Chapter 5
GABA, Glycine, and Glutamate Co-Release at Developing Inhibitory Synapses

Deda C. Gillespie and Karl Kandler

Abstract Neurobiologists have long classified synaptic phenotype by a single neurotransmitter released at that synapse. Research over the past two decades has made it clear, however, that the classification of neurons and synapses as purely GABAergic, or even as purely inhibitory or excitatory, is no longer valid. In this chapter we review evidence showing that inhibitory synapses co-release multiple inhibitory neurotransmitters, and that some classical inhibitory synapses also release excitatory neurotransmitters. As multiple transmitter release is particularly prevalent at immature synapses, we pay special attention to developmental plasticity in considering possible mechanisms and functions for release of these seemingly antagonistic neurotransmitters.

List of Abbreviations

ACh	acetylcholine
AMPAR	amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ATP	adenosine triphosphate
CN	cochlear nucleus
GABA	gamma-aminobutyric acid
$GABA_AR$	GABA (A) receptor
$GABA_BR$	GABA (B) receptor
GAD	glutamic acid decarboxylase
GlyR	glycine receptor
GLYT2	glycine transporter 2
IPSC	inhibitory postsynaptic current
LSO	lateral superior olive
mIPSC	miniature inhibitory postsynaptic current
MNTB	medial nucleus of the trapezoid body

D.C. Gillespie (✉)
Department of Psychology, Neuroscience & Behaviour, McMaster University, Hamilton, ON L8S 4K1, Canada
e-mail: dgillespie@mcmaster.ca

R. Gutierrez (ed.), *Co-Existence and Co-Release of Classical Neurotransmitters*,
DOI 10.1007/978-0-387-09622-3_5,

mPSC	miniature postsynaptic current
MSO	medial superior olive
NMDAR	N-methyl D-aspartic acid receptor
P*n*	postnatal day *n*
SPN	superior paraolivary nucleus
VGAT	vesicular GABA transporter
VGLUT2	vesicular glutamate transporter 2
VGLUT3	vesicular glutamate transporter 3
VIAAT	vesicular inhibitory amino acid transporter

5.1 Introduction

Classically, many neuroscientists have used Dale's principle as a first-order descriptor of neuronal phenotype. Although referred to as the idea that the same neurotransmitter is released from all terminals of a neuron (Eccles 1964), this principle has been reduced and simplified into the widely accepted dogma: "one neuron-one neurotransmitter." In fact, many students still learn this version of Dale's principle, despite a significant and growing number of studies that have provided convincing counterexamples to invalidate this overly simplified view of neuron and synapse. In this chapter we will focus on a subset of the evidence supporting a more nuanced view of the synapse: release of multiple transmitters at inhibitory synapses. We will first consider co-release of the classic small amino acid neurotransmitters GABA and glycine and we will then consider release of GABA or glycine with other neurotransmitters, in particular glutamate. We will pay special attention to a synapse in the auditory brainstem where the three major fast neurotransmitters of the brain—GABA, glycine and glutamate—are all released during a developmentally significant period, and we will consider potential hypotheses for the function of multiple transmitter release.

5.1.1 Co-release of GABA and Glycine

Early evidence that fast inhibitory synapses might use multiple neurotransmitters came from immunohistochemical studies at the light and electron microsopic levels, which showed colocalization of markers for GABA and glycine in cerebellum, spinal cord, auditory brainstem, dorsal cochlear nucleus, and oculomotor nucleus, among others (Triller et al. 1987; Ottersen et al. 1988; Todd and Sullivan 1990; Helfert et al. 1992; Kolston et al. 1992; Wentzel et al. 1993; Juiz et al. 1996).

5.1.1.1 Spinal Cord and Brainstem

More recent studies have presented physiological evidence for concomitant release of GABA and glycine. Using whole-cell patch clamp recordings in acute slices of neonatal spinal cord, Jonas et al. (1998) recorded simultaneously from interneurons and postsynaptic presumed motor neurons. By stimulating the presynaptic

interneuron to induce monosynaptic unitary inhibitory postsynaptic currents (IPSCs), and applying antagonists of $GABA_A$ or glycine receptors ($GABA_A$Rs or GlyRs), the authors showed that the unitary IPSC comprises two components, a strychnine-sensitive component with fast kinetics characteristic of GlyR-mediated currents and a bicuculline-sensitive component with slower kinetics characteristic of $GABA_A$R-mediated currents (Fig. 5.1). Physiologically, the most convincing evidence for co-release of GABA and glycine from single vesicles comes from

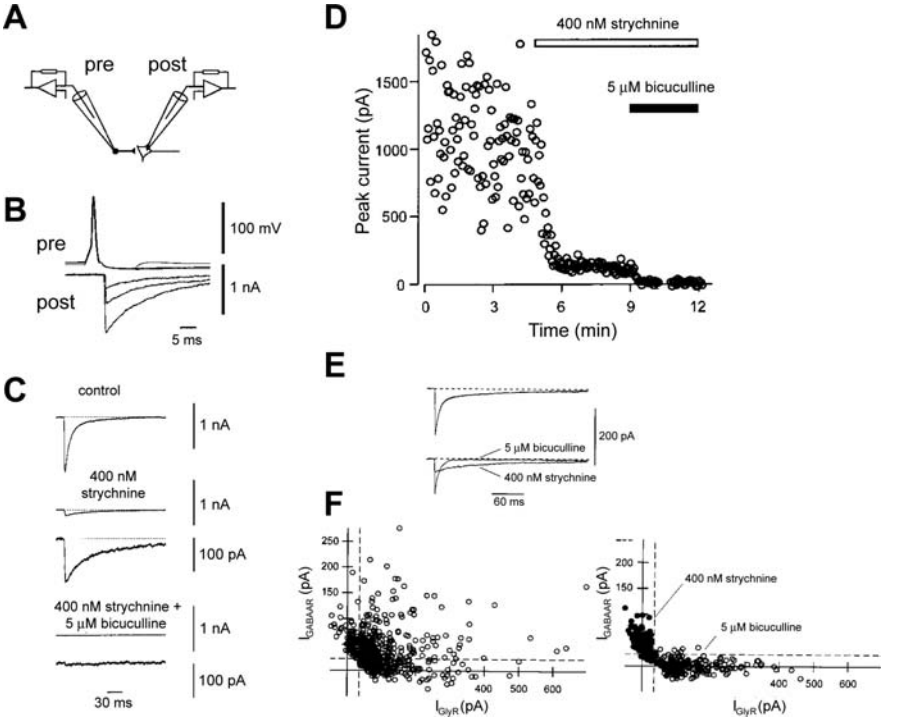

Fig. 5.1 GABA-glycine co-release at immature synapses in the spinal cord. **(a)** Schematic illustration of simultaneous whole-cell recordings from a presynaptic interneuron and a postsynaptic motoneuron. (**b)** Examples of three individual inhibitory postsynaptic currents in the motoneuron (post) evoked by three presynaptic action potentials in the interneuron (pre) (traces are overlaid). (**c, d)** Unitary postsynaptic currents are mediated by both glycine and GABA receptors. The GlyR antagonist strychnine strongly reduces postsynaptic responses but leaves a component that is blocked by the $GABA_A$R antagonist bicuculline. C) Illustrates the average of 3–10 single sweeps. In D) the peak amplitudes of single responses are plotted against time before and during antagonist application. (**e)** Glycine and GABA components of miniature IPSCs can be distinguished by their decay times. Flunitrazepam, which prolongs specifically $GABA_A$R-mediated currents, increases the decay times of $GABA_A$R- mediated currents. Average miniature IPSCs in control conditions (upper traces) and in the presence of bicuculline or strychnine (lower traces). **(f)** Scatter plots of the amplitudes of the $GABA_A$R-mediated component against amplitudes of the GlyR-mediated component of mIPSCs without (left plot) and in the presence of antagonists (right plot). Points falling outside the dashed lines indicate individual mIPSCs with dual GABA and glycine components. *Adapted with permission from Jonas et al.* 1998.

analysis of miniature IPSCs (mIPSCs), as each mIPSC is generally considered to be a single quantal event resulting from the release of the contents of a single synaptic vesicle. Miniature IPSCs recorded at the spinal interneuron-motor neuron synapse exhibit mixed components, distinguished by differing receptor pharmacology and kinetics (Fig. 5.1e-f). A subset of mIPSCs is mediated purely by $GABA_A$Rs, and a larger subset purely by GlyRs, whereas nearly half of all mIPSCs are mediated by both $GABA_A$Rs and GlyRs (points above and to right of dotted lines in Fig. 5.1f, left panel). The group of mixed mIPSCs comprising both GABAergic and glycinergic components indicates that spinal interneurons' terminals contain synaptic vesicles that include both GABA and glycine, and confirms that GABA and glycine are packaged together in individual synaptic vesicles from which they are co-released. Co-release of GABA and glycine is likely to be a common property of developing inhibitory synapses, as other groups subsequently have revealed mixed GABA and glycine release onto functional GlyRs and $GABA_A$Rs at the sympathetic preganglionic neurons of spinal cord lamina X, dorsal horn laminae I-II, and abducens and hypoglossal motoneuron synapses (O'Brien and Berger 1999; Keller et al. 2001; Russier et al. 2002; Seddik et al. 2007).

5.1.1.2 Auditory Brainstem

The lateral superior olive (LSO), a binaural nucleus in the auditory brainstem that computes interaural level differences (Boudreau and Tsuchitani 1968) (Fig. 2a), receives a prominent inhibitory input from the medial nucleus of the trapezoid body (MNTB; Moore and Caspary 1983, Caspary and Finlayson 1991). As in the spinal cord, early electron microscopic studies of immunoreactivity for the amino acid neurotransmitters in the LSO showed label for both GABA and glycine in the same synaptic terminals, and pointed to the possibility that GABA and glycine might both be released from single synapses onto principal neurons of the LSO (Helfert et al. 1992) (Fig. 5.2b).

