process controlled by the early depolarizing function of glycine, because postsynaptic GlyR clustering is prevented by pharmacologically blocking either GlyRs themselves or voltage-activated Ca^{2+} channels (Kirsch and Betz, 1998). Based on these and numerous other studies, the role of GABA and glycine release in the establishment of neuronal circuits during development is generally considered a trophic action (Ben-Ari, 2002; Owens and Kriegstein, 2002). However, in this context it is important to point out that GABA is also thought to act as a trophic substance early in development before synaptic communication is established (Represa and Ben-Ari, 2005). Consequently, the earliest effects of GABA on neuronal development may be due to a diffuse mode of action mediated by nonsynaptic and nonvesicular release (Demarque et al., 2002).

The generation of GABAergic synapses during development precedes the establishment of functional glutamatergic inputs (Chen et al., 1995; Walton et al., 1993), and the initially excitatory action of GABA and glycine during development may therefore be a self-limiting process that establishes early network activity and serves as a mechanism to avoid excitotoxicity (Ben-Ari, 2002). However, the roles of GABA and glycine in neuronal network development may not always be equivalent, because in the brainstem and spinal cord, where both neurotransmitters are used, a developmental transition from $GABA_AR$ to GlyR-mediated responses occurs postnatally (Gao et al., 2001). This shift from GABAergic to glycinergic neurotransmission, whose molecular basis still remains to be resolved, does not appear to be caused solely by postsynaptic changes in receptor composition, since in synapses of the auditory brainstem presynaptic changes were shown to occur (Nabekura et al., 2004).

To characterize the vesicular transporter systems of inhibitory synapses and their role in central nervous system development, we generated mice deficient for Viaat and characterized their phenotype with respect to the generation and function of GABAergic and glycinergic synapses and the functional maturation of GABAergic and glycinergic synaptic transmission.

Results

Viaat-Deficient Mice Die In Utero and Exhibit a Cleft Palate and Omphalocele

We generated Viaat knockout mice (KOs) through homologous recombination in embryonic stem cells (Figures 1A–1C). Viaat KO mice die between embryonic day (E)18.5 and birth, exhibit a hunched posture, and at E17.5 (Figure 1D), the age chosen for experimental analysis, and are completely immobile and stiff. Stiffness is most likely a symptom of overexcitation; however, a lack of mobility was also noted at earlier embryonic stages (E15.5–E16.5) and is also consistent with an absence of excitatory drive, which in the developing nervous system is initially provided at least partially by GABA (Ben-Ari, 2002). Homozygous KO animals have a cleft palate (Figure 1E), which was also described for KOs of the 67 kDa isoform, but not the 65 kDa isoform, of the main GABA-synthesizing enzymes glutamic acid decarboxylase (GAD)67 and GAD65 (Asada et al., 1997; Condie et al., 1997). Apparently, closure of the palate roof requires vesicular GABA release, which indicates that the cleft palate in Viaat and GAD67 KOs is due to immobility of the tongue, rather than to elimination of a trophic effect of GABA in the palate. In addition, Viaat KOs display an omphalocele, an abdominal defect where the intestine is not withdrawn into the abdominal cavity during embryonic development (Figures 1D and 1E). Interestingly, this defect is also seen in embryos deficient for KCC2 (Hubner et al., 2001), the K^+-Cl^- cotransporter responsible for extrusion of Cl^- during neuronal maturation, whose expression determines the developmental shift of GABAergic and glycinergic synaptic input from excitatory to inhibitory (Balakrishnan et al., 2003; Rivera et al., 1999).

Because GABA is thought to have trophic effects during neuronal development (Represa and Ben-Ari, 2005), we examined Nissl-stained serial brain sections and spinal cord sections of E17.5 Viaat KO and wild-type (wt). We found that neither the brain nor the spinal cord of Viaat KOs showed any morphological abnormalities (Figures 1F–1I). These observations are in line with studies on GAD65 and GAD67 double KOs (Ji et al., 1999), where no evidence of abnormal brain histogenesis was detectable.

The Absence of Viaat Results in Reduced GAD65/67 Levels and Altered Subcellular Distribution of GAD67

While Viaat KO spinal cord sections showed no histological defects, with immunofluorescence staining, a decreased synaptic and increased somatic labeling for GAD67 (Figures 2A and 2B) was readily apparent in the spinal cord of Viaat KO animals. In contrast to this, we found no significant difference in the GAD65 staining between wt and KO spinal cord (Figures 2C and 2D). We next looked for changes in the protein expression levels of general synaptic markers as well as markers that are specific for GABAergic/glycinergic synapses (Figure 3). The only alteration detected in KO animals was a reduction of GAD65 and GAD67 levels in spinal cord, but not whole brain (Figures 2E and 3). To confirm and quantify the reduction of GAD65 and GAD67 levels in spinal cord, total GAD65 and GAD67 protein levels in E17.5 whole brain and spinal cord were measured using detection of fluorescently labeled secondary antibodies with normalization to β-tubulin levels. Total GAD65 and GAD67 protein levels in spinal cord were significantly reduced to 65% and 64% of wt levels, respectively (Figure 2E). However, there was no significant change of GAD protein levels in KO brain.

The most likely interpretation of these findings is that GAD65 and GAD67 are associated with Viaat, either directly or as part of a protein complex, as has been suggested for GAD65 (Jin et al., 2003). Absence of the transporter leads to a reduction of protein levels of both GAD isoforms and to mislocalization of the remaining cellular GAD67 pool. GAD65, which unlike GAD67 is palmitoylated (Kanaani et al., 2002), appears to be less dependent on Viaat for synaptic targeting. Although GAD67 generally has a more cytosolic distribution than GAD65

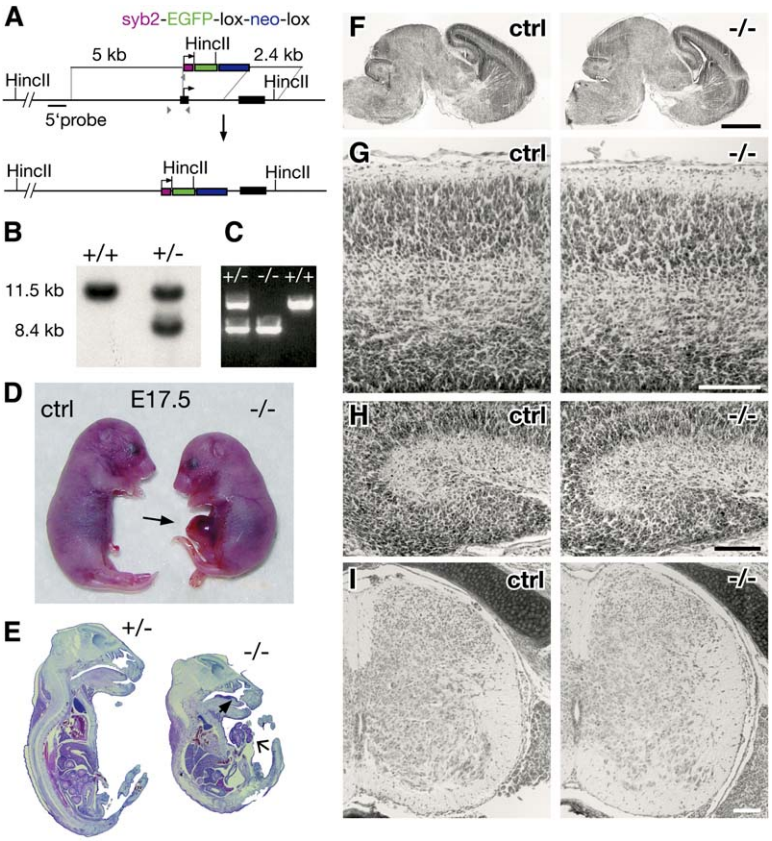

Figure 1. Viaat KO Mice

(A) Targeting vector and Viaat locus before and after homologous recombination. Coding exon 1 and part of intron 1 were replaced by a synaptobrevin 2-enhanced green fluorescent protein minigene and a loxP flanked neomycin resistance cassette (syb2-EGFP-lox-neo-lox). The syb2-EGFP fusion protein is not expressed in the Viaat KO mice or heterozygotes, not even after removal of the neo cassette, presumably because intron 1 contains transcription factor sites critical for expression from the Viaat locus. The position of HincII restriction sites and the 5′ Southern probe are indicated. Gray arrowheads indicate the position of the PCR primers used for genotyping.

