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GABA Vesicles at Synapses: Are There 2 Distinct Pools?

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Abstract

Fast synaptic inhibition in the neocortex is mediated by the neurotransmitter GABA, acting on GABA_A receptors. Neurotransmitters, including GABA, are stored in synaptic vesicles at presynaptic nerve terminals. A long-held assumption has been that evoked and spontaneous neurotransmissions draw on the same pools of vesicles. We review the evidence from FM1-43 studies supporting the contention that at least 2 distinct pools of GABA vesicles are present at inhibitory synapses in the rat neocortex. FM1-43 uptake during spontaneous vesicle endocytosis labels a vesicle pool within neocortical inhibitory nerve terminals that is released much more slowly (“reluctant” pool) than those vesicles loaded by electrical stimulation of afferent fibers or hyper-kalemic solutions. These multiple pools may play diverse roles in such processes as long-term depression and/or potentiating of inhibitory synaptic transmission, homeostatic plasticity of inhibitory activity, or developmental changes in inhibitory synaptic transmission.

Keywords

recycling pools; GABA; FM1-43; inhibition; neocortex

Local cortical circuits are formed by synapses between pyramidal cells, as well as by synaptic connections among interneurons and pyramidal cells. In the neocortex, excitatory synapses are pre-dominant, with 85% of synaptic connections within the gray matter being excitatory (Braitenberg and Schuz 1991; Douglas and others 1995). Experimental and modeling studies have shown that such circuits containing extensive recurrent excitatory connections are inherently unstable (Douglas and others 1995; Nelson and Turrigiano 1998; Sompolinsky and Shapley 1997). Feedback inhibition provided by GABAergic interneurons provides a stabilizing influence and prevents run-away excitation. Fast synaptic inhibition in the neocortex is mediated by the neurotransmitter GABA, acting on GABA_A receptors. Following the arrival of an action potential at a GABAergic nerve terminal, high (mM) concentrations of GABA are released into the cleft, producing rapid, transient activation of postsynaptic receptors (Nusser and others 2001; Mozrzymas and others 2003). Factors determining the postsynaptic response, including the amplitude and time course of the GABA transient, subunit composition of the receptor and receptor number have received considerable attention (Mody and Pearce 2004). Control of the presynaptic release machinery has not been as extensively examined. A prominent feature of GABAergic synaptic transmission, in addition to action potential dependent transmitter release, is spontaneous quantal neurotransmitter release. Miniature inhibitory postsynaptic currents (mIPSCs) produced by this mechanism provide a “perpetual” background inhibition (Otis and others 1991). The development of methods for visualizing exocytosis from individual GABAergic synaptic boutons (Brager and others 2003; Axmacher

and others 2004) has raised questions about the composition and number of presynaptic vesicular pools underlying spontaneous versus evoked transmission.

Neurotransmitters, including GABA, are stored in synaptic vesicles at presynaptic nerve terminals. Soon after del Castillo and Katz (1954) proposed the quantal hypothesis, electrophysiological studies at the neuromuscular junction showed that only a fraction of the total number of vesicles stored were available for release (Liley and North 1953; Elmqvist and Quastel 1965). Studies of central synapses have also supported the existence of functionally distinct pools of vesicles (Zucker and Regehr 2002). Traditionally, these pools have been called the readily releasable pool (RRP) and the reserve pool (RP). The RRP is defined as those quanta that are released by short depolarizations (Schneggenburger and others 1999) or upon application of hypertonic solutions (Stevens and Tsujimoto 1995; Rosenmund and Stevens 1996). During repetitive nerve stimulation, the RRP is depleted, and RP vesicles are then mobilized to the active zone (Elmqvist and Quastel 1965; Richards and others 2003). A basic tenant of the quantal hypothesis is that release can be evoked by action potential invasion of the presynaptic terminal or can occur spontaneously via low probability random fusion of primed synaptic vesicles in the absence of an action potential (Murthy and Stevens 1999; Prange and Murphy 1999). A long-held assumption is that both mechanisms drew upon the same pools of vesicles.