Fig. 5.2 (continued) **(c)** MNTB-LSO synapses switch from being mainly GABAergic in newborn animals to being mainly glycinergic around hearing onset. Examples illustrate MNTB-elicited postsynaptic currents obtained in whole-cell recordings of LSO neurons in slices from 4-day-old (P4) and 14-day- old (P14) gerbils. At P4, postsynaptic currents are strongly reduced by the $GABA_A$R antagonist bicuculline (BIC) but only slightly affected by the GlyR antagonist strychnine. At P14, bicuculline has little effect whereas strychnine almost completely abolishes the responses. **(d)** Developmental down-regulation of $GABA_A$Rs and up-regulation of GlyRs in the LSO. Immunoreactivity for the β2, 3 subunits of the $GABA_A$R decreases in the gerbil from P4 to P14 while immunoreactivity for the α 1 subunit increases in the rat LSO from P4 to P10. **(e)** Co-release of GABA and glycine from single synaptic vesicles in isolated LSO neurons. Traces show examples of miniature PSCs that are glycinergic (fast decay), GABAergic (slow decay), and mixed GABA/glycine (fast and slow decay components). The plot shows changes in the proportion of the three types of mPSCs during development. In newborn rats (P1–2) most mPSCs are GABAergic whereas at P16–17 most mPSCs are glycinergic. Mixed GABA/glycine mPSCS are encountered at all ages investigated. **(f)** Immunoreactivity for glycine increases from P5 to P18 but immunoreactivity for glutamic acid decarboxylase (GAD) decreases, indicating a developmental decrease in the presynaptic release of GABA. *Adapted with permission from: B) Helfert et al.* 1992; *C, D) Kotak et al.* 1998; *D) Friauf et al. 1997; E,F) Nabekura et al.* 2004.

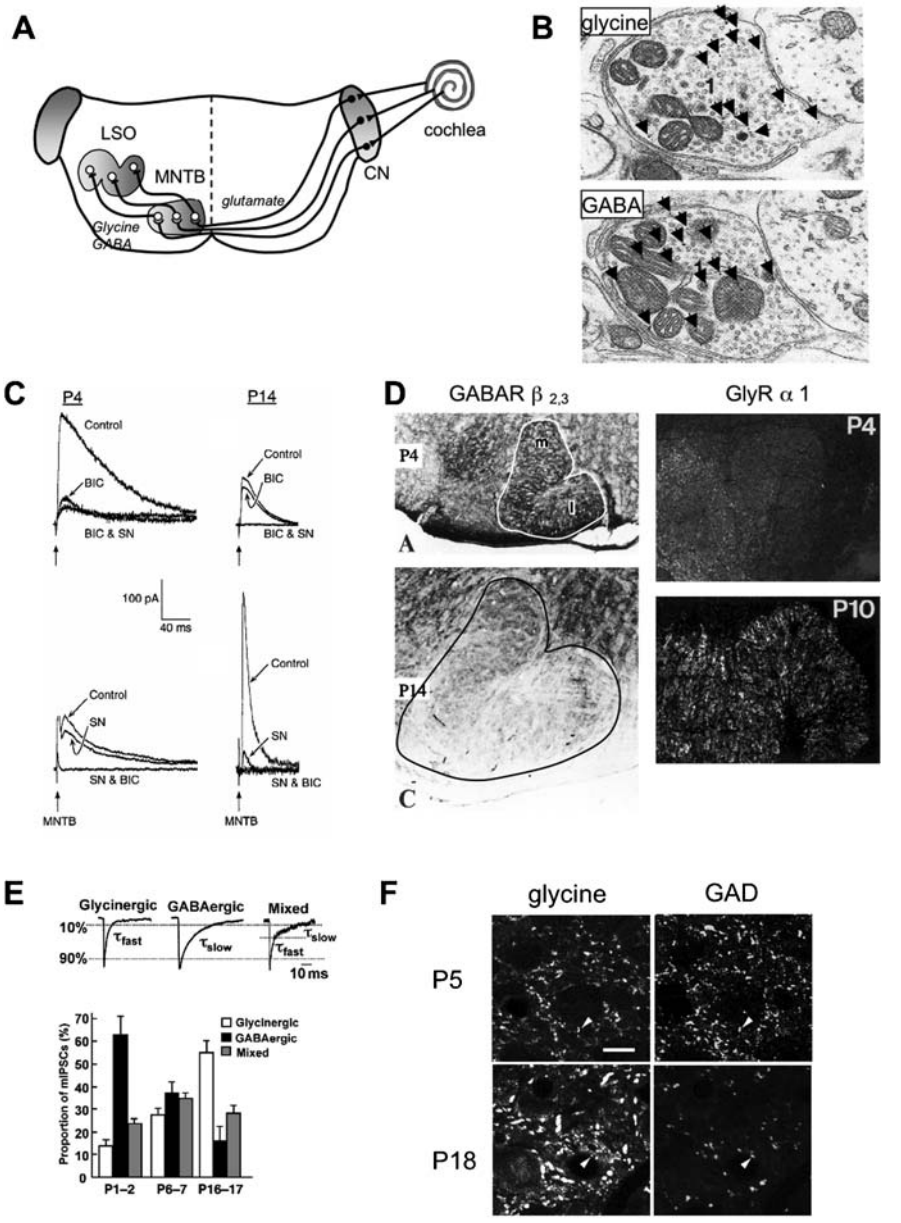

Fig. 5.2 GABA-glycine co-release at developing auditory synapses. **(a)** Schematic illustration of the inhibitory MNTB-LSO pathway in the auditory brainstem. Neurons in the medial nucleus of the trapezoid body (MNTB) receive glutamatergic inputs from the contralateral cochlear nucleus (CN). MNTB neurons give rise to a tonotopically organized inhibitory pathway to the lateral superior olive (LSO). This pathway is glycinergic in mature animals but during development is primarily GABAergic. **(b)** Electron micrographs of an individual terminal from serial sections immunolabeled for glycine and GABA. The terminal labels positively for both glycine and GABA. Gold particles that tag immunopositive sites.

Further physiological and histological studies not only corroborated coincident GABA and glycine release, but also showed that the relative balance of GABAergic and glycinergic components in the MNTB-LSO response shifts during early development. This has been shown in acute brain slices, using whole-cell recordings from LSO principal neurons to measure their physiological response to electrical stimulation of the MNTB. Using the receptor antagonists strychnine and bicuculline to separate the GlyR and $GABA_AR$ components of the response to electrical stimulation of the MNTB shows that in early neonatal (postnatal day 4; P4) gerbil, a majority of the synaptic current is mediated by $GABA_ARs$, whereas by P14 the majority of the current passes through GlyRs (Kotak et al. 1998) (Fig. 5.2c). This developmental shift from a primarily GABAergic to primarily glycinergic response is accompanied by a shift in the population of receptors expressed postsynaptically in the dendrites of LSO neurons (Fig. 5.2d). Immunoreactivity for $GABA_AR$ subunits is relatively high in the postsynaptic membrane of LSO neurons at P4 but decreases over the next 10 days. The inverse pattern is seen for markers of GlyR, as immunoreactivity for the GlyR-associated protein gephyrin increases between P4 and P14 (Korada and Schwartz 1999). Because transcripts for the GlyR subunit $\alpha 2$ are present only at low levels in the LSO throughout postnatal development, whereas transcripts for the GlyR subunit $\alpha 1$ increase over the first few weeks (Piechotta et al. 2001), this increased expression of GlyRs is probably determined largely by addition of the GlyR subunit $\alpha 1$. The decrease in $GABA_AR$ expression, concomitant with an increase in GlyR expression, causes a striking decrease in the $GABA_AR$/GlyR ratio between P4 and P14.

The developmental progression from primarily GABAergic to primarily glycinergic transmission at the developing MNTB-LSO synapse, which has now been confirmed in several studies (Henkel and Brunso-Bechtold 1998; Kullmann and Kandler 2001; Kullmann et al. 2002), results not only from a shift in receptor expression, but also from a shift in neurotransmitter release. In order to isolate and closely examine spontaneous mIPSCs, Nabekura et al. (2004) mechanically dissociated LSO principal cells along with adherent presynaptic (MNTB) terminals and then distinguished the GABAergic and glycinergic components of spontaneous mIPSCs using receptor pharmacology and kinetics. They found a mixed population of mIPSCs: at birth the majority of mIPSCs were purely GABAergic, with the remainder split between purely glycinergic and mixed gly/GABA mIPSCs; at one week the three populations were roughly equal in proportion, and at two weeks the majority of the mIPSCs were purely glycinergic. Although mixed gly/GABA mIPSCs (and hence mixed gly/GABA vesicles) were present at all ages, a clear developmental trend was seen, shifting from predominantly GABA release toward predominantly glycine release (Fig. 5.2e). Over the same period, immunolabeling for glycine increases in presynaptic terminals, while immunolabeling for the GABA marker glutamic acid decarboxylase (GAD) decreases (Fig. 5.2f). Thus, the physiological shift from GABAergic to glycinergic transmission (Kotak et al. 1998) is due to both a shift in postsynaptic receptor expression (Korada and Schwartz 1999) and a shift in vesicle content (Nabekura et al. 2004). Whether the

downregulation of $GABA_AR$ expression and the decrease in GABA content of synaptic vesicles occur simultaneously, or whether one event leads—or even induces—the other, is not known. Additionally, the mechanism that accounts for the decrease in GABA-containing synaptic vesicles is an open question, as indeed is the mechanism that determines whether synaptic vesicles contain GABA, glycine, or both.

The progression from GABAergic to glycinergic phenotype at the MNTB-LSO synapse is mirrored at other synapses in the auditory brainstem. For example, in the nearby medial superior olive (MSO), synapses in the inhibitory MNTB-MSO pathway, which are nearly exclusively glycinergic in the adult, also exhibit a prominent GABAergic component during the first postnatal week (Smith et al. 2000). Inhibitory synapses within the MNTB also show a mixed glycinergic and GABAergic phenotype during early development, switching to exclusively glycinergic by P25 (Awatramani et al. 2005). Finally, the switch from GABAergic to glycinergic function is not limited to information transfer in the feed-forward direction and the shift in receptor expression is not limited to the postsynaptic membrane. At the well-known Calyx of Held synapse in the MNTB, activation of presynaptic GlyRs normally causes increased transmitter release (Turecek and Trussell 2001). Before approximately P11, however, this GlyR modulation of glutamate release is largely absent (Turecek and Trussell 2002), and glutamate release is enhanced instead by activation of presynaptic $GABA_ARs$.