(B) Genomic Southern blot with 5′ probe after HincII digestion of stem cell DNA. Homologous recombination of the Viaat targeting vector results in a shift of a wt 11.5 kb HincII fragment to 8.4 kb.

(C) Genomic PCR used for genotyping Viaat litters with 622 bp for wt and 382 bp for the targeted locus.

(D) Viaat KO E17.5 embryo with littermate. Note the omphalocele (arrow) of the KO pup.

(E) Hematoxylin and eosin stained sagittal section of E17.5 Viaat KO animal with heterozygous littermate for comparison. Note the hunched posture, cleft palate (closed arrow), and omphalocele containing the gut in the KO animal (open arrow).

(F–I) Nissl-stained E17.5 brain and spinal cord sections from Viaat KOs and littermate control animals. (F) Parasagittal brain sections. Panels (G) and (H) are from coronal brain sections. (G) Cortical layering appears to be normal in Viaat KO animals. (H) Hippocampus. (I) Spinal cord. Scale bar (F), 1 mm. Scale bar (G–I), 100 μm.

(Solimena et al., 1993), our data imply that the synaptic GAD67 pool is directly associated with Viaat containing synaptic vesicles. The absence of significant differences in GAD65/67 protein levels and GAD67 localization (data not shown) in whole brain may be attributable to the relative developmental lag in synaptogenesis in the E17.5 brain compared to spinal cord. There was no evidence of an impairment of synapse formation in Viaat KO neurons cultured from brain and spinal cord as judged by staining with pre- and postsynaptic markers (GAD65, synapsin, GlyR, gephyrin, $GABA_AR\beta$, and neuroligin 2; Figure 6 and data not shown).

Transmitter Release from GABAergic Striatal and Hippocampal Viaat KO Neurons Is Either Completely Eliminated or Drastically Reduced

To examine the functional role of Viaat in inhibitory synaptic transmission, we used single-neuron autaptic cultures (Pyott and Rosenmund, 2002) from E17.5 Viaat KO embryos and wt or heterozygous littermate controls. We detected no significant differences between cultures from wt and heterozygous animals in these and all subsequent experiments, and therefore pooled the data from both genotypes, designating them as wt in all electrophysiological experiments. All electrophysiological data are from recordings of autaptic cells. We first focused on cultures from striatum, which predominantly contain neurons that release GABA. In general, only nonglutamatergic neurons, identified by the insensitivity of their evoked synaptic responses to the AMPA receptor antagonist NBQX (10 μM), were analyzed in detail. While all NBQX-insensitive wt neurons (n = 97) showed robust inhibitory postsynaptic currents (IPSCs) that were blocked by the $GABA_AR$ antagonist bicuculline (10 μM), the majority (122/210; 58%) of nonglutamatergic Viaat KO neurons showed no detectable evoked response (Figure 4A). The remaining 88 cells showed a drastically reduced response amplitude (0.25 ± 0.04 nA, compared to 7.50 ± 0.47 nA in wt neurons; Figure 4A).

To confirm that the residual transmission observed in KO cells was $GABA_AR$ mediated, we examined the effects of the $GABA_AR$ and glycine receptor (GlyR) antagonists bicuculline and strychnine, respectively (Figure 4B). The complete inhibition of the residual current by bicuculline, almost no inhibition at 1 μM strychnine, and approximately 50% inhibition at 3 μM strychnine are consistent with a purely GABA-mediated IPSC (Jonas et al., 1998). In addition, we measured responses to exogenously applied GABA and the glutamate receptor agonist kainate to determine whether postsynaptic receptor sensitivity was affected. No significant differences between wt and Viaat KO neurons were detected

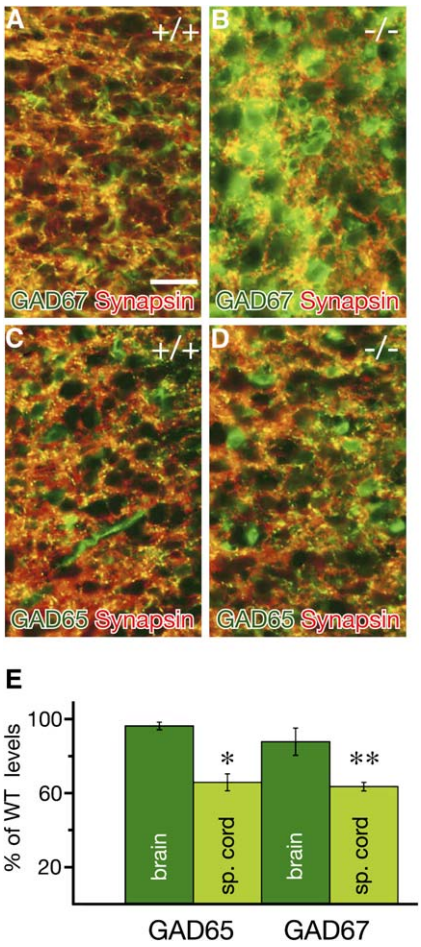

Figure 2. GAD Localization and Protein Levels

(A–D) Parasagittal sections of E17.5 ventral spinal cord. (A) GAD67 (green) shows partial colocalization with synapsin (red) in wt spinal cord. (B) In KO spinal cord, GAD67 is mostly found in the cell somata, with very little colocalization with synapsin. (C) GAD65 (green) shows partial colocalization with synapsin in wt spinal cord. (D) GAD65 staining in KO spinal cord is indistinguishable from the staining in wt samples.

(E) GAD65 and GAD67 protein levels are reduced in spinal cord but not whole brain of Viaat KO animals. GAD protein levels of three animals per genotype were analyzed with quantitative immunoblots, and wt GAD levels set to 100% (*$p < 0.05$, **$p < 0.01$). Scale bar, 25 μM.