The hypothesis that evoked and spontaneous neurotransmissions use independent pools of vesicles was directly tested at excitatory synapses by Sara and others (2005). These studies made use of the styrl dye imaging method (Cochilla and others 1999). The basic approach is illustrated in Figure 1. When synapses between cultured hippocampal neurons were loaded with styrl amphipathic fluorescent dyes (e.g., FM1-43, FM2-10) using either electrical or high potassium stimulation, fluorescent puncta were labeled intensely and destained with the typical biphasic pattern previously reported by others (Klingauf and others 1998). When exposed to the styrl dye in the presence of TTX, vesicles that spontaneously fused with the plasma membrane—releasing neurotransmitter loaded with the dye upon subsequent endocytosis. Surprisingly, when spontaneously loaded puncta were destained with high potassium or electrical stimulation, a slow monophasic destaining time course was observed (Sara and others 2005). This led to the conclusion that spontaneously endocytosed vesicles constituted a unique vesicle pool that was “reluctant” to release its content. These findings, although at variance with other findings in cultured hippocampal neurons (Prange and Murphy 1999; Groemer and Klingauf 2007), challenged some basic tenets of the quantal hypothesis of neurotransmission. In a recent study, we used multiphoton excitation microscopy of FM1-43 fluorescence to directly probe for the existence of distinct vesicular pools at more mature inhibitory synapses in acute rat cortical slices (Mathew and others 2008). Here, we review the evidence supporting the contention that at least 2 distinct pools of GABA vesicles are present at inhibitory synapses.

Using FM dye labeling to study inhibitory synapses in acute brain slices from mature animals presents several challenges. The first is the problem of incomplete FM dye washout in brain slices, causing high levels of background fluorescence. This has been dealt with via the use of fluorescence quenching (Pyle and others 1999) and 2-photon imaging (Zakharenko and others 2001; Axmacher and others 2004; Tyler and others 2006). The second issue is the identification of inhibitory versus excitatory terminals. Because FM dyes indiscriminately label all active terminals with recycling synaptic vesicles, anatomical or functional methods for identifying inhibitory synapses are needed. GABAergic nerve terminals in the hippocampus have been identified on the basis of insensitivity to blockade by adenosine, the location in the stratum pyramidale where glutamatergic synapses are rare, and colocalization with GAD65-eGFP–labeled boutons in slices from transgenic mice (Brager and others 2003; Axmacher and others 2004). In our studies on the neocortex, we took advantage of the fact that parvalbumin-positive, fast-spiking GABAergic interneurons synapse onto pyramidal cell somata and proximal

dendrites (Kawaguchi and Kubota 1998a, 1998b). A photomicrograph of parvalbumin staining in the neocortex is shown in Figure 2A, where cell bodies of pyramidal cells are outlined by parvalbumin-positive puncta. An FM1-43-labeled slice, imaged by multiphoton excitation microscopy, is shown in Figure 2B. Cell bodies and proximal dendrites are extensively labeled. These puncta represent presumptive inhibitory terminals and were used for analyses of FM1-43 fluorescence intensity and destaining kinetics. These images provide suggestive correlative data that somatic puncta represent GABAergic nerve terminals.

Glutamate, but not GABA, release in the hippocampus is inhibited by adenosine (Lambert and Teyler 1991; Thompson and others 1992). To further identify cortical perisomatic terminals as inhibitory, we tested their sensitivity to adenosine. Under whole-cell voltage-clamp conditions, bath application of 50 μ M adenosine had no effect on evoked IPSC amplitude (Figure 3A), whereas the amplitude of evoked EPSCs was significantly reduced (Figure 3B). Similar experiments were done using FM1-43 staining. Boutons in neocortical slices were loaded using 10-Hz stimulation in control saline and destained in the presence (red) or absence (blue) of 50 μ M adenosine. This treatment did not affect destaining in proximal puncta surrounding pyramidal cell somata (Figure 3C). In comparison, adenosine inhibited FM1-43 destaining of distal, presumably glutamatergic, puncta (Figure 3D). This is strong evidence that proximal perisomatic puncta are inhibitory, whereas more distal puncta are excitatory.