5.1.2 VGAT and Co-release of GABA/Glycine

The molecular basis for the inhibitory phenotype of MNTB-LSO synapses, as at all synapses that release inhibitory amino acids, is expression of the vesicular GABA transporter (VGAT; also known as vesicular inhibitory amino acid transporter, VIAAT). VGAT, which is localized to synaptic vesicles of glycinergic and GABAergic neurons, was first identified as a proton-coupled high-affinity GABA transporter that also transports glycine, though with lower affinity (McIntire et al. 1997; Sagne et al. 1997; Chaudhry et al. 1998). The only known vesicular transporter for inhibitory amino acids, VGAT underlies co-release of GABA and glycine (Wojcik et al. 2006). Because GABA and glycine share the same vesicular transporter, the mechanism that specifies whether vesicles are GABA- or glycinergic is unknown, though one possibility is that the relative abundance of glycine and GABA in synaptic vesicles is determined by the availability of glycine in the presynaptic terminal. At least *in vitro?* a glycinergic phenotype can be achieved in cell lines by coexpression of VGAT with GLYT2, a membrane-bound, high-affinity, Na^+-coupled, glycine uptake transporter expressed in glycinergic neuronal terminals (Liu et al. 1993; Zafra et al. 1995; Spike et al. 1997; Aubrey et al. 2007), and this mechanism may also regulate GABA/glycine vesicular content in the MNTB terminals. In the LSO, GLYT2 is expressed in presumed presynaptic terminals and GLYT2

expression levels increase during the first two postnatal weeks (Friauf et al. 1999), the same period during which the glycinergic component of MNTB-LSO synapses increases. Because, however, GLYT2 in the LSO is already present prenatally (Friauf et al. 1999)—when MNTB-LSO synapses are predominantly GABAergic—GLYT2 expression alone cannot account for the switch from predominantly GABA- to predominantly glycine-containing vesicles.

5.1.3 Functional Role for Co-release of GABA and Glycine in Developing Auditory Brainstem

Although the progression from release of mixed GABA and glycine to release of glycine alone is common in several areas during development, it is currently not known whether this developmental change is primarily a non-functional epiphenomenon reflecting other developmental processes (such as the maturation of glycine transporters) or whether early GABAergic signaling is important in establishing glycinergic networks. A number of reasons have been proposed for why early GABAergic transmission might be developmentally significant.

5.1.3.1 Trophic Actions of GABA

GABAergic neurotransmission appears to have trophic effects on several early developmental processes including synaptogenesis (for reviews, see Owens and Kriegstein 2000; Represa and Ben-Ari 2005). Although the possible trophic role of GABA is controversial, and may primarily be due to the depolarizing effect GABA exerts during early development, GABAergic neurotransmission is a common feature at many nominally non-GABAergic synapses during development (Ben-Ari et al. 1997, rev; Overstreet-Wadiche et al. 2005). If depolarization is the critical feature of early putative trophic effects of GABA, then is glycine, which also induces depolarization during early development, able to accomplish the same task? Glycine might be sufficiently depolarizing for this scenario, but if longer depolarizations were required, GABAergic transmission would be more effective, due to the slower kinetics of $GABA_A$Rs. Results from a VGAT-knockout mouse (Wojcik et al. 2006), however, showing that synaptogenesis and postsynaptic receptor clustering can occur in the absence of vesicular GABA release, argue against a critical trophic role for GABA in synapse formation.

5.1.3.2 Receptor Kinetics

At excitatory synapses in which activity-dependent mechanisms strengthen or weaken synapses, the timing of inputs relative to postsynaptic membrane depolarizations can determine both the direction and the amplitude of the plasticity (Bi and Poo 1998). This dependence on timing means that the width of the depolarization window, which is itself determined by the kinetics of response to

neurotransmitter, can influence synaptic plasticity. For example, decay times for NMDAR-mediated currents in many systems are long early in development and decrease with age (Hestrin 1992; Carmignoto and Vicini 1992), following a timecourse that corresponds to a decrease in developmental plasticity (Crair and Malenka 1995). The early expression of subunits that confer slower kinetics may lengthen the postsynaptic membrane depolarization, increasing the window for coincidence detection and allowing developing circuits access to mechanisms of synaptic plasticity during a period of long synaptic delays and low conduction velocities. Our understanding of plasticity at developing inhibitory synapses is more rudimentary (Gaiarsa et al. 2002, rev; Woodin et al. 2003; Haas et al. 2006), but if glycinergic synapses do undergo analogous timing-dependent plasticity, it is possible that the slower kinetics of $GABA_A$Rs might be better suited to mediating plasticity at the relatively slow speeds of synaptic transmission and action potential conduction of developing circuits. In this receptor kinetics hypothesis, synapses could be established using the slower kinetics of $GABA_A$Rs; the subsequent replacement of $GABA_A$Rs with GlyRs over time would cause the maturing synapse to switch to a predominantly fast glycinergic phenotype.

5.1.3.3 Receptor Clustering

An alternate scenario posits that $GABA_A$Rs are required to establish initial receptor clusters at developing synapses, and that with maturity $GABA_A$Rs are replaced within the synapse by GlyRs. Although much about the development of inhibitory synapses in general is still unknown, the GlyR- and $GABA_A$R- associated protein gephyrin is understood to be critical for clustering GlyRs at functional synapses, as gephyrin-deficient mice lack postsynaptic GlyRs clusters (Feng et al. 1998). Although loss of gephyrin also results in reduced $GABA_A$R clusters (Kneussel et al. 1999), $GABA_A$Rs that include certain receptor subunits can cluster independently of gephyrin (Kneussel et al. 2001). The additional finding that $GABA_A$R clusters can induce associated gephyrin clustering in cultured hippocampal cells over a period of several hours (Levi et al. 2004) correlates with a clustering function for early GABAergic transmission. The clustering hypothesis would assume that for some reason (as might occur, for example, with developmental regulation of splice variants for gephyrin or the gephyrin-binding GlyR β subunit; Paarmann et al. 2006; Oertel et al. 2007), gephyrin is not available to mediate the clustering and maturation of GlyRs during early developmental stages. In the absence of gephyrin, the postsynaptic components of developing inhibitory synapses could nevertheless be established by $GABA_A$R clustering. $GABA_A$R clusters in these nascent synapses would then induce gephyrin clustering, and the gephyrin clusters would in turn seed and organize GlyR clusters. Together with a subsequent loss of $GABA_A$Rs, this increase in functional GlyRs would effect the switch from GABAergic to glycinergic postsynaptic phenotype.

5.1.4 Function of Co-release at Mature Synapses

Although synapses that are mixed GABA- and glycin- ergic during development become primarily glycinergic in the adult, a GABAergic component remains in some cases (Chery and De Koninck 2000; Russier et al. 2002). In the LSO, the large-scale shift in proportion of GABA to glycine release and the upregulation of GlyRs are consistent with a developmental role for GABA. The presence of a lingering GABAergic component (Helfert et al. 1992; Nabekura et al. 2004), however, is consistent with an additional role of GABA in the physiology of the mature synapse. For example, the presence of two transmitters with differing kinetics could allow the synapse to use a greater range of IPSC shapes that might be informationally relevant (Russier et al. 2002). Alternatively, co-packaging of two transmitters could allow a single vesicle released from a presynaptic terminal to activate receptors in distinct neuronal subpopulations. For example, Golgi cells in the cerebellar granular layer release both glycine and GABA, but onto distinct postsynaptic targets: glycine acts on GlyRs on unipolar brush cells, whereas GABA acts on granule cells (Dugue et al. 2005). At present, two such separate targets are unknown in the LSO, as is also the relative location of GlyRs and $GABA_A$Rs. More generally, it has been suggested that synapses using vesicles with varying proportions of two neurotransmitters could achieve a more finely graded range of information transfer than that generally achieved with single-neurotransmitter quantal release (Somogyi 2006). Because GABA and glycine share the same vesicular transporter, however, at GABA/glycinergic synapses this scenario would require involvement of other mechanisms, such as GLYT2 (Aubrey et al. 2007), to regulate glycine concentrations in presynaptic terminals and to control the GABA:glycine ratio in synaptic vesicles. Finally, it is possible that GABA co-released in the adult LSO does not reach postsynaptic receptors, but acts only on presynaptic $GABA_B$Rs. Precedence for this idea exists in the response to glycine and GABA co-release in the adult spinal cord, where release fails to activate postsynaptic $GABA_A$Rs, but does activate postsynaptic GlyRs and presynaptic $GABA_B$Rs (Chery and De Koninck 2000). Although the expression of presynaptic $GABA_B$Rs at the MNTB-LSO synapse is not well understood, $GABA_B$Rs have been shown to modulate both glutamate and glycine release at other synapses in the auditory brainstem (Isaacson 1998, Lim et al. 2000; though note also presynaptic GlyRs and $GABA_A$Rs in developing MNTB (Turecek and Trussell 2001).

5.2 Dual Release of GABA or Glycine and Other Neurotransmitters

The discovery that GABA and glycine can be co-released at many synapses has necessitated a change in the stereotypical model of the inhibitory synapse, but because GABA and glycine are both fast inhibitory neurotransmitters, it has

not forced a complete rethinking of the inhibitory synapse. More surprising have been studies suggesting that GABA, and/or glycine, is released with quite different neurotransmitters at several synapses. The following represent a subset of a growing list of such examples.

5.2.1 Release of Multiple Transmitters in the Retina

GABA or glycine co-release with other neurotransmitters appears to be a common theme in amacrine cells of the retina. One population of glycine-immunoreactive amacrine cells is immunoreactive for the vesicular glutamate transporter VGLUT3 (Johnson et al. 2004; Haverkamp and Wassle 2004), and is thought to release glutamate vesicularly. It is unclear whether glycine functions primarily as a neurotransmitter in these synapses, though the lack of VGAT in these cells suggests that any synaptic release is likely via a membrane transporter; regardless, as glycine and glutamate are not both vesicularly released they are unlikely to be co-released from single vesicles.

A second retinal population, the starburst amacrine cells, release both ACh and GABA. Although at these synapses GABA may be released either vesicularly or via reversal of a membrane transporter, it is unlikely to be released together with ACh from single vesicles (Vaney et al. 1988; O'Malley and Masland 1989; O'Malley et al. 1992; Zheng et al. 2004). The role of cholinergic amacrine cells in generating spontaneous retinal waves that drive early visual plasticity (Meister et al. 1991; Feller et al. 1996; Hooks and Chen 2006; Huberman et al. 2006), suggests that release of GABA in the cholinergic amacrine network may affect developmental refinement by shaping spontaneous retinal activity (Wang et al. 2007).