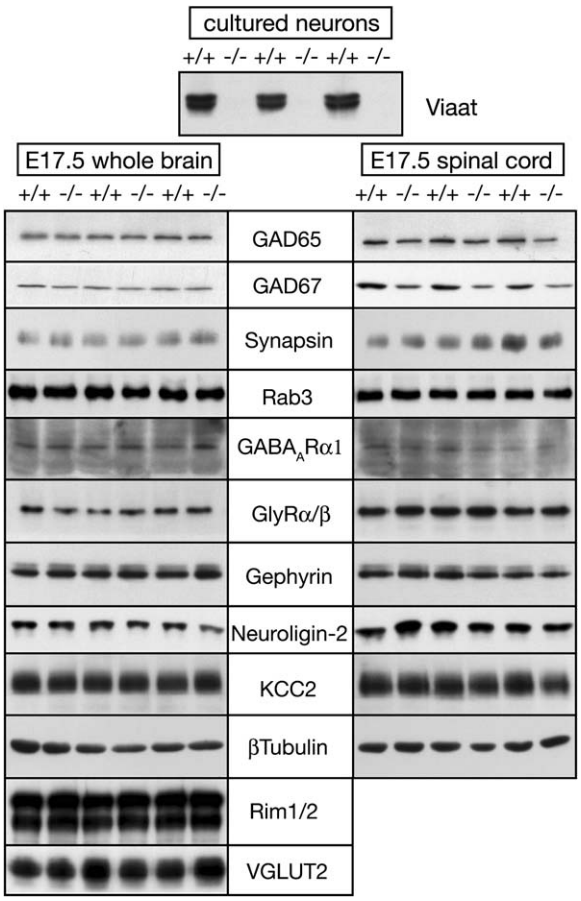

Figure 3. Immunoblot Analysis of E17.5 Whole Brain and Spinal Cord

GAD65 and GAD67 levels were reduced in spinal cord, but not in whole-brain extracts of Viaat KO mice. Note that several commercial Viaat antibodies tested on E17.5 protein extracts gave extremely high background staining. To demonstrate the absence of Viaat protein in Viaat KOs, we therefore performed immunoblots on lysate prepared form cultured neurons allowed to mature in vitro.

(Figure 4C), indicating normal postsynaptic sensitivity to GABA and glutamate.

In addition to the experiments in striatal cultures, we also examined excitatory postsynaptic current (EPSC) and IPSC amplitudes in hippocampal cultures. While evoked EPSC amplitudes as well as spontaneous miniature EPSC (mEPSC) amplitudes, which correspond to the spontaneous release of single vesicles, were indistinguishable in wt and Viaat KO neurons (data not shown), nonglutamatergic neurons derived from Viaat KO mice showed again either no synaptic response (n = 9) or very small and bicuculline-sensitive IPSC responses (n = 4). In contrast, all nonglutamatergic neurons in the wt group displayed robust IPSC amplitudes. These observations are fully consistent with the findings in the striatal cells.

Reduced Spontaneous Release Frequency and Quantal Size in Viaat KO Neurons with Residual GABA Release

To determine whether the residual GABA release in Viaat KO neurons is vesicular, we recorded miniature IPSCs (mIPSCs) from wt and KO cells (Figure 4D). KO cells that were not completely silent showed a drastic reduction in the mIPSC frequency (wt: 4.18 ± 0.29 Hz; KO: 0.12 ± 0.032 Hz), and the amplitude of detectable mIPSCs was reduced to 40% of wt levels (wt: 54.20 ± 2.59 pA; KO: 21.81 ± 1.35 pA). As for all other electrophysiological experiments, the mean mIPSC wt values represent pooled data from wt and heterozygous cultures. Although in 2-week-old heterozygous animals the amount of total brain Viaat protein was reduced to 45% ± 5% of wt levels, comparison of mIPSC amplitudes from cultured heterozygous and wt neurons revealed only a slightly reduced mIPSC amplitude in the heterozygous neurons, and the difference was statistically not significant (data not shown). The percentage of KO cells from which mIPSCs could be recorded was slightly lower than those with detectable evoked responses (31% versus 42%); however, this can be explained by

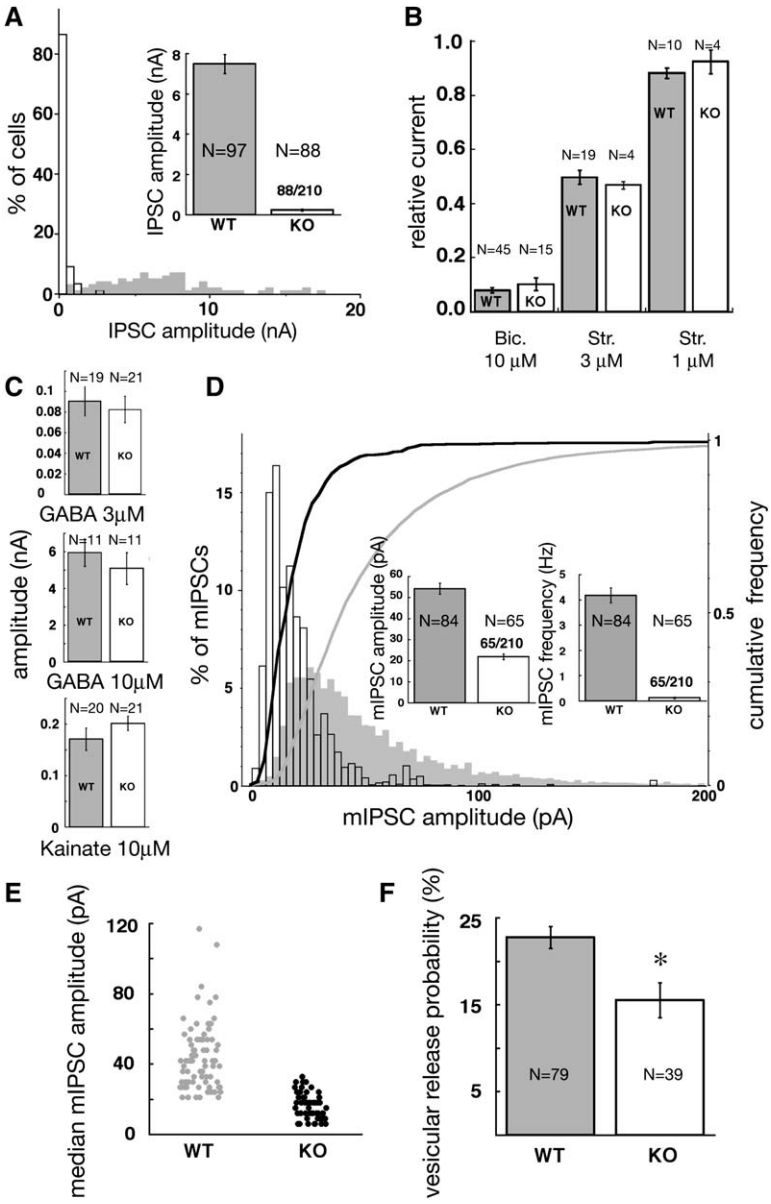

Figure 4. GABAergic IPSC and mIPSCs in Striatal Neurons

(A) IPSC amplitude distribution. The majority of nonglutamatergic KO cells were silent, but from 88 out of 210 KO neurons, reduced IPSCs were recorded. (Inset) Mean amplitude of wt and responding KO cells.

(B) Remaining relative currents (I_{inhib}/I) in the presence of bicuculline (Bic.) and strychnine (Str.) did not differ significantly between wt and KO neurons.

(C) Exogenous application of GABA and the glutamate receptor agonist kainate elicited responses from KO cells that were indistinguishable from wt responses.

(D) mIPSC amplitude distribution. The majority of KO cells were silent. However, from 31% of cells, mIPSCs of reduced amplitude size were detected at low frequencies (inset).

(E) Median mIPSC amplitudes of individual wt and KO neurons show a clear separation between wt mIPSC sizes and mIPSC sizes of KO cells with residual release.

