



Glycinergic transmission at auditory brainstem synapses: The impact of glycine recycling and vesicle refilling

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Kaizen

is a Japanese term meaning continuous improvement. Developing a little further every day through small improvements. It reflects the idea that growth is a constant and natural process.

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Summary

Inhibitory transmission in the auditory brainstem is essential for sound source localization. Glycinergic synapses between the medial nucleus of the trapezoid body (MNTB) and the lateral superior olive (LSO) process interaural level differences and reliably transmit inhibitory signals, even during minutes of high-frequency activity. Recycling of synaptically released glycine is crucial for reliable inhibitory transmission at MNTB-LSO synapses. Glycine transporter 2 (GlyT2) mediates glycine (re)uptake into MNTB axon terminals. Glycine is (re)filled into the synaptic vesicles (SV) with the vesicular inhibitory amino acid transporter (VIAAT), driven by a proton gradient generated by vacuolar (H⁺)-ATPase (V-ATPase). Loss of GlyT2 or V-ATPase inhibition impairs transmission but does not cause complete failure, suggesting the presence of additional mechanisms supporting glycine (re)uptake and SV (re)filling. Potential candidates are the alanine-serine-cysteine-1 transporter (Asc-1) glycine (re)uptake and the Na⁺/H⁺ exchanger (NHE) for SV (re)filling. However, their contribution to inhibitory transmission at MNTB-LSO synapses under high-frequency activity remains largely unclear.

In the first part of my thesis, I investigated the contribution of Asc-1 in wildtype (WT) and GlyT2 knock-out (KO) mice. Immunohistochemistry in the auditory brainstem confirmed Asc-1 expression in both genotypes. Whole-cell patch-clamp recordings from LSO neurons were performed while stimulating MNTB axons (Protocol 1 and 2). In KO mice, synaptic strength and vesicle replenishment were severely impaired. In WT mice, synaptic transmission remained stable and was only affected after sustained activity. In the second part, I examined synaptic strength during very prolonged activity (Protocol 3) under pharmacological inhibition of GlyT2 and/or Asc-1 in WT. While synaptic transmission and SV replenishment remained reliable under GlyT2 or Asc-1 inhibition, combined blockade led to impaired transmission and reduced SV (re)filling and replenishment. In the third part of my thesis, I investigated SV (re)filling by performing and reanalyzing data under V-ATPase and/or NHE inhibition. Transmission, SV (re)filling and replenishment were reduced. In addition, NHE inhibition affected action potential conduction fidelity, suggesting a non-specific inhibition.

Collectively, my results imply that Asc-1 inhibition reduces glycinergic transmission at MNTB-LSO synapses. In KO, the remaining transmission is maintained by Asc-1-mediated glycine (re)uptake. This demonstrates that Asc-1, in addition to GlyT2, serves as a key glycine source at MNTB-LSO synapses. Inhibition of SV (re)filling reduces glycinergic transmission, highlighting the essential role of V-ATPase in maintaining reliable transmission at MNTB-LSO synapses. The used NHE inhibitor EIPA is not selective for NHE6 and interferes with AP conduction. Due to these unspecific effects, the functional contribution to synaptic performance could not be directly assessed.

Zusammenfassung

Die inhibitorische Übertragung im auditorischen Hirnstamm ist für die Lokalisierung von Schallquellen unerlässlich. Glycinerge Synapsen zwischen dem medialen Kern des Trapezoidkörpers (MNTB) und der lateralen superioren Olive (LSO) verarbeiten interaurale Pegelunterschiede und übertragen hemmende Signale zuverlässig, selbst während Minuten mit hoher Frequenzaktivität. Die Wiederverwertung von synaptisch freigesetztem Glycin ist entscheidend für eine zuverlässige hemmende Übertragung an den MNTB-LSO-Synapsen. Der Glycintransporter 2 (GlyT2) vermittelt die (Wieder-)Aufnahme von Glycin in die Axonendigungen des MNTB. Glycin wird dann über den vesikulären inhibitorischen Aminosäuretransporter (VIAAT) unter Antrieb eines durch die vakuoläre (H^+)-ATPase (V-ATPase) erzeugten Protonengradienten in die synaptischen Vesikel (SV) (wieder) aufgefüllt. Der Verlust von GlyT2 oder die Hemmung der V-ATPase beeinträchtigt die Übertragung, führt jedoch nicht zu einem vollständigen Ausfall, was auf das Vorhandensein zusätzlicher Mechanismen hindeutet, die die Glycin (Wieder-)Aufnahme und die (Wieder-)Füllung der SV unterstützen. Mögliche Kandidaten sind der Alanin-Serin-Cystein-1 Transporter (Asc-1) für die Glycin (Wieder-)Aufnahme und der Na^+/H^+ -Austauscher (NHE) für die SV (Wieder-)Befüllung. Ihr Beitrag zur hemmenden Übertragung an MNTB-LSO-Synapsen bei hoher Aktivität ist jedoch weitgehend unklar.