Yet a third retinal amacrine synapse comprises both dopaminergic and GABAergic elements. At synapses between the dopaminergic amacrine cell and the AII amacrine cell, immunoreactivity for GABA colocalizes with immunoreactivity for the dopamine marker tyrosine hydroxylase (Wulle and Wagner 1990). In addition, VGAT and the dopamine-associated vesicular monoamine transporter 2 are both expressed presynaptically, while $GABA_A$Rs are expressed postsynaptically (Contini and Raviola 2003), suggesting that at these synapses both dopamine and GABA are released as functional neurotransmitters.

5.2.2 Release of Multiple Transmitters in Other Brain Areas

Retinal amacrine cells are not the only neurons that may share cholinergic and GABAergic phenotypes, as subpopulations of neurons in the dorsal horn and basal forebrain likely release both GABA and ACh (Todd 1991, Tkatch et al. 1998). GlyRs and nAChRs are found together in the same postsynaptic membranes in chick ciliary ganglion, though glycine and ACh are not truly co-released at this synapse, as ACh undergoes vesicular release and glycine is released by reversal of the glycine transporter GLYT1 (Tsen et al. 2000). In culture, a

majority of dorsal horn laminae I-III neurons co-release GABA and ATP, which probably acts on presynaptic receptors to modulate transmitter release (Jo and Schlichter 1999; Hugel and Schlichter 2003). Moving up the neuraxis, brainstem medullary raphe neurons exhibit markers correlating with release of serotonin, glutamate and GABA (Stornetta et al. 2005). Even more surprising is a recent study suggesting that the developing neuromuscular junction can express an array of phenotypes ranging from cholinergic, to glutamatergic, to glycinergic or GABAergic (Borodinsky and Spitzer 2007, see chapter 3 in this volume).

Immuno studies at the light and electron microscopic level showing GABA-immunoreactivity in glutamatergic mossy fiber terminals of hippocampus (Ottersen and Storm-Mathisen 1984; Sandler and Smith 1991) initially appeared to pose a paradox, as they suggested the possibility of co-release of excitatory and inhibitory neurotransmitters. More recently, these findings have been validated and expanded on by physiological studies showing GABA release from glutamatergic hippocampal mossy fibers in young brains (Walker et al. 2001) or after epileptic activity (Gutierrez 2000), a phenomenon discussed in a separate chapter of this volume (Gutierrez, Chapter 10).

5.2.3 Release of GABA, Glycine, and Glutamate in Auditory Brainstem

A surprise in the co-release field was that developing synapses in the MNTB-LSO projection, already shown to co-release GABA and glycine (Nabekura et al. 2004), also release glutamate as a third, and seemingly opposing, neurotransmitter (Gillespie et al. 2005) (Fig. 5.3b). The authors used whole-cell voltage-clamp recordings in acute slices of auditory brainstem to demonstrate dual release of GABA/glycine and glutamate in the LSO in response to photo-uncaging of glutamate in MNTB (to focally activate MNTB cell bodies) or to single-fiber electrical stimulation of MNTB fibers (Fig. 5.3c). Additional evidence came from immunocytochemistry showing that markers for GABAergic (VGAT) and glutamatergic (VGLUT3) transmission colocalize within the LSO in synaptic terminals of the MNTB (Fig. 5.3d, e). Just as GABA release at MNTB-LSO synapses predominates initially and declines during postnatal life, so too glutamate release is highest during the first postnatal week and declines thereafter. In the LSO, VGLUT3 expression is highest during the first two weeks, after which it declines rapidly (Gillespie et al. 2004; Blaesse et al. 2005). Glutamatergic transmission at these synapses is mediated by ionotropic glutamate receptors, largely by NMDARs. These data provide the first physiological evidence for glutamate release from GABA/glycine terminals in the mammalian brain and offer physiological support for earlier anatomical studies suggesting that glutamate and glycine might both be released at MNTB-LSO synapses (Glendenning et al. 1991; Helfert et al. 1992) (Fig. 5.3a).

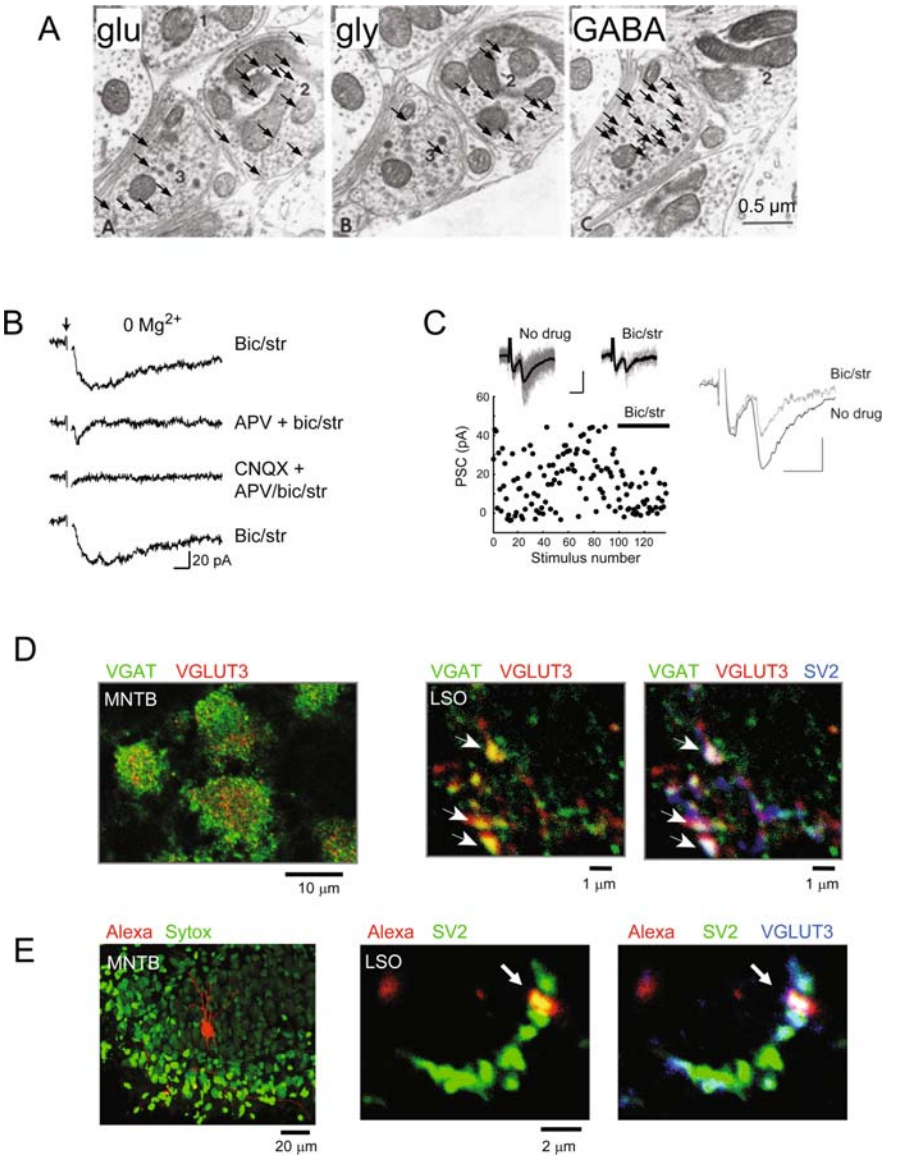

Fig. 5.3 Glutamate co-release at developing GABA/glycine auditory synapses. **(a)** Electron micrographs of an individual terminal from serial sections immunolabeled for glutamate, glycine, and GABA. Terminal 3 is immunoreactive for all three neurotransmitters, terminal 2 for glutamate and glycine. Arrows point to gold particles that tag immunopositive sites. **(b)** Blocking $GABA_A$Rs and GlyRs with bicuculline and strychnine uncovers a MNTB-elicited glutamatergic response in LSO neurons. In these recordings, magnesium was excluded from the bath to unblock NMDA receptors. Glutamatergic responses are mediated by NMDA and AMPA receptors, as they are partially blocked by the NMDAR antagonist APV and completely blocked by addition of the AMPAR antagonist CNQX. **(c)** Single

A second auditory brainstem nucleus, the superior paraolivary nucleus (SPN) also receives a prominent inhibitory input from the MNTB (Banks and Smith 1992). Early expression of VGLUT3 in the SPN is at least as striking as that in the LSO, and immunohistochemistry in the SPN shows a decrease in VGLUT3 expression that parallels the decrease in the LSO (Gillespie et al. 2004; Boulland et al. 2004; Blaesse et al. 2005). Although the synaptic circuitry of the SPN, and even the role of this nucleus in auditory processing, is still poorly understood and controversial (Dehmel et al. 2002; Behrend et al. 2002; Kulesza et al. 2003), the high neonatal expression levels of VGLUT3 and the subsequent developmental decline of VGLUT3 expression suggest that, similar to what has been proposed for the LSO, VGLUT3 also plays an important role in the development of SPN circuitry.

5.2.4 VGLUT3 and Co-release of Glutamate

The basis for glutamate release from MNTB terminals is almost certainly expression of the vesicular glutamate transporter 3 (VGLUT3), as immunohistochemistry for vesicular transporters has revealed high levels of VGLUT3 expression in MTNB cell bodies and in MNTB synaptic terminals within the LSO (Gillespie et al. 2005). The colocalization of immunofluorescence for VGLUT3 with that for VGAT in identified synaptic terminals in the LSO is consistent with the idea that individual terminals release both glutamate and GABA/glycine. VGLUT3 is a relatively rare vesicular glutamate transporter whose function has been a puzzle since its first description, when it was found to be expressed at many non-glutamatergic synapses (Fremeau et al. 2002, Gras et al. 2002, Schafer et al. 2002, Takamori et al. 2002, Seal and Edwards 2006). The temporal correlation of glutamatergic synaptic transmission with high levels of VGLUT3 expression in the LSO constituted the first experimental corroboration for the hypothesis that VGLUT3 in fact underlies vesicular glutamate release at nominally non-glutamatergic synapses.