(F) The vesicular release probability of KO neurons was reduced compared to wt.

the fact that the smaller KO mIPSCs are more likely to escape detection than a small evoked response. Plotting the median mIPSC amplitude of individual wt and KO cells (Figure 4E) showed a clear separation between the size of KO and wt mIPSCs, indicating that vesicles in KO cells are not filled to wt levels. As the majority of KO neurons were silent, and the mIPSC frequency dramatically reduced in the remainder, the Viaat KO neurons most likely release not only partially filled but mostly empty vesicles, as has been shown to be the case for deletion mutants of other vesicular transporters (Croft et al., 2005; Wojcik et al., 2004). The vesicular release probability of Viaat KO neurons with detectable evoked IPSCs was also reduced (Figure 4F) (wt: 23% ± 1.3%; KO: 16% ± 2%), and hence is likely to contribute to the observed reduction in IPSC amplitude.

Since Viaat is the only vesicular transporter for GABA described to date, the fact that vesicular GABA release is still detectable in some KO neurons is unexpected. The combination of reduced IPSC and mIPSC size in Viaat KO neurons is reminiscent of the reduction in EPSC and mEPSC size, which we previously found in neurons lacking the vesicular glutamate transporter VGLUT1 (Wojcik et al., 2004). While VGLUT2 is likely to be responsible for the residual release in VGLUT1 KO neurons (Fremeau et al., 2004a; Wojcik et al., 2004), our analysis of Viaat KO neurons uncovers the presence of a Viaat-independent vesicular uptake mechanism for GABA.

Transmitter Release from GABAergic/Glycinergic Spinal Cord Neurons from Viaat KOs Is Either Completely Eliminated or Drastically Reduced

To investigate whether the absence of Viaat also affects vesicular glycine release, we studied spinal cord neurons from E15.5 to E17.5 embryos in autaptic cultures (Figure 5). In embryonic spinal cord, $GABA_AR$-mediated neurotransmission predominates, whereas GlyR-mediated transmission increases dramatically after birth

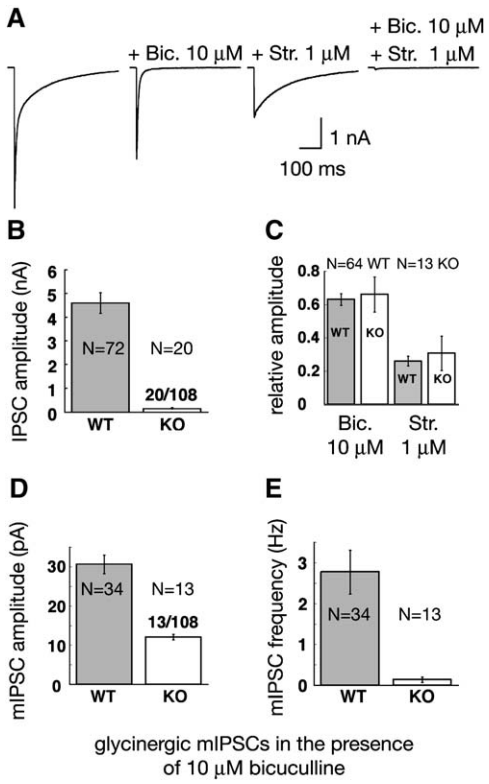

Figure 5. Glycinergic/GABAergic IPSCs and mIPSCs in Spinal Cord Neurons (DIV12–20)

(A) Raw traces of pharmacologically separated evoked $GABA_A$R- and GlyR-mediated currents from an autaptic spinal cord neuron. Bicuculline (Bic.), strychnine (Str.), and both drugs together were applied sequentially to each neuron, with intervening washout steps of 1 min.

(B) The majority of nonglutamatergic KO spinal cord neurons were silent. However, from 20 out of 108 cells, mixed glycinergic/GABAergic IPSCs of a reduced amplitude were recorded.

(C) In spite of the drastic difference in the size of KO and wt spinal cord IPSCs, their relative inhibition by bicuculline and strychnine was identical. The remaining relative amplitude was calculated as the ratio of the currents recorded with and without inhibitor (I_{inhib}/I) and did not differ between wt and KO cells.

(D) Glycinergic mIPSCs of reduced amplitude size were recorded in 13 out of 108 KO neurons in the presence of 10 μM bicuculline.

(E) Glycinergic mIPSCs in KO cells were detected at drastically reduced frequencies in the presence of 10 μM bicuculline.

(Gao et al., 2001). We found that in autaptic spinal cord cultures this developmental switch was partially recapitulated. In young cultures (DIV8–9) most neurons were purely GABAergic (data not shown), whereas in older cultures (DIV12–20) most neurons displayed a mixed GABAergic/glycinergic phenotype (Figures 5A and 5C). Mixed IPSC amplitudes were recorded from wt and Viaat KO spinal cord neurons. The majority of nonglutamatergic KO cells (88/108) were silent. However, in 20 out of 108 cells we observed IPSCs, albeit with a more than 90% reduction in amplitude (wt: 4.6 ± 0.44 nA; KO: 0.15 ± 0.02 nA; Figure 5B). The GABAergic and glycinergic components of the current were separated based on their sensitivity to bicuculline and strychnine, respectively (Figures 5A and 5C). Surprisingly, the relative sensitivities of the IPSCs of wt and KO neurons to strychnine and bicuculline were indistinguishable from each other. In both groups, approximately 65% of the response was mediated by glycine release (Figure 5C). Thus, despite the drastic reduction in vesicular neurotransmitter content, the ratio of the remaining vesicular GABA and glycine in the absence of Viaat was unchanged.

Reduced Spontaneous Release Frequency and Quantal Size in Viaat KO Neurons with Residual Glycine Release

Given that mIPSC amplitudes in GABAergic Viaat KO neurons are reduced (Figure 4D), we expected a similar phenotype in glycinergic neurons. Glycinergic mIPSCs were recorded from spinal cord neurons in the presence of 10 μM bicuculline (Figures 5D and 5E). In KO cells that were not completely silent, we detected glycinergic mIPSCs at a mean frequency of 0.14 ± 0.06 Hz, whereas the wt glycinergic mIPSC frequency was 2.77 ± 0.54 Hz (Figure 5E). The amplitude of the detectable glycinergic mIPSCs in KO cells was reduced to 39% of the wt amplitude (wt: 30.61 ± 2.39 pA; KO: 12.05 ± 0.69 pA; Figure 5D), an effect that is very similar to the mIPSC amplitude reduction found in GABAergic striatal neurons.

Normal Clustering of GlyR in Viaat KO Spinal Cord Neurons

The postsynaptic clustering of GlyR has been described as an activity-dependent process requiring the activation of the receptors themselves (Kirsch and Betz, 1998). Since the majority of Viaat KO spinal cord neurons are silent in culture, we wanted to determine whether GlyR clustering was affected in our cultures. We observed no differences between GlyR immunoreactivity in Viaat KO and wt confluent spinal cord cultures. GlyR receptor puncta were directly apposed to presynaptic terminals identified by Viaat immunoreactivity in wt neurons (data not shown) and GAD65 staining in both wt (Figures 6A–6C) and KO neurons (Figures 6D–6F). We chose DIV20/21 autaptic spinal cord neurons to quantify GlyR-positive synapses to avoid possible confounding effects of competition between synapses from silent cells and cells with residual release that might take place in confluent cultures. The average density of autaptic GlyR/GAD65-positive synapses in KO neurons was 2.2 ± 0.2 per 10 μm^2, compared to 2.1 ± 0.2 per 10 μm^2 in control neurons (Figure 6G). Synaptic clustering of GlyRs therefore did not appear to be impaired in autaptic spinal cord neurons, although 81% of neurons in the spinal cord cultures showed no detectable evoked or spontaneous electrophysiological activity (Figure 5). However, the interpretation of these data is compromised by the fact that neuron cell culture medium contains glycine, and tonic and diffuse activation of GlyRs may be sufficient to induce postsynaptic clustering. Since blocking the receptors pharmacologically was shown to lead to receptor internalization (Kirsch and Betz, 1998), we decided to determine in situ whether Viaat KO animals showed any defects in GlyR clustering. We were unable to detect any significant punctate GlyR immunoreactivity in either wt or KO spinal cord at E17.5. However, gephyrin clusters, whose appearance precedes and is thought to determine GlyR clustering in vivo and in vitro (Colin et al., 1998; Feng et al., 1998; Kirsch et al., 1993), were readily apparent and similarly frequent in both wt and KO spinal