Once we established that proximal FM1-43 puncta represent GABAergic terminals, we determined whether separate pools of vesicles contribute to evoked versus spontaneous release. To do this, we used protocols similar to those of Sara and others (2005) and, in addition, tested whether presynaptic receptors could modulate these pools. FM1-43 loading was accomplished using 900 afferent stimuli at 10 Hz. After dye washout, 10-Hz stimulation was used to evoke FM1-43 destaining (Figure 4A, left). Under control conditions, FM1-43 destaining proceeded exponentially (Figure 4B). In the presence of 250 nM kainate, which is known to facilitate IPSCs in the neocortex (Mathew and Hablitz 2008), increases in the destaining rate were observed (Figure 4B). A common feature of inhibitory synapses is the spontaneous release of neurotransmitters in the absence of action potentials (Otis and others 1991). As discussed above, it has been suggested that a distinct vesicle pool underlies spontaneous glutamate release in cultured hippocampal neurons (Sara and others 2005). We tested if a similar “reluctant” pool of GABAergic vesicles was demonstrable. FM1-43 uptake was allowed to proceed by spontaneous vesicular recycling for 15 minutes in the presence of TTX (Figure 4A, middle). Clearly resolved FM1-43 puncta outlining pyramidal neuron somata, similar to those observed after electrical- or high K^+ -induced labeling, were observed after dye washout. A slow, linear destaining of this spontaneously loaded pool, reminiscent of the kinetic behavior of the “reluctant” vesicle pool, was observed when spontaneous unloading occurred in the presence of TTX (Figure 4C). Spontaneously loaded and unloaded vesicles had destaining profiles best fit by a linear regression. The rate of destaining from the spontaneous pool was increased by KA, although still linear in nature. These results suggest that the activity-dependent and -independent (reluctant) pools are both subject to modulation by kainate.

Two interesting findings were obtained when terminals were loaded with FM1-43 by spontaneous activity in the absence of TTX and subsequently destained by a 10-Hz afferent stimulation (Figure 4A, right). In this situation, destaining followed a complex time course, and kainate was not able to alter the rate of FM1-43 destaining (Figure 4D). The initial exponential rate of destaining most likely reflects a release from vesicles that were loaded following action potential-dependent release (i.e., following a spontaneous IPSC). Presumably, the subsequent linear rate is due to the release of vesicles loaded independently of action potentials (i.e., following a miniature IPSC). Our interpretation is that a release from vesicle pools which are loaded and unloaded using matching dye-loading protocols is subject to modulation via activation of kainate receptors. When vesicle pools are loaded and then

subsequently unloaded by mismatching protocols, that is, spontaneous or evoked, kainate modulation does not occur. The mechanism underlying this phenomenon is still uncertain, but the findings underscore the complexity of vesicular pool composition and its regulation.

Dopamine inhibits evoked IPSCs in the neocortex, presumably by acting on D1-like presynaptic receptors (Gonzalez-Islas and Hablitz 2001). A direct presynaptic effect has, however, never been demonstrated. Figure 5A shows the effect of bath application of dopamine on FM1-43 destaining rates in perisomatic puncta. Using a protocol similar to that in Figure 4A (electrical loading and unloading), the rate of destaining was clearly slowed in the presence of dopamine, indicating a decrease in GABA release. When spontaneous dye loading and unloading in the presence of TTX was used, a slow linear release, associated with a “reluctant” pool of vesicles, was again observed. In this case, a slowing in the presence of dopamine was observed. Overall, these results suggest that there are 2 pools of GABAergic vesicles and their release properties can be both positively (kainate) and negatively (dopamine) regulated by neuromodulators.

In summary, we used FM1-43 dye loading, via spontaneous or activity-dependent vesicle recycling, and multiphoton microscopy to dissect different vesicular pools of GABA in acute neocortical slices. Our results present the first evidence for different vesicle pools depending on the loading paradigms. Consistent with the observations of Sara and others (2005) in hippocampal excitatory terminals, FM1-43 uptake during spontaneous vesicle endocytosis labels a vesicle pool within neocortical inhibitory nerve terminals that is released much more slowly than those vesicles loaded by electrical or high K^+ stimulation. These results support the view that distinct vesicle pools exist in central presynaptic terminals of inhibitory synapses, which are engaged during different levels of neuronal activity and the ensuing vesicle-recycling process. Differential effects of neuromodulators on evoked and miniature synaptic currents have been reported in cerebellar stellate cells (Kondo and Marty 1998) and hippocampal neurons (Pitler and Alger 1992; Scanziani and others 1992, 1993). It would be interesting to know if neuromodulator effects on action potential–dependent and action potential–independent transmitter release at these synapses were examples of actions on different vesicular pools. Likewise, the role of multiple pools on such processes as long-term depression and/or potentiation of inhibitory synaptic transmission (Gaiarsa and others 2002), homeostatic plasticity of inhibitory activity (Mody 2005), and developmental changes in inhibitory synaptic transmission deserves further consideration.