Fig. 5.3 (continued) GABA/glycinergic MNTB axons can co-release glutamate. Activation of single MNTB axons by minimal stimulation elicits postsynaptic currents in LSO neurons (black trace is average of grey, overlaid responses) that are only partially blocked by bicuculline and strychnine. **(d)** Expression of the vesicular glutamate transporter 3 (VGLUT3) in the MNTB-LSO pathway. MNTB neurons are immunopositive for both the vesicular GABA transporter (VGAT) and VGLUT3. In the LSO, VGAT co-labels with VGLUT3 in small clusters, which also label for the synaptic vesicle protein 2 (SV2). Arrows point to presumed presynaptic endings (SV2-positive) that also label with both VGLUT3 and VGAT. **(e)** Identified MNTB terminals in the LSO express VGLUT3. A single MNTB neuron was filled with the dye Alexa 568 (red). In the LSO an Alexa-filled terminal of this neuron is identified by expression of SV2 (yellow). This terminal also expresses VGLUT3 (white). *Adapted with permission from: A) Helfert et al.* 1992; *B-E) Gillespie et al.* 2005. (*See* Color Plate 3)

5.2.4.1 Possible Glutamate Co-release at GABA/Glycinergic or Other Synapses in Other Brain Areas

The expression of VGLUT3 in many nominally non-glutamatergic synapses suggests that glutamate co-release may be a widespread phenomenon in the mammalian brain (Fremeau et al. 2002; Gras et al. 2002; Schafer et al. 2002; Takamori et al. 2002; Herzog et al. 2004; Somogyi et al. 2004; Gabellec et al. 2007). VGLUT3 expression correlates with markers for non-glutamatergic synapses within certain restricted neuronal populations, and in other cases within a restricted temporal window. For example, VGLUT3 mRNA and protein are found at high levels in the developing cerebellar nuclei, where VGAT and VGLUT3 colocalize in synaptic terminals of presumed Purkinje cells (Boulland et al. 2004; Gras et al. 2005), and where VGLUT3 is down-regulated during the first postnatal weeks. Like the MNTB-LSO synapses, these cerebellar synapses are inhibitory in the adult; unlike the MNTB-LSO synapses, glutamate release at these developing synapses has not (yet?) been demonstrated. Nevertheless, an attractive hypothesis is that VGLUT3 supports an early glutamate release that is important for establishing these inhibitory synapses.

VGLUT3 is not the only vesicular glutamate transporter found in close proximity to GABA release machinery, as both mRNA and protein for both vesicular glutamate transporter 2 (VGLUT2) and glutamic acid decarboxylase (GAD) appear to colocalize in neurons of the anteroventral periventricular nucleus of the preoptic area (Ottem et al. 2004). The expression of both VGLUT2 and VGAT in the same cells further suggests that these neurons may release both GABA and glutamate, though at present no physiological evidence supports this prediction.

5.2.5 Functional Role for Coincident Release of Glutamate, GABA, and Glycine

Glutamate release at GABA/glycinergic MNTB-LSO terminals is a new finding and the functional role of this triple release is unknown. Understanding the role of the inhibitory MNTB-LSO synapse in auditory processing, and what is known about synaptic refinement in the LSO, may cast light on this question.

Sound localization and binaural detection of signal in noise depend on the precise tonotopic alignment of inputs to the principal cells of the LSO. Neurons of the ipsilateral cochlear nucleus (CN) project directly to the LSO where they form glutamatergic synapses (Cant and Casseday 1986, Wu and Kelly 1992). Neurons from the contralateral CN make glutamatergic synapses onto principal cells of the MNTB (Smith et al. 1991), which in turn make inhibitory synapses onto LSO neurons (Moore and Caspary 1983, Caspary and Finlayson 1991). Both the excitatory and the inhibitory projections are tonotopic, but in order to establish an adult LSO in which individual principal neurons receive

inhibitory and excitatory inputs responding to the same frequency of sound, each projection also must achieve a precise tonotopic match with the complementary projection.

Why might developing GABA/glycinergic MNTB-LSO synapses release glutamate? Several converging strands of evidence lead to the hypothesis that glutamate co-release plays a critical role in developmental plasticity and refinement of the MNTB-LSO pathway. During LSO development, glutamatergic transmission at MNTB-LSO synapses is developmentally regulated, as is also the expression level of VGLUT3, the protein that presumably supports this glutamate release. In addition, the period when glutamatergic transmission is most prominent corresponds to the period of major functional refinement in the MNTB-LSO projection by synapse elimination (Kim and Kandler 2003). Additionally, decreased glutamatergic transmission and VGLUT3 expression persist for a short period after hearing onset, during a time of increased sharpness in the frequency tuning and alignment of excitatory and inhibitory responses of LSO neurons (Sanes and Rubel 1988). Furthermore, glutamate released at MNTB-LSO synapses activates postsynaptic NMDA receptors (NMDARs), the subtype of ionotropic glutamate receptor closely linked to induction of synaptic plasticity in a variety of excitatory and inhibitory synapses (Kullmann et al. 2000, rev). Finally, the peak of the period of glutamatergic transmission corresponds to the peak period when GABA and glycine are still depolarizing in the LSO (Kandler and Friauf 1995). The depolarizing action of GABA and glycine may be functionally relevant, as it could provide the critical step necessary for NMDAR-dependent plasticity: it could relieve the voltage-sensitive magnesium block of NMDARs, thus allowing co-released glutamate to activate NMDARs.

The temporal correlation of these three transient developmental periods: (1) major functional refinement, (2) glutamate release onto NMDARs, and (3) depolarizing action of GABA and glycine—supports the hypothesis that glutamate release from MNTB terminals onto NMDAR-containing LSO dendrites participates in synaptic refinement in this system. This could occur at one (or both) of two stages: either (a) functional refinement of the MNTB-LSO pathway that occurs before hearing onset and is thought to be directed by spontaneous patterned activity from the cochlea (Lippe 1994; Kros et al. 1998; Beutner and Moser 2001), or (b) subsequent fine-tuning of CN-LSO and MNTB-LSO pathway alignment that may be guided by auditory experience after hearing onset (Sanes and Rubel 1988; Echteler et al. 1989).

During the first stage of synaptic refinement in the MNTB-LSO pathway, local GABA/glycinergic depolarization could relieve the Mg^{++} block of NMDARs (Leinekugel et al. 1997), inducing Ca-influx through NMDA receptors at active MNTB-LSO synapses. In many developing systems, NMDAR-mediated calcium influx is essential for excitatory synaptic plasticity, such as elimination of glutamatergic synapses (Rabacchi et al. 1992, Kakizawa et al. 2000) or insertion of AMPA receptors at "silent" synapses (Isaac et al. 1997, Liao et al. 1999). In the LSO, NMDAR-mediated Ca-influx could play a role in clustering or insertion of GABA and glycine receptors (Kano et al. 1992, Otis

et al. 1994; Charpier et al. 1995; Kirsch and Betz 1998; Moss and Smart 2001). Ca-dependent and/or NMDAR-dependent plasticity at inhibitory synapses has been demonstrated in a number of inhibitory systems (Kano et al. 1992, Komatsu 1994, Oda et al. 1995, McLean et al. 1996, Wang and Stelzer 1996, Caillard et al. 1999, Ouardouz and Sastry 2000), although the route by which NMDARs are activated in the absence of synaptically released glutamate has remained an open question. Glutamate release, accompanied by release of depolarizing GABA/glycine, provides an answer to this question by allowing inhibitory synapses to access NMDARs and their downstream machinery of synaptic plasticity, independent of glutamate from other sources. Although at LSO-MNTB synapses a form of LTD can be induced through non-NMDAR-dependent mechanisms involving $GABA_BRs$ (Kotak et al. 2001, Chang et al. 2003), additional forms of activity-dependent synaptic plasticity may also occur at this synapse, hypothetically induced through glutamate release and activation of NMDARs.

Finer scale synaptic refinement during the initial period after hearing onset presents a different problem. Despite our ever-deepening understanding of synaptic plasticity at individual synapses, it has generally been difficult—with some exceptions (e.g., Lien et al. 2006, Nugent et al. 2007)—to determine how the finely tuned coordinate refinement of inhibitory and excitatory inputs to a single neuron might occur. It is tempting to speculate that the reduced levels of VGLUT3 expression, glutamate release, and NMDAR activation that remain in the auditory brainstem after hearing onset might play a role in stimulus-driven alignment of glutamatergic and GABA/glycinergic inputs. By the time of hearing onset, GABA and glycine are hyperpolarizing in the LSO (Kandler and Friauf 1995; Ehrlich et al. 1999), and the major period of functional refinement in the MNTB-LSO pathway is complete (Kim and Kandler 2003), although anatomical refinement occurs during the first few days after hearing onset (Sanes and Siverls 1991). One specific hypothesis for NMDAR-mediated alignment is that the CN-LSO pathway could signal to MNTB-LSO synapses through back-propagating action potentials—or through sufficiently strong depolarization arising in nearby excitatory (CN-LSO) synapses—that relieve the Mg^{++}-block at NMDARs in MNTB-LSO synapses. Simultaneous relief of Mg^{++} block and release of glutamate from MNTB terminals would activate NMDARs in MNTB-LSO synapses, allowing the postsynaptic neuron to detect coincident input from the excitatory CN-LSO pathway and the inhibitory MNTB-LSO pathway. In this strategy, late finescale refinement mediated by NMDAR activation would likely occur not at the excitatory CN-LSO synapses, but rather at the GABA/glycinergic MNTB-LSO synapses. An alternative, reversed, scenario is that glutamate spillover from MNTB-LSO synapses reaches nearby CN-LSO synapses to allow NMDARs in the CN-LSO synapses to detect coincident inputs. Although we know very little about the locations of developing synaptic inputs on LSO principal cells, this scenario would more strongly depend on parameters such as reuptake and the physical locations of excitatory and inhibitory synapses (Rusakov and Kullmann 1998). These additional constraints on NMDAR activation appear to make this scenario less

generally applicable than one dependent on backpropagating spiking activity. Nevertheless, both options offer models for how inputs of opposite sign might signal each other through the postsynaptic neuron to achieve coordinated refinement of excitatory and inhibitory synapses.

5.2.6 *Molecular Basis for Release of Glutamate with GABA and Glycine*

For the strong version of this glutamate-in-inhibitory-plasticity hypothesis to hold in the most limiting case, we might expect that glutamate and GABA/glycine would be released from the same synapse even when stimulation of the presynaptic terminal resulted in release of only a single vesicle. This would require that GABA/glycine and glutamate be packaged together in individual synaptic vesicles. This limiting case version of the hypothesis predicts the existence of individual synaptic vesicles whose membranes contain both VGLUT3 and VGAT. Immuno-EM methods are unfortunately insufficiently precise to answer this question (see e.g. Bergersen et al. 2003), though paired MNTB-LSO recordings or recordings of spontaneous mIPSCs at the MNTB-LSO synapse could offer insight into this question.