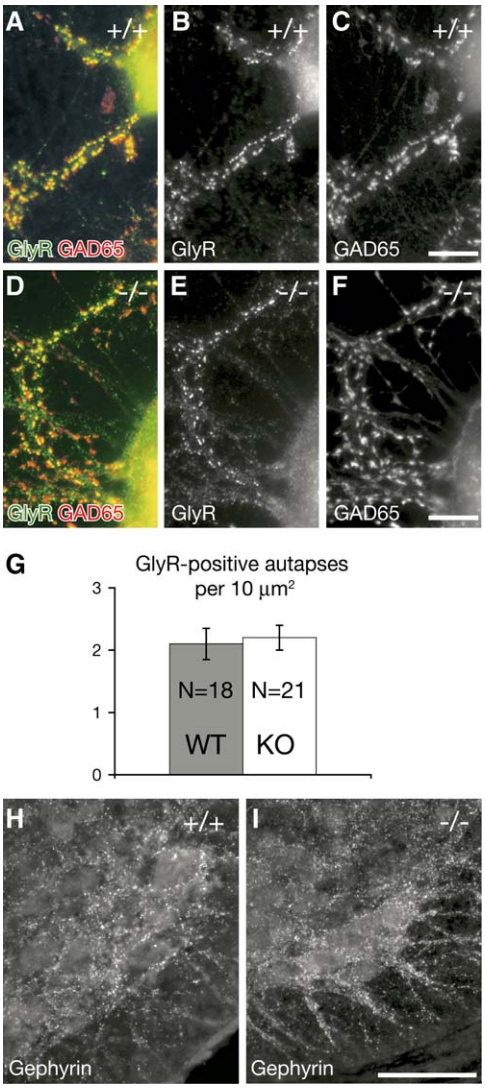

Figure 6. GlyR and Gephyrin Clustering

(A–F) Spinal cord neurons from confluent cultures at DIV28. (A) Wild-type synaptic GlyR clusters (green) apposed to GAD65 (red) positive presynaptic terminals in DIV28 cultured spinal cord neurons. (B) Wild-type synaptic GlyR clusters. (C) Wild-type synaptic GAD65 staining. (D) KO synaptic GlyR clusters (green) apposed to GAD65 (red) positive presynaptic terminals in DIV28 cultured spinal cord neurons. (E) KO synaptic GlyR clusters. (F) KO synaptic GAD65 staining.

(G) Quantification of GlyR/GAD65-positive synapses in DIV20–21 autaptic spinal cord neurons.

(H and I) Transverse sections of E17.5 ventral spinal cord. (H) Wild-type E17.5 spinal cord transverse section, ventral region, with gephyrin clusters on somata and dendrites of motor neurons. (I) KO E17.5 spinal cord transverse section, ventral region, with gephyrin clusters decorating motor neuron somata and dendrites.

Scale bar (A–F), 10 μm; scale bar (H and I), 50 μm.

cord (Figures 6H and 6I). Gephyrin clusters in the ventral spinal cord decorate the somata and dendrites of motor neurons (Figures 6H and 6I).

Viaat KO Animals Show Normal KCC2 Expression and Function

The shift from the excitatory to inhibitory action of GABA and glycine during neuronal maturation is thought to be an activity-dependent event that reflects the depolarization-induced upregulation of KCC2 expression. Since the initial proposal that GABA itself is responsible for the induction of KCC2 expression (Ganguly et al., 2001), this mechanism of induction has been disputed on the basis of contradictory findings (Ludwig et al., 2003; Titz et al., 2003). The Viaat KO mice present a valuable tool to address this unresolved issue.

Because the Viaat and KCC2 KOs show the same abdominal malformation, we hypothesized that vesicular GABA release may indeed act upstream of KCC2 induction. However, when we determined KCC2 mRNA levels in E17.5 whole brain and spinal cord by RNase protection assay, we found no significant changes in Viaat KO animals (brain: 102% ± 8% of wt; spinal cord: 98% ± 15% of wt). Similarly, protein levels of KCC2 were not reduced in whole brain and spinal cord of Viaat KO embryos (brain: 106% ± 11% of wt; spinal cord: 107% ± 8% of wt). Since a possible effect on KCC2 induction is most likely to be apparent in the brain regions that mature first and have comparatively high levels of KCC2 protein at E17.5, we also quantified KCC2 protein levels separately for the medulla and midbrain as well as the hypothalamus/thalamus. We found no differences in KCC2 levels in these regions that were statistically significant (medulla: 125% ± 16% of wt, midbrain: 125% ± 26% of wt, hypothalamus/thalamus: 99% ± 9% of wt). To examine the possibility of locally restricted effects on KCC2 expression, we performed immunofluorescence analyses of coronal brain sections and transverse spinal cord sections of E17.5 Viaat KO embryos and littermate controls. We found the KCC2 expression pattern in Viaat KO brain and spinal cord to be indistinguishable from KCC2 expression in littermate controls (Figures 7A–7H). Hence, at least in the central nervous system, vesicular GABA release is not necessary for induction of KCC2 expression. However, one caveat to consider is that the death of the KO mice may occur before the main upregulation of KCC2 levels takes place.

We therefore turned to the analysis of the Cl^- reversal potential (E_{Cl}^-) in cultured striatal neurons as a measure of KCC2 activity (Figure 8). Although the relationship between KCC2 expression levels and functional KCC2 protein may be linear only in situ and not in cultured neurons (Khirug et al., 2005), we assumed that impaired KCC2 expression would still be reflected in the intraneuronal Cl^- concentration at some point during maturation. E_{Cl}^- was determined with gramicidin perforated-patch recordings of neurons between DIV6 and DIV14. The concentration of intracellular Cl^- available for passage through Cl^- channels was calculated using the Nernst equation. Because the residual GABA release found in some Viaat KO neurons could be sufficient for the induction of KCC2 expression, we included only silent neurons in these analyses. E_{Cl}^- became more negative with increasing age of the neurons, as would be expected for a decrease in the intracellular Cl^- concentration during maturation, with no detectable difference between wt and KO neurons (Figures 8B–8D). Furthermore, no significant changes of KCC2 protein levels were detected in Viaat KO striatal cultures (KCC2 protein: 116% ± 10% of wt), demonstrating that neither KCC2 induction nor KCC2 function are impeded in the absence of Viaat. To exclude the possibility that tonic activation of GlyRs