Acknowledgments

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Figure 1.

Schematic representation of loading of presynaptic vesicle pools with the styryl dye FM1-43. An inhibitory synapse, expressing presynaptic kainate and dopamine receptors, is shown under resting conditions. GABA-containing vesicles are shown in the presynaptic terminal and GABA_A receptors in the postsynaptic membrane. FM1-43 is applied to the extracellular solution and taken up into presynaptic terminals during synaptic vesicle recycling engaged by spontaneous or evoked synaptic vesicle fusion with the presynaptic membrane (the cartoon shows an action potential invading the preterminal). Synaptic vesicles within the presynaptic terminals remain labeled with FM1-43 after dye removal from the extracellular solution and extensive washout (~30 minutes). The steady-state fluorescence intensity of these FM1-43-labeled terminals at rest is directly proportional to the synaptic vesicle endocytosis. A second round of afferent stimulation can be used to evoke synaptic vesicle fusion and discharge of FM1-43 dye (along with the neurotransmitter) out to the extracellular space, which is measured as a time-dependent decrease in fluorescence intensity (after background subtraction and bleaching correction). Alternatively, FM1-43 destaining can be measured during spontaneous vesicle fusion. The initial rate of FM1-43 destaining is directly proportional to synaptic vesicle exocytosis (Zakharenko and others 2001; Tyler and others 2006).

Figure 2.

Visualization of inhibitory terminals in rat neocortical slices. (A) Staining with an antibody against parvalbumin. Immunoreactivity detected using a biotinilated secondary. The photomicrograph shows multiple labeled boutons outlining the cell body of a layer III pyramidal cell. (B) An image, obtained with multiphoton excitation microscopy, of labeling with the styrl dye FM1-43. Dye-containing puncta surround pyramidal cell somas and proximal dendrites. Scale bars indicate 10 μm. Modified from Mathew and others 2008 (used with permission).

Figure 3.

Glutamate, but not GABA, release is inhibited by adenosine. (A) Examples of whole-cell patch-clamp recordings of IPSCs evoked before (blue) and after (red) bath application of 50 μ M adenosine. Ten trials were averaged in each case. Adenosine had no effect on IPSC amplitude. (B) A similar experiment in another neuron where EPSCs were evoked before (blue) and after (red) adenosine (50 μ M) application. Adenosine significantly reduced EPSC amplitudes. (C) Examples of FM1-43 destaining evoked by electrical stimulation in perisomatic inhibitory terminals. Destaining was similar in the presence (red) and absence (blue) of 50 μ M adenosine. (D) Similar experiment as in C but destaining examined in distally located puncta on dendrites. Destaining was virtually blocked in the presence of adenosine (red). Modified from Mathew and others 2008 (used with permission).

Figure 4.

FM1-43 destaining kinetics and modulation by kainic acid. (A) Schematic showing loading and unloading protocols used. (B) Examples of destaining in response to 10-Hz electrical stimulation. Rapid destaining (blue) was accelerated in the presence of kainic acid (red). (C) Monotonic slow destaining was observed when vesicles were spontaneously loaded and subsequently spontaneously unloaded in the presence of TTX. The destaining rate was significantly increased in the presence of kainic acid. (D) Experiments in which vesicles were loaded by spontaneous activity and then unloaded with electrical stimulation. Destaining had fast and slow components which were both insensitive to kainic acid. Modified from Mathew and others 2008 (used with permission).

Figure 5.

Dopamine inhibition of GABA release. FM1-43 dye was loaded by (A) electrical stimulation or (B) spontaneous activity in the presence of TTX. Destaining of perisomatic inhibitory terminals was examined under control conditions (blue) and after bath application of 30 μ M dopamine. (A) Destaining kinetics with electrical stimulation was significantly decreased by dopamine. (B) Spontaneous destaining was also decreased by dopamine.