Alternatively, glutamate and GABA/glycine may be packaged in distinct populations of synaptic vesicles. This would not necessarily invalidate the hypothesis that glutamate release plays a central role in activity-dependent plasticity at glycinergic synapses. Separate vesicle populations with distinct spatial distributions could participate differently in transmission and plasticity, or release probabilities for GABA/glycinergic vesicles relative to those for glutamatergic vesicles—perhaps via differential expression of distinct synaptotagmin isoforms (Xu et al. 2007)—could be adjusted to maximize the probability of glutamate release within a certain range of firing rates. Under this scenario, the distribution of mPSCs seen at the MNTB-LSO synapses would include at least some purely glutamatergic and/or some purely GABA/glycinergic mPSCs.

Regardless of whether VGLUT3, glutamate release and NMDAR activation mediate plasticity at MNTB-LSO synapses, it will be of great interest to determine whether VGLUT3 and VGAT are in fact inserted in the membrane of the same synaptic vesicles. This possibility seems unlikely, but it may not be preposterous. Although in *Drosophila* a single vesicular glutamate transporter is sufficient to load a glutamatergic vesicle (Daniels et al. 2006), a given mammalian synaptic vesicle may contain approximately 10 transporters (Takamori et al. 2006). Furthermore, two distinct vesicular transporter types, VGLUT1 and VGLUT2, have been found coexpressed in the same synaptic vesicles in the developing hippocampus (Herzog et al. 2006). It may be possible in the developing LSO, by extension, that VGLUT3 and VGAT are transiently co-expressed in the same synaptic vesicles and that single synaptic vesicles contain both excitatory and inhibitory classical fast neurotransmitters.

5.3 Summary

Evidence gleaned over the past decade has forced us to dramatically change our picture of inhibitory synapses. In the first place, nominally inhibitory synapses do not always inhibit their postsynaptic neurons; at early periods, GABA and glycinergic synapses in many parts of the nervous system are depolarizing and even excitatory. Inhibitory information transfer is not strictly unidirectional; presynaptic GABARs and glyRs can modulate release of GABA and glycine. Glycinergic synapses do not always release (much) glycine; immature glycinergic synapses in brainstem and spinal cord in fact release primarily GABA. And finally, at the synapse formerly known as glycinergic, the GABA/glycinergic MNTB-LSO synapse of the auditory brainstem, "inhibitory" synapses do not release solely inhibitory neurotransmitters; nascent glycinergic synapses release the inhibitory neurotransmitters GABA and glycine and the excitatory neurotransmitter glutamate. These findings have forced us to begin to see the inhibitory synapse as a much more complex and exciting unit than it previously appeared.

These findings also force us to consider what the function of multiple transmitter release might be. Of particular interest is the triple release of glutamate, GABA and glycine in the developing MNTB-LSO pathway. This pathway exhibits a rich repertoire of developmental changes, including synapse elimination and strengthening, during the period corresponding to glutamate release and NMDAR activation. The MNTB-LSO pathway has been seen as an elegant model system for understanding the mechanisms by which inhibitory circuits are assembled and refined. With its precisely converging tonotopic projections from excitatory and inhibitory pathways, the LSO offers an exceptionally well-organized model for delving into questions of inhibitory circuit development and of the more complex coordinated refinement of inhibitory and excitatory inputs. The unexpected discovery that the "purely inhibitory" pathway is not so pure has forced us to redraw our model system, but it has also opened up new and exciting research directions.

References

Aubrey KR, Rossi FM, Ruivo R, Alboni S, Bellenchi GC, Le Goff A, Gasnier B, Supplisson S (2007) The transporters GlyT2 and VIAAT cooperate to determine the vesicular glycinergic phenotype. J Neurosci 27:6273–6281

Awatramani GB, Turecek R, Trussell LO (2005) Staggered development of GABAergic and glycinergic transmission in the MNTB. J Neurophysiol 93:819–828

Banks MI, Smith PH (1992) Intracellular recordings from neurobiotin-labeled cells in brain slices of the rat medial nucleus of the trapezoid body. J Neurosci 12:2819–2837

Behrend O, Brand A, Kapfer C, Grothe B (2002) Auditory response properties in the superior paraolivary nucleus of the gerbil. J Neurophysiol 87:2915–2928

Ben-Ari Y, Khazipov R, Leinekugel X, Caillard O, Gaiarsa JL (1997) GABAA, NMDA and AMPA receptors: a developmentally regulated 'ménage à trois'. Trends Neurosci 20:523–529

Bergersen L, Ruiz A, Bjaalie JG, Kullmann DM, Gundersen V (2003) GABA and GABAA receptors at hippocampal mossy fibre synapses. Eur J Neurosci 18:931–941
Beutner D, Moser T (2001) The presynaptic function of mouse cochlear inner hair cells during development of hearing. J Neurosci 21:4593–4599
Bi GQ, Poo MM (1998) Synaptic modifications in cultured hippocampal neurons: dependence on spike timing, synaptic strength, and postsynaptic cell type. J Neurosci 18:10464–10472
Blaesse P, Ehrhardt S, Friauf E, Nothwang HG (2005) Developmental pattern of three vesicular glutamate transporters in the rat superior olivary complex. Cell Tissue Res 320:33–50
Borodinsky LN, Spitzer NC (2007) Activity-dependent neurotransmitter-receptor matching at the neuromuscular junction. Proc Natl Acad Sci USA. 104:335–340
Boudreau JC, Tsuchitani C (1968) Binaural interaction in the cat superior olive S segment. J Neurophysiol 31:442–454
Boulland JL, Qureshi T, Seal RP, Rafiki A, Gundersen V, Bergersen LH, Fremeau RT Jr, Edwards RH, Storm-Mathisen J, Chaudhry FA (2004) Expression of the vesicular glutamate transporters during development indicates the widespread corelease of multiple neurotransmitters. J Comp Neurol 480:264–280
Caillard O, Ben-Ari Y, Gaiarsa JL (1999) Mechanisms of induction and expression of long-term depression at GABAergic synapses in the neonatal rat hippocampus. J Neurosci 19:7568–7577
Cant NB, Casseday JH (1986) Projections from the anteroventral cochlear nucleus to the lateral and medial superior olivary nuclei. J Comp Neurol 247:457–476
Carmignoto G, Vicini S (1992) Activity-dependent decrease in NMDA receptor responses during development of the visual cortex. Science 258:1007–1011
Caspary DM, Finlayson PG (1991) Superior olivary complex: functional neuropharmacology of the principal cell types. In Neurobiology of hearing: the central auditory system (ed. RA Altschuler et al), 141–161
Chang EH, Kotak VC, Sanes DH (2003) Long-term depression of synaptic inhibition is expressed postsynaptically in the developing auditory system. J Neurophysiol 90:1479–1488
Charpier S, Behrends JC, Triller A, Faber DS, Korn H l (1995) "Latent" inhibitory connections become functional during activity-dependent plasticity. Proc Natl Acad Sci 92:117–120
Chaudhry FA, Reimer RJ, Bellocchio EE, Danbolt NC, Osen KK, Edwards RH, Storm-Mathisen J (1998) The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. J Neurosci 18:9733–9750
Chery N, De Koninck Y (2000) GABA(B) receptors are the first target of released GABA at lamina I inhibitory synapses in the adult rat spinal cord. J Neurophysiol 84:1006–1011
Contini M, Raviola E (2003) GABAergic synapses made by a retinal dopaminergic neuron. Proc Natl Acad Sci USA. 100:1358–1363
Crair MC, Malenka RC (1995) A critical period for long-term potentiation at thalamocortical synapses. Nature 375:325–328
Daniels RW, Collins CA, Chen K, Gelfand MV, Featherstone DE, DiAntonio A (2006) A single vesicular glutamate transporter is sufficient to fill a synaptic vesicle. Neuron 49:11–16
Dehmel S, Kopp-Scheinpflug C, Dorrscheidt GJ, Rubsamen R (2002) Electrophysiological characterization of the superior paraolivary nucleus in the Mongolian gerbil. Hear Res 172:18–36
Dugue GP, Dumoulin A, Triller A, Dieudonne S (2005) Target-dependent use of co-released inhibitory transmitters at central synapses. J Neurosci 25:6490–6498
Eccles JC (1964) The physiology of synapses. Springer, Berlin
Echteler, SM, Arjmand, E, Dallos, P (1989) Developmental alterations in the frequency map of the mammalian cochlea. Nature 341:147–149
Ehrlich I, Lohrke S, Friauf E (1999) Shift from depolarizing to hyperpolarizing glycine action in rat auditory neurones is due to age-dependent Cl- regulation. J Physiol 520:121–137

Feller MB, Wellis DP, Stellwagen D, Werblin FS, Shatz CJ (1996) Requirement for cholinergic synaptic transmission in the propagation of spontaneous retinal waves. Science 272:1182–1187

Feng G, Tintrup H, Kirsch J, Nichol MC, Kuhse J, Betz H, Sanes JR (1998) Dual requirement for gephyrin in glycine receptor clustering and molybdoenzyme activity. Science 282:1321–1324

Fremeau RT Jr, Burman J, Qureshi T, Tran CH, Proctor J, Johnson J, Zhang H, Sulzer D, Copenhagen DR, Storm-Mathisen J, Reimer RJ, Chaudhry FA, Edwards RH (2002) The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. Proc Natl Acad Sci USA. 99:14488–14493

Friauf E, Aragon C, Lohrke S, Westenfelder B, Zafra F (1999) Developmental expression of the glycine transporter GLYT2 in the auditory system of rats suggests involvement in synapse maturation. J Comp Neurol 412:17–37

Gabellec MM, Panzanelli P, Sassoe-Pognetto M, Lledo PM (2007) Synapse-specific localization of vesicular glutamate transporters in the rat olfactory bulb. Eur J Neurosci 25:1373–1383

Gaiarsa JL, Caillard O, Ben-Ari Y (2002) Long-term plasticity at GABAergic and glycinergic synapses: mechanisms and functional significance. Tr Neurosci 25: 564–570