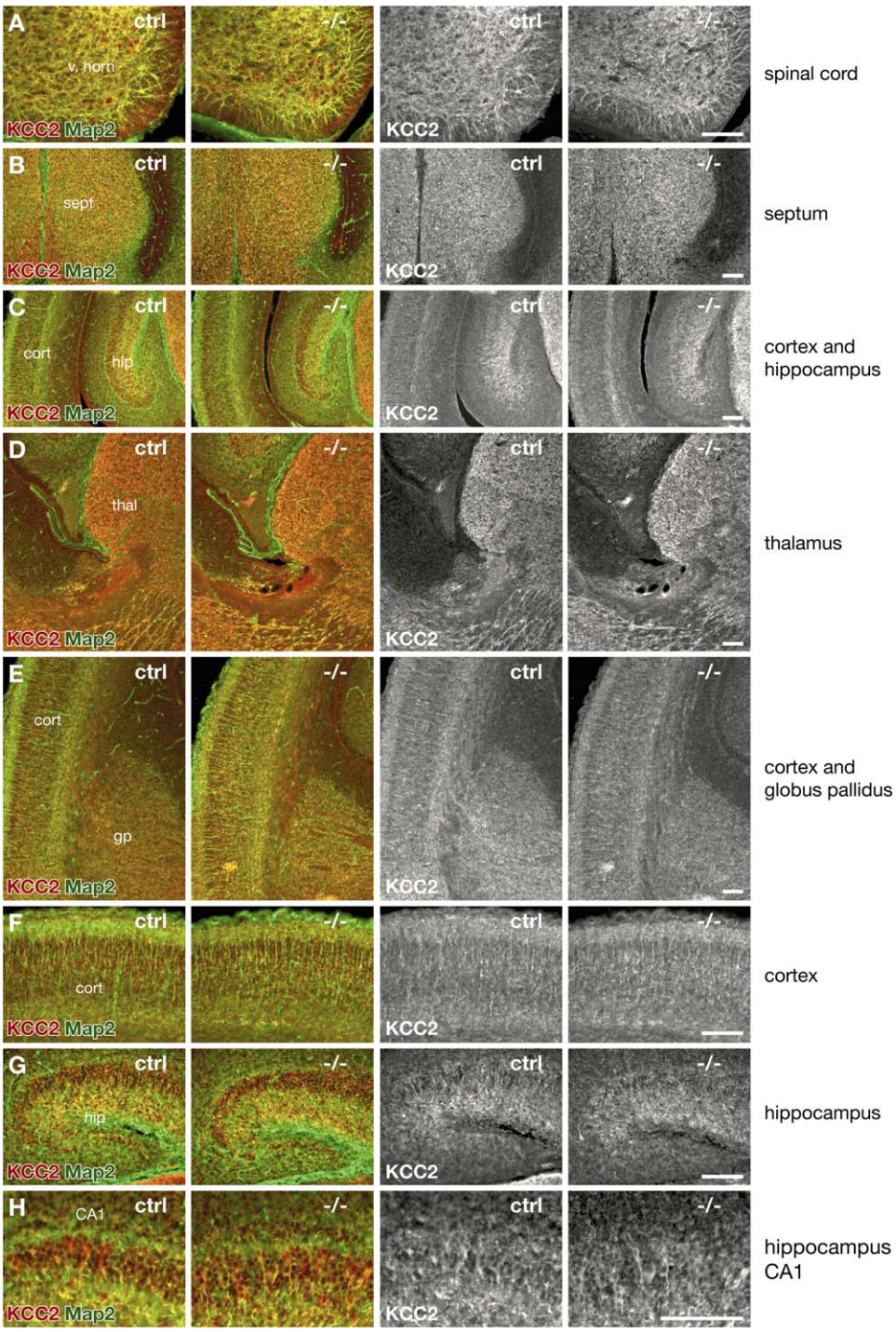

Figure 7. Identical Distribution of KCC2 Staining in E17.5 Viaat KO and Littermate Control Brain and Spinal Cord

Red immunofluorescent labeling represents KCC2 staining: the Map2 antibody (green) was used as a counterstain. Grayscale images on the right show KCC2 labeling alone. (A) Spinal cord, ventral horn. Note the KCC2 labeling in cell bodies and processes. (B) Septum. (C) Hippocampus and cortex. (D) Thalamus. (E) Cortex and globus pallidus. (F) Cortex. (G) Hippocampus. (H) Hippocampus, CA1. Note the KCC2 labeling outlining individual cell bodies and processes. v. horn, ventral horn; sept, septum; cort, cortex; hip, hippocampus; thal, thalamus; gp, globus pallidus. Scale bar, 100 μm.

through glycine present in the cell culture medium induces expression of KCC2, we also compared the intracellular Cl^- concentration and KCC2 protein levels of wt and KO neurons raised in the presence of 1 μM strychnine, but found no significant differences (data not shown).

Discussion

Corelease and Viaat-Independent Release of GABA and Glycine

The present study on Viaat-deficient mice provides direct evidence for vesicular corelease of GABA and

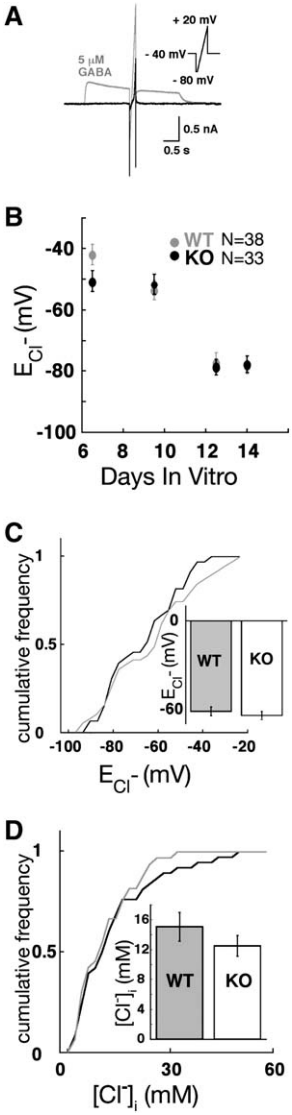

Figure 8. Gramicidin Perforated-Patch Recordings in Cultured Striatal Neurons

(A) Raw traces of the current in presence and absence of 5 μM GABA during voltage ramps from −80 mV to +20 mV. Voltage ramps were repeated three times and the chloride reversal potential (E_{Cl-}) determined. Only those cells where E_{Cl-} remained stable (±5 mV) were included in the analysis, and the concentration of intracellular Cl^- ($[Cl^-]_i$) available for passage through Cl^- channels was calculated using the Nernst equation.

(B) E_{Cl-} decreases with the maturation of the neurons in both wt and KO cultures.

(C) Cumulative representation of the E_{Cl-} determined for all cells recorded between DIV6 and DIV14. (Inset) Mean E_{Cl-} values do not differ between wt and KO cells.

(D) Cumulative representation of the internal Cl^- concentration ($[Cl^-]_i$) calculated for all cells. (Inset) Mean $[Cl^-]_i$ values of wt and KO values show no significant difference.

glycine from spinal cord neurons and shows that this corelease is due to the fact that GABA and glycine are sequestered into synaptic vesicles by the same transporter protein, Viaat. However, our analyses also indicate that at least one other transporter or transport mechanism is able to import GABA and glycine into synaptic vesicles, because some Viaat-deficient neurons still show measurable vesicular release of the two transmitters (Figures 4 and 5). The unchanged vesicular GABA/glycine ratio in Viaat KO spinal cord neurons indicates that a candidate transporter or transport mechanism should have a Viaat-like substrate specificity, but current databases contain no evidence of a transporter with high sequence homology to Viaat. Since half of the striatal and most spinal cord neurons of Viaat KO mice were silent (Figures 4 and 5), this putative transporter should be present only in a subset of neurons. Furthermore, because quantal size was reduced in Viaat KO cells, but not in heterozygous cells, this second GABA/glycine transporter would have to be either less efficient or present in a significantly lower copy number than Viaat. However, because the residual release detected in Viaat KO cells is minimal, the putative carrier is unlikely to represent a bona fide vesicular GABA/glycine transporter, and three alternative explanations might be considered. First, in the absence of Viaat, GABA and glycine may accumulate in the cytosol of the terminal and therefore be taken up by one or more vesicular transporters whose main cargo is usually another substrate. Second, minimal vesicular GABA and glycine uptake may occur via a transporter-independent mechanism, for instance during endocytosis. Third, the observed residual currents may be due to gating of $GABA_A$Rs and GlyRs by other amino acids, such as β-alanine or taurine. We consider the latter scenario to be the least likely because of the low affinity of these amino acids for $GABA_A$Rs and GlyRs (Jonas et al., 1998).

Definition of GABAergic and Glycinergic Neuronal Phenotypes

Our data show that the specification of GABAergic and glycinergic neurons is more complex and mechanistically distinct from that of the glutamatergic phenotype, where the presence of the VGLUTs alone is the determining factor (Bellocchio et al., 2000; Takamori et al., 2000). Since Viaat is the major vesicular transporter for both GABA and glycine, the development of a GABAergic versus a glycinergic phenotype is critically dependent on the relative levels of GABA and glycine available for vesicular uptake. A purely glycinergic phenotype should develop in the absence of GAD65/67, and in the presence of Viaat and the neuronal plasma membrane glycine transporter Glyt2, whose expression levels regulate quantal size at glycinergic synapses (Gomeza et al., 2003). However, genetic deletion of Glyt2 only reduces quantal glycine release and does not eliminate it (Gomeza et al., 2003), indicating that in mouse spinal cord intracellular glycine levels are sufficient for limited uptake by Viaat even in the absence of Glyt2. Although this may not be the case for all GABAergic cell types (Kalloniatis et al., 1996), the question arises as to how a purely GABAergic phenotype is determined. The expression of GAD65/67 and plasma membrane GABA transporters (GAT-1/2/3/4) alone would not suffice as a determinant of a pure GABAergic phenotype in neurons where glycine levels are sufficiently high for uptake by Viaat and would therefore be expected to result in mostly mixed-release terminals. Here, the close association of Viaat and GAD65 as well as GAD67 (Figure 2) appears to be essential to favor GABA uptake and thereby shift the balance toward the GABAergic phenotype.

The developmental transition from GABA to glycine release would then be achieved through downregulation of GAD65/67. This type of negative correlation between GAD65/67 expression and the development of a glycinergic phenotype has been demonstrated for synapses of the auditory brainstem (Nabekura et al., 2004). Interestingly, the percentage of mixed GABA/glycine release has been reported to remain relatively constant during perinatal spinal cord development (Gao et al., 2001). This mixed GABAergic/glycinergic phenotype, which is predicted to result from limited expression of GAD65/67 and plasma membrane GABA transporters, is likely be of functional relevance because corelease of GABA and glycine can provide an autoregulatory feedback mechanism through activation of presynaptic $GABA_BR$ (Lim et al., 2000), which would not be available for glycine release alone.

Gephyrin and GlyR Clustering in Viaat KO Neurons

In cultured immature spinal cord neurons, the postsynaptic clustering of gephyrin and the subsequent accumulation of GlyRs in these clusters has been shown to require GlyR activation, concomitant membrane depolarization, gating of voltage-dependent Ca^{2+} channels, and Ca^{2+} influx (Kirsch and Betz, 1998). However, in the Viaat KO mice, we found no evidence that gephyrin clustering on motor neurons in the ventral horn of the spinal cord was impaired (Figure 6), indicating that gephyrin clusters form even in the absence of GABA or glycine release. Unfortunately, the majority of gephyrin clusters in spinal cord at E17.5 does not yet correspond to mature glycinergic synapses, since we were not able to detect significant punctate GlyR immunoreactivity. This finding is consistent with a study showing that in newborn rat spinal cord the number of gephyrin clusters exceeds that of GlyR clusters, and a 1:1 correspondence is not achieved until postnatal day 10 (Colin et al., 1998).

Since the Viaat KO mice die between E18.5 and birth, we were unable to investigate the full maturation of glycinergic synapses in situ. In our spinal cord cultures, about 80% of neurons were silent, and the remainder showed dramatically reduced activity, yet we detected no difference in the density of GlyR-positive synapses between Viaat KO and wt neurons. This finding demonstrates that synaptic GlyR clustering does not require synaptic release of glycine, but we cannot exclude that the glycine present in the culture medium contributes to the maturation of glycinergic synapses in cultured spinal cord neurons, for example by inducing ectopic GlyR clusters that are subsequently recruited to synapses.

GABA/Glycine Release and KCC2 Expression

In addition to the maturation of postsynaptic specializations at glycinergic synapses, the developmental upregulation of KCC2 expression is a second developmental process for which a requirement for the depolarizing action of GABA and glycine has been described. In this context, different studies on cultured neurons came to different conclusion as to whether $GABA_AR$ activation is required for KCC2 induction (Ganguly et al., 2001) or whether this process proceeds even if $GABA_AR$ and GlyR are blocked pharmacologically (Ludwig et al., 2003; Titz et al., 2003).

At first glance, the presence of an omphalocele in Viaat and KCC2 KO mice (Hubner et al., 2001), a phenotype which is also caused by mutations affecting Pitx2 (Katz et al., 2004), a transcription factor with recognition sites in the Viaat promoter (Ebihara et al., 2003), is suggestive of a functional link between vesicular GABA/glycine release and KCC2 activity. However, we were unable to establish a causal link between vesicular GABA/glycine release and KCC2 expression in the brain or the Cl^- equilibrium potential E_{Cl-} and, by inference, KCC2 function, in our autaptic cell culture system. Although GABA released via nonvesicular mechanisms (Demarque et al., 2002) could potentially still mediate the induction of KCC2 expression in Viaat KO embryos, our data indicate that KCC2 expression is not regulated by GABA or glycine-mediated membrane depolarization. A scenario that nonetheless assumes a functional link as the reason for the shared phenotype is that the inhibitory action of GABA/glycine release may be necessary for the proper withdrawal of the embryonic gut during development, a requirement that could not be met in the absence of either Viaat or KCC2. The fact that GAD65/67 double KOs do not show the same defect (Ji et al., 1999) might argue against this explanation, but GAD-independent GABA synthesis pathways are known to exist (Seiler, 1980) and could provide limited GABA levels in the absence of GAD65/67. Alternatively, GABA/glycine release might upregulate KCC2 expression in a gut-specific, locally restricted manner, as is seen in turtle retina (Leitch et al., 2005), or Viaat and KCC2 might act in parallel pathways during gut development, involving processes that are not related to GABA/glycine release or Cl^- transport. However, KCC2 expression in embryonic gut or umbilical cord has not been demonstrated (Hubner et al., 2004) and is therefore likely to be rather low or restricted to an extremely small population of cells.

Conclusion

Our analysis demonstrates that Viaat is the main vesicular transporter for both GABA and glycine and thus explains how neurons in the mammalian central nervous system achieve corelease of these two transmitters. Apart from Viaat, neurons in the central nervous system appear to contain an as yet unknown vesicular transport system for GABA and glycine with Viaat-like substrate specificity. This transport system is responsible for the residual GABA and glycine release activity of Viaat KO neurons.

Our analyses uncovered no evidence for a role of GABA as a trophic substance. Both major developmental defects seen in Viaat KOs, failures in gut withdrawal and palate closure, can be understood as defects secondary to loss of GABA/glycine-mediated neurotransmission and complete paralysis. Furthermore, in contrast to current hypotheses, synaptogenesis and the functional maturation of GABAergic and glycinergic neurons were not impaired in Viaat KOs. Most notably, the formation of glycinergic postsynaptic specializations and the developmental induction of KCC2 expression, both processes that are thought to be dependent upon synaptic glycinergic and GABAergic activity, were normal in Viaat KOs despite an almost complete shutdown of GABAergic and glycinergic synaptic transmission.