Gillespie DC, Cihil K, Kandler K (2004) Developmental expression patterns of the vesicular glutamate transporters VGLUT1–3 in the auditory brainstem. Soc Nsci Abstr 947.12

Gillespie DC, Kim G, Kandler K (2005) Inhibitory synapses in the developing auditory system are glutamatergic. Nat Neurosci 8:332–338

Glendenning KK, Masterton RB, Baker BN, Wenthold RJ (1991) Acoustic chiasm. III: Nature, distribution, and sources of afferents to the lateral superior olive in the cat. J Comp Neurol 310:377–400

Gras C, Herzog E, Bellenchi GC, Bernard V, Ravassard P, Pohl M, Gasnier B, Giros B, El Mestikawy S (2002) A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. J Neurosci 22:5442–5451

Gras C, Vinatier J, Amilhon B, Guerci A, Christov C, Ravassard P, Giros B, El Mestikawy S (2005) Developmentally regulated expression of VGLUT3 during early post-natal life. Neuropharmacology 49:901–911

Gutierrez R (2000) Seizures induce simultaneous GABAergic and glutamatergic transmission in the dentate gyrus-CA3 system. J Neurophysiol 84:3088–3090

Haas JS, Nowotny T, Abarbanel HD (2006) Spike-timing-dependent plasticity of inhibitory synapses in the entorhinal cortex. J Neurophysiol 96:3305–3313

Haverkamp S, Wassle H (2004) Characterization of an amacrine cell type of the mammalian retina immunoreactive for vesicular glutamate transporter 3. J Comp Neurol 468:251–263

Helfert RH, Juiz JM, Bledsoe SC Jr, Bonneau JM, Wenthold RJ, Altschuler RA (1992) Patterns of glutamate, glycine, and GABA immunolabeling in four synaptic terminal classes in the lateral superior olive of the guinea pig. J Comp Neurol 323:305–325

Henkel CK, Brunso-Bechtold JK (1998) Calcium-binding proteins and GABA reveal spatial segregation of cell types within the developing lateral superior olivary nucleus of the ferret. Microsc Res Tech 41:234–245

Herzog E, Gilchrist J, Gras C, Muzerelle A, Ravassard P, Giros B, Gaspar P, El Mestikawy S (2004) Localization of VGLUT3, the vesicular glutamate transporter type 3, in the rat brain. Neuroscience 123:983–1002

Herzog E, Takamori S, Jahn R, Brose N, Wojcik SM (2006) Synaptic and vesicular co-localization of the glutamate transporters VGLUT1 and VGLUT2 in the mouse hippocampus. J Neurochem 99:1011–1018

Hestrin S (1992) Developmental regulation of NMDA receptor-mediated synaptic currents at a central synapse. Nature 357:686–689

Hooks BM, Chen C (2006) Distinct roles for spontaneous and visual activity in remodeling of the retinogeniculate synapse. Neuron 52:281–291

Huberman AD, Speer CM, Chapman B (2006) Spontaneous retinal activity mediates development of ocular dominance columns and binocular receptive fields in v1. Neuron 52:247–254
Hugel S, Schlichter R (2003) Convergent control of synaptic GABA release from rat dorsal horn neurones by adenosine and GABA autoreceptors. J Physiol 551:479–489
Isaac JT, Crair MC, Nicoll RA, Malenka RC (1997) Silent synapses during development of thalamocortical inputs. Neuron 18:269–280
Isaacson JS (1998) GABAB receptor-mediated modulation of presynaptic currents and excitatory transmission at a fast central synapse. J Neurophysiol 80:1571–1576
Jo YH, Schlichter R (1999) Synaptic corelease of ATP and GABA in cultured spinal neurons. Nat Neurosc. 2:241–245
Johnson J, Sherry DM, Liu X, Fremeau RT Jr, Seal RP, Edwards RH, Copenhagen DR (2004) Vesicular glutamate transporter 3 expression identifies glutamatergic amacrine cells in the rodent retina. J Comp Neurol 477:386–398
Jonas P, Bischofberger J, Sandkuhler J. (1998) Corelease of two fast neurotransmitters at a central synapse. Science 281:419–424
Juiz JM, Helfert RH, Bonneau JM, Wenthold RJ, Altschuler RA l (1996) Three classes of inhibitory amino acid terminals in the cochlear nucleus of the guinea pig. J Comp Neurol 373:11–26
Kakizawa S, Yamasaki M, Watanabe M, Kano M (2000) Critical period for activity-dependent synapse elimination in developing cerebellum. J Neurosci 20:4954–4961
Kandler K, Friauf E (1995) Development of glycinergic and glutamatergic synaptic transmission in the auditory brainstem of perinatal rats. J Neurosci 15:6890–6894
Kano M, Rexhausen U, Dresse, J, Konnerth A (1992) Synaptic excitation produces a long-lasting rebound potentiation of inhibitory synaptic signals in cerebellar Purkinje cells. Nature 356:601–604
Keller AF, Coull JA, Chery N, Poisbeau P, De Koninck Y (2001) Region-specific developmental specialization of GABA-glycine cosynapses in laminas I-II of the rat spinal dorsal horn. J Neurosci 21:7871–7880
Kim GS, Kandler K (2003) Elimination and strengthening of glycinergic/GABAergic connections during tonotopic map formation. Nat Neurosci 6:282–290
Kirsch J, Betz H (1998) Glycine-receptor activation is required for receptor clustering in spinal neurons. Nature 392:717–720
Kneussel M, Brandstatter JH, Gasnier B, Feng G, Sanes JR, Betz H (2001) Gephyrin-independent clustering of postsynaptic GABA(A) receptor subtypes. Mol Cell Neurosci 17:973–982
Kneussel M, Brandstatter JH, Laube B, Stahl S, Muller U, Betz H (1999) Loss of postsynaptic GABA(A) receptor clustering in gephyrin-deficient mice. J Neurosci 19:9289–9297
Kolston J, Osen KK, Hackney CM, Ottersen OP, Storm-Mathisen J (1992) An atlas of glycine- and GABA-like immunoreactivity and colocalization in the cochlear nuclear complex of the guinea pig. Anat Embryol (Berl) 186:443–465
Komatsu Y (1994) Age-dependent long-term potentiation of inhibitory synaptic transmission in rat visual cortex. J Neurosci 14:6488–6499
Korada S, Schwartz IR (1999) Development of GABA, glycine, and their receptors in the auditory brainstem of gerbil: a light and electron microscopic study. J Comp Neurol 409:664–681
Kotak VC, DiMattina C, Sanes DH (2001) GABA(B) and Trk receptor signaling mediates long-lasting inhibitory synaptic depression. J Neurophysiol 86:536–540
Kotak VC, Korada S, Schwartz IR, Sanes DH (1998) A developmental shift from GABAergic to glycinergic transmission in the central auditory system. J Neurosci 18:4646–4655
Kros CJ, Ruppersberg JP, Rusch A (1998) Expression of a potassium current in inner hair cells during development of hearing in mice. Nature 394:281–284
Kulesza RJ Jr, Spirou GA, Berrebi AS (2003) Physiological response properties of neurons in the superior paraolivary nucleus of the rat. J Neurophysiol 89:2299–2312.
Kullmann DM, Asztely F, Walker MC (2000) The role of mammalian ionotropic receptors in synaptic plasticity: LTP, LTD and epilepsy. Cell Mol Life Sci 57:1551–1561

Kullmann PH, Kandler K (2001) Glycinergic/GABAergic synapses in the lateral superior olive are excitatory in neonatal C57B1/6 J mice. Brain Res Dev Brain Res 131:143–147
Kullmann PH, Ene FA, Kandler K (2002) Glycinergic and GABAergic calcium responses in the developing lateral superior olive. Eur J Neurosci 15:1093–1104
Leinekugel X, Medina I, Khalilov I, Ben-Ari Y, Khazipov R (1997) Ca^{2+} oscillations mediated by the synergistic excitatory actions of $GABA_A$ and NMDA receptors in the neonatal hippocampus. Neuron 18:243–255
Levi S, Logan SM, Tovar KR, Craig AM (2004) Gephyrin is critical for glycine receptor clustering but not for the formation of functional GABAergic synapses in hippocampal neurons. J Neurosci 24:207–217
Liao D, Zhang X, O'Brien R, Ehlers MD, Huganir RL (1999) Regulation of morphological postsynaptic silent synapses in developing hippocampal neurons. Nat Neurosci 2:37–43
Lien CC, Mu Y, Vargas-Caballero M, Poo MM (2006) Visual stimuli-induced LTD of GABAergic synapses mediated by presynaptic NMDA receptors. Nat Neurosci 9:372–380
Lim R, Alvarez FJ, Walmsley B (2000) GABA mediates presynaptic inhibition at glycinergic synapses in a rat auditory brainstem nucleus. J Physiol 525:447–459
Lippe WR (1994) Rhythmic spontaneous activity in the developing avian auditory system. J Neurosci 14:1486–1495
Liu QR, Lopez-Corcuera B, Mandiyan S, Nelson H, Nelson N (1993) Cloning and expression of a spinal cord- and brain-specific glycine transporter with novel structural features. J Biol Chem 268:22802–22808
McIntire SL, Reimer RJ, Schuske K, Edwards RH, Jorgensen EM (1997) Identification and characterization of the vesicular GABA transporter. Nature 389:870–876
McLean HA, Caillard O, Ben-Ari Y, Gaiarsa JL (1996) Bidirectional plasticity expressed by GABAergic synapses in the neonatal rat hippocampus. J Physiol 496:471–477
Meister M, Wong RO, Baylor DA, Shatz CJ (1991) Synchronous bursts of action potentials in ganglion cells of the developing mammalian retina. Science 252:939–943
Moore MJ, Caspary DM (1983) Strychnine blocks binaural inhibition in lateral superior olivary neurons. J Neurosci 3:237–242
Moss, SJ, Smart, TG (2001) Constructing inhibitory synapses. Nat Rev Neurosci 2:240–250
Nabekura J, Katsurabayashi S, Kakazu Y, Shibata S, Matsubara A, Jinno S, Mizoguchi Y, Sasaki A, Ishibashi H (2004) Developmental switch from GABA to glycine release in single central synaptic terminals. Nat Neurosci 7:17–23
Nugent FS, Penick EC, Kauer JA (2007) Opioids block long-term potentiation of inhibitory synapses. Nature 446:1086–10890
O'Brien JA, Berger AJ (1999) Cotransmission of GABA and glycine to brain stem motoneurons. J Neurophysiol 82:1638–1641
Oda Y, Charpier S, Murayama Y, et al (1995) Long-term potentiation of glycinergic inhibitory synaptic transmission. J Neurophysiol 74:1056–1074
Oertel J, Villmann C, Kettenmann H, Kirchhoff F, Becker CM (2007) A novel glycine receptor beta subunit splice variant predicts an unorthodox transmembrane topology Assembly into heteromeric receptor complexes. J Biol Chem 282:2798–2807
O'Malley DM, Masland RH (1989) Co-release of acetylcholine and gamma-aminobutyric acid by a retinal neuron. Proc Natl Acad Sci USA. 86:3414–3418
O'Malley DM, Sandell JH, Masland RH (1992) Co-release of acetylcholine and GABA by the starburst amacrine cells. J Neurosci 12:1394–408
Otis TS, De Koninck Y, Mody I (1994) Lasting potentiation of inhibition is associated with an increased number of gamma-aminobutyric acid type A receptors activated during miniature inhibitory postsynaptic currents. Proc Natl Acad Sci USA. 91:7698–7702
Ottem EN, Godwin JG, Krishnan S, Petersen SL (2004) Dual-phenotype GABA/glutamate neurons in adult preoptic area: sexual dimorphism and function. J Neurosci 24:8097–8105

Ottersen OP, Storm-Mathisen J (1984) Glutamate- and GABA-containing neurons in the mouse and rat brain, as demonstrated with a new immunocytochemical technique. J Comp Neurol 229:374–392

Ottersen OP, Storm-Mathisen J, Somogyi P (1988) Colocalization of glycine-like and GABA-like immunoreactivities in Golgi cell terminals in the rat cerebellum: a postembedding light and electron microscopic study. Brain Res 450:342–353

Ouardouz M, Sastry BR (2000) Mechanisms underlying LTP of inhibitory synaptic transmission in the deep cerebellar nuclei. J Neurophysiol 84:1414–1421

Overstreet-Wadiche L, Bromberg DA, Bensen AL, Westbrook GL (2005) GABAergic signaling to newborn neurons in dentate gyrus. J Neurophysiol 94:4528–4532

Owens DF, Kriegstein AR (2002) Is there more to GABA than synaptic inhibition? Nat Rev Neurosci 3:715–727

Paarmann I, Schmitt B, Meyer B, Karas M, Betz H (2006) Mass spectrometric analysis of glycine receptor-associated gephyrin splice variants. J Biol Chem 281:34918–34925

Piechotta K, Weth F, Harvey RJ, Friauf E (2001) Localization of rat glycine receptor alpha1 and alpha2 subunit transcripts in the developing auditory brainstem. J Comp Neurol 438:336–352

Rabacchi S, Bailly Y, Delhaye-Bourchaud N, Mariani J (1992) Involvement of the N-methyl D-aspartate (NMDA) receptor in synapse elimination during cerebellar development. Science 256:1823–1825

Represa A, Ben-Ari Y (2005) Trophic actions of GABA on neuronal development. Trends Neurosci 28:278–283

Rusakov DA, Kullmann DM (1998) Extrasynaptic glutamate diffusion in the hippocampus: ultrastructural constraints, uptake, and receptor activation. J Neurosci 18:3158–3170

Russier M, Kopysova IL, Ankri N, Ferrand N, Debanne D (2002) GABA and glycine co-release optimizes functional inhibition in rat brainstem motoneurons in vitro. J Physiol 541:123–137

Sagne C, El Mestikawy S, Isambert MF, Hamon M, Henry JP, Giros B, Gasnier B (1997) Cloning of a functional vesicular GABA and glycine transporter by screening of genome databases. FEBS Lett 417:177–183

Sandler R, Smith AD (1991) Coexistence of GABA and glutamate in mossy fiber terminals of the primate hippocampus: an ultrastructural study. J Comp Neurol 303:177–192

Sanes DH, Rubel EW (1988) The ontogeny of inhibition and excitation in the gerbil lateral superior olive. J Neurosci 8:682–700

Sanes DH, Siverls V (1991) Development and specificity of inhibitory terminal arborizations in the central nervous system. J Neurobiol 22:837–854

Schafer MK, Varoqui H, Defamie N, Weihe E, Erickson JD (2002) Molecular cloning and functional identification of mouse vesicular glutamate transporter 3 and its expression in subsets of novel excitatory neurons. J Biol Chem 277:50734–50748

Seal RP, Edwards RH. (2006) The diverse roles of vesicular glutamate transporter 3. Handb Exp Pharmacol (175):137–150

Seddik R, Schlichter R, Trouslard J (2007) Corelease of GABA/glycine in lamina-X of the spinal cord of neonatal rats. Neuroreport 18:1025–1029

Smith AJ, Owens S, Forsythe ID (2000) Characterisation of inhibitory and excitatory post-synaptic currents of the rat medial superior olive. J Physiol 529:681–698

Smith PH, Joris PX, Carney LH, Yin TCT (1991) Projections of physiologically characterized globular bushy cell axons from the cochlear nucleus of the cat. J Comp Neurol 304:387–407

Somogyi J (2006) Functional significance of co-localization of GABA and Glu in nerve terminals: a hypothesis. Curr Top Med Chem 6:969–973

Somogyi J, Baude A, Omori Y, Shimizu H, El Mestikawy S, Fukaya M, Shigemoto R, Watanabe M, Somogyi P (2004) GABAergic basket cells expressing cholecystokinin contain vesicular glutamate transporter type 3 (VGLUT3) in their synaptic terminals in hippocampus and isocortex of the rat. Eur J Neurosci 19:552–569

Spike RC, Watt C, Zafra F, Todd AJ (1997) An ultrastructural study of the glycine transporter GLYT2 and its association with glycine in the superficial laminae of the rat spinal dorsal horn. Neuroscience 77:543–551

Stornetta RL, Rosin DL, Simmons JR, McQuiston TJ, Vujovic N, Weston MC, Guyenet PG (2005) Coexpression of vesicular glutamate transporter-3 and gamma-aminobutyric acidergic markers in rat rostral medullary raphe and intermediolateral cell column. J Comp Neurol 492:477–494

Takamori S, Malherbe P, Broger C, Jahn R (2002) Molecular cloning and functional characterization of human vesicular glutamate transporter 3. EMBO Rep 3:798–803

Takamori S, Holt M, Stenius K, Lemke EA, Gronborg M, Riedel D, Urlaub H, Schenck S, Brugger B, Ringler P, Muller SA, Rammner B, Grater F, Hub JS, De Groot BL, Mieskes G, Moriyama Y, Klingauf J, Grubmuller H, Heuser J, Wieland F, Jahn R (2006) Molecular anatomy of a trafficking organelle. Cell 127:831–846

Tkatch T, Baranauskas G, Surmeier DJ (1998) Basal forebrain neurons adjacent to the globus pallidus coexpress GABAergic and cholinergic marker mRNAs. Neuroreport 9:1935–1939

Todd AJ (1991) Immunohistochemical evidence that acetylcholine and glycine exist in different populations of GABAergic neurons in lamina III of rat spinal dorsal horn. Neuroscience 44:741–746

Todd AJ, Sullivan AC (1990) Light microscope study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat. J Comp Neurol 296:496–505

Triller A, Cluzeaud F, Korn H (1987) gamma-Aminobutyric acid-containing terminals can be apposed to glycine receptors at central synapses. J Cell Biol 104:947–956

Tsen G, Williams B, Allaire P, Zhoru YD, Ikonomov O, Kondova I, Jacob MH (2000) Receptors with opposing functions are in postsynaptic microdomains under one presynaptic terminal. Nat Neurosci 3:126–132

Turecek R, Trussell LO (2001) Presynaptic glycine receptors enhance transmitter release at a mammalian central synapse. Nature 411:587–590

Turecek R, Trussell LO (2002) Reciprocal developmental regulation of presynaptic ionotropic receptors. Proc Natl Acad Sci USA. 99:13884–13889

Vaney DI, Young HM (1988) GABA-like immunoreactivity in cholinergic amacrine cells of the rabbit retina. Brain Res. 438:369–373

Walker MC, Ruiz A, Kullmann DM (2001) Monosynaptic GABAergic signaling from dentate to CA3 with a pharmacological and physiological profile typical of mossy fiber synapses. Neuron 29:703–715

Wang CT, Blankenship AG, Anishchenko A, Elstrott J, Fikhman M, Nakanishi S, Feller MB (2007) GABA(A) receptor-mediated signaling alters the structure of spontaneous activity in the developing retina. J Neurosci 27:9130–9140

Wang, JH, Stelzer, A (1996) Shared calcium signalling pathways in the induction of long-term potentiation and synaptic disinhibition in CA1 pyramidal cell dendrites. J Neurophysiol 75:1687–1702

Wentzel PR, De Zeeuw CI, Holstege JC, Gerrits NM (1993) Colocalization of GABA and glycine in the rabbit oculomotor nucleus. Neurosci Lett 164:25–29

Wojcik SM, Katsurabayashi S, Guillemin I, Friauf E, Rosenmund C, Brose N, Rhee JS (2006) A shared vesicular carrier allows synaptic corelease of GABA and glycine. Neuron 50:575–587

Woodin MA, Ganguly K, Poo MM (2003) Coincident pre- and postsynaptic activity modifies GABAergic synapses by postsynaptic changes in Cl- transporter activity. Neuron 39:807–820

Wu SH, Kelly JB (1992) Synaptic pharmacology of the superior olivary complex studied in mouse brain slice. J Neurosci 12:3084–3097

Wulle I, Wagner HJ. (1990) GABA and tyrosine hydroxylase immunocytochemistry reveal different patterns of colocalization in retinal neurons of various vertebrates. J Comp Neurol 296:173–178

Xu J, Mashimo T, Sudhof TC (2007) Synaptotagmin-1, -2, and -9: Ca(2 +) sensors for fast release that specify distinct presynaptic properties in subsets of neurons. Neuron 54:567–581

Zafra F, Gomeza J, Olivares L, Aragon C, Gimenez C (1995) Regional distribution and developmental variation of the glycine transporters GLYT1 and GLYT2 in the rat CNS. Eur J Neurosci 7:1342–1352

Zheng JJ, Lee S, Zhou ZJ (2004) A developmental switch in the excitability and function of the starburst network in the mammalian retina. Neuron 44:851–864