Unfortunately, our constitutive Viaat KOs die between E18.5 and birth, which prevented us from analyzing the maturation of the GABAergic and glycinergic transmitter systems in later phases of development. Future analyses of the full spectrum of effects controlled by vesicular GABA and glycine release in the maturing nervous system will require the generation of conditional Viaat KO mice.

Experimental Procedures

Generation of Viaat KO Mice

The coding region of the Viaat gene between the start codon and an XbaI site in the first intron was replaced with a synaptobrevin 2-enhanced green fluorescence protein (syb2-EGFP) minigene followed by a loxP flanked neomycin resistance (neo) cassette through homologous recombination in embryonic stem cells (129/ola). Genomic regions used in construction of the targeting vector were identified through screening of a lambda fixII 129/Sv phage library (Stratagene). In addition to the 5 kb 5′ arm, the 2.4 kb 3′ arm, and the 2.5 kb syb2-EGFP-neo cassette, two thymidine kinase gene cassettes for negative selection were included in the targeting vector (Figure 1A). The syb2-EGFP fusion protein is not expressed in the Viaat KO mice, not even after cre-mediated removal of the neo cassette. Control animals were wild-type or heterozygous littermates of Viaat KO mice. Animal care was in accordance with the institutional guidelines.

Protein Analysis

Protein levels in mouse E17.5 brain and spinal cord homogenate as well as lysate prepared from cultured neurons were assessed by immunoblotting using the following primary antibodies: rabbit-anti-GAD65 (AB5082), mouse-anti-GAD67 (MAB5406), rabbit-anti-VGAT, rabbit-anti-$GABA_AR\alpha1$, guinea-pig-anti-VGLUT2 (all from Chemicon), mouse-anti-synapsin1, mouse-anti-GlyR(C.4a), mouse-anti-Rab3 (all from Synaptic Systems), mouse-anti-gephyrin, mouse-anti-Rim1/2 (both from BD Transduction Laboratories), mouse-anti-β-Tubulin (Sigma), rabbit-anti-neuroligin 2 (Varoqueaux et al., 2004), and rabbit-anti-KCC2 (raised against the C terminus). Peroxidase-coupled secondary antibodies to mouse and rabbit IgG (Jackson ImmunoResearch) and guinea-pig IgG (Acris) were used, followed by visualization through enhanced chemiluminescence (Amersham Pharmacia Biotech). Protein levels of GAD65, GAD67, and KCC2 were quantified using detection of fluorescently labeled secondary antibodies (IRDye 800, Odyssey) with an Odyssey infrared scanner (LI-COR Biotechnology). Expression levels were normalized to β-tubulin levels, and values are expressed as mean ± standard error. Significance was assessed using Student's t test.

RNA Analysis

Total RNA from E17.5 brain and spinal cord was prepared with Trizol reagent (Invitrogen).

KCC2 RNA levels of E17.5 brain and spinal cord were quantified by RNase protection assay (RPA) using the RPA III kit (Ambion) in combination with chemiluminescence detection (Pierce). The KCC2 probe was amplified from a mouse brain cDNA library (Clontech) using the primers 5′-GCTGGTGCTGGTGCGTGTGGAC-3′ and 5′-CGAGGTGGCCGGCTGTAGTTTC-3′. KCC2 RNA levels were normalized to cyclophilin mRNA levels. Values are expressed as mean ± standard error.

Morphological Analysis

Brain and spinal cord samples were immersion fixed (4% paraformaldehyde) and either cryoprotected (30% sucrose) and frozen or dehydrated and embedded in paraffin. For morphological comparison, frozen brain sections and paraffin-embedded spinal cord sections were stained with thionin and cresyl violet, respectively. For immunofluorescence analysis, frozen sections were stained using mouse-anti-GAD67 (MAB5406) and mouse-anti-GAD65 (MAB351R) antibodies (Chemicon) and rabbit-anti-synapsin antiserum (Synaptic Systems). To assess the distribution of KCC2 expression, rabbit-anti-KCC2 (raised against the C terminus) was used together with mouse-anti-Map2 (MAB3418, Chemicon). The incubation with primary antibodies was followed by detection with IgG-coupled Alexa Fluor 488 and Alexa Fluor 555 dyes, respectively (Molecular Probes). For immunodetection of gephyrin in E17.5 spinal cord with mouse-anti-gephyrin (Synaptic Systems), the spinal cord sections were boiled in 0.01 M citrate buffer (pH 6) prior to incubation with the antibody. Cultured neurons were stained with mouse-anti-GlyR(C.4a) (Synaptic Systems) and rabbit-anti-GAD65 (AB5082), followed by detection with IgG-coupled Alexa Fluor 488 and Alexa Fluor 633, respectively. False-color images were obtained with a conventional epifluorescence microscope (Olympus BX61).

Quantification of Synaptic GlyR Clustering

For quantification of GlyR-positive synapses, autaptic spinal cord neurons stained with mouse-anti-GlyR(C.4a) (Synaptic Systems) and rabbit-anti-GAD65 (AB5082) were photographed (Olympus BX61) and the images analyzed using analySIS^B (Olympus) software. Primary and secondary dendrites were outlined, and GlyR/GAD65-positive synapses were counted. Values are expressed as mean number of synapses per 10 μm^2 ± standard error.

Electrophysiology and Cell Culture

Microisland cultures of E17.5 striatal and hippocampal neurons and E15.5 to E17.5 spinal cord neurons were prepared according to published procedures (Pyott and Rosenmund, 2002). Experiments were performed using neuron cultures between DIV6 and 20. All extracellular solutions were applied with a custom-built fast flow system consisting of an array of flow pipes controlled by a stepper motor that allows complete and rapid solution exchange with time constants of approximately 30 ms (Pyott and Rosenmund, 2002; Rosenmund et al., 1995). Nonglutamatergic cells were identified by the insensitivity of their evoked synaptic response to the AMPA and kainate receptor antagonist NBQX (1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide, 10 μM). To distinguish between GABAergic and glycinergic IPSCs, the respective antagonists bicuculline and strychnine (bicuculline 10 μM and strychnine at 1 μM and 3 μM) were used. No tonic currents were detected during the application of bicuculline or strychnine in striatal or spinal cord cultures. Comparison of the effects of 1 μM strychnine, 3 μM strychnine, and 10 μM bicuculline was performed in striatal neurons. IPSCs were recorded sequentially during the application of bicuculline and strychnine as well as in the presence of both antagonists; the intervening wash steps with bath solution lasted 1 min. Postsynaptic GABA and AMPA receptor sensitivity was tested with the application of GABA at 3 μM or 10 μM, and 10 μM kainate, respectively. Recordings of mIPSCs were done in the presence of 300 nM tetrodotoxin, and for detection of glycinergic mIPSCs 10 μM bicuculline was applied. Gramicidin perforated-patch-clamp recordings were performed as described (Rhee et al., 1994), with 10 μg/ml gramicidin in the pipette solution. Vesicular release probability was measured as the ratio between IPSC charge and the size of the vesicle pool released in response to application of hypertonic sucrose solution (Pyott and Rosenmund, 2002), and statistical significance was assessed by unpaired Kolmogorov-Smirnov comparison. Data acquisition and analyses were done as previously described (Pyott and Rosenmund, 2002), with values expressed as mean ± standard error.

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