Im ersten Teil meiner Arbeit habe ich den Beitrag von Asc-1 in Wildtyp (WT) und GlyT2 Knockout (KO) Mäusen untersucht. Immunhistochemische Untersuchungen im auditorischen Hirnstamm bestätigten die Expression von Asc-1 in beiden Genotypen. Es wurden Whole-Cell-Patch-Clamp-Aufzeichnungen von LSO-Neuronen durchgeführt, während MNTB-Axone stimuliert wurden (Protokoll 1 und 2). Bei KO Mäusen waren die synaptische Stärke und die Vesikelauffüllung stark beeinträchtigt. In WT Mäusen blieb die synaptische Übertragung stabil und wurde erst nach anhaltender Aktivität beeinträchtigt. Im zweiten Teil untersuchte ich die synaptische Stärke während sehr langer Aktivität (Protokoll 3) unter pharmakologischer Hemmung von GlyT2 und/oder Asc-1 in WT. Während die synaptische Übertragung und die SV (Wieder-)Auffüllung unter GlyT2 oder Asc-1 Hemmung zuverlässig blieben, führte eine kombinierte Blockade zu einer beeinträchtigten Übertragung und einer verminderten SV (Wieder-)Füllung. Im dritten Teil meiner Arbeit untersuchte ich die SV (Wieder-)Füllung unter V-ATPase und/oder NHE-Hemmung. Die synaptische Übertragung und die SV (Wieder-)Füllung waren reduziert. Darüber hinaus beeinträchtigte die NHE-Hemmung die Weiterleitung der Aktionspotenziale, was auf eine unspezifische Hemmung hindeutet.

Zusammenfassend lässt sich deuten, dass die Hemmung von Asc-1 die glycinerge Übertragung an MNTB-LSO-Synapsen reduziert. In KO wird die verbleibende Übertragung

durch die Asc-1-vermittelte Glycin (Wieder)Aufnahme aufrechterhalten. Dies zeigt, dass Asc-1 neben GlyT2 als wichtige Glycinquelle an MTNB-LSO-Synapsen dient. Die Hemmung der SV (Wieder)Füllung reduziert die glycinerge Übertragung, was die wesentliche Rolle von V-ATPase für die Aufrechterhaltung einer zuverlässigen Übertragung an MNTB-LSO Synapsen unterstreicht. Der verwendete NHE-Inhibitor EIPA ist nicht selektiv für NHE6 und beeinträchtigt die AP-Leitung. Aufgrund dieser unspezifischen Effekte konnte der funktionelle Beitrag zur synaptischen Leistung nicht direkt bewertet werden.

1 Introduction

1.1 Inhibitory glycinergic transmission

Synaptic transmission is the fundamental process by which neurons communicate and transfer information. This process occurs at the contact side between neurons, called synapses which consist of a pre- and postsynaptic part. Presynaptic terminals contain synaptic vesicles (SVs; Südhof, 1999). These SVs can divide into different vesicle pools. The active zone docks and primes a small number of SVs, known as the readily releasable pool (RRP; Rizzoli & Betz, 2004). The remaining SVs are part of the recycling pool or the reserve pool (Rizzoli & Betz, 2005; Alabi & Tsien, 2012). SVs from the RRP are replenished from the recycling pool and consist of newly endocytosed and (re)filled SVs (Denker & Rizzoli, 2010). Action potentials (APs) propagate along axons to presynaptic terminals, where they trigger the opening of voltage-gated calcium (Ca^{2+}) channels, this results in an influx of Ca^{2+} ions and initiates SV exocytosis and neurotransmitter (NT) release (Südhof, 2013; Silva *et al.*, 2021). The NT can be excitatory (e.g. glutamate) or inhibitory (e.g. glycine and GABA). My thesis focuses on inhibitory synapses and consequently on inhibitory NT (Figure 1.1).

Glycine, the simplest amino acid, is a major inhibitory NT in the central nervous system (Aprison & Werman, 1965). The spinal cord and brainstem have a high density of glycinergic inhibitory synapses (Poyatos *et al.*, 1997; Legendre, 2001). NT release into the synaptic cleft, leads to an increase in the glycine concentration in the synaptic cleft to ~3 mM (Beato, 2008). Glycine binds to postsynaptic glycine receptors (GlyR), which leads to chloride (Cl^-) ions influx and hyperpolarization of the postsynaptic membrane. This reduces neuronal excitability by increasing the threshold of AP generation (Betz *et al.*, 2001; Lynch, 2004). Regulation of intracellular and extracellular glycine levels is essential for maintaining reliable transmission. Glycine transporters contribute to this regulation by mediating (re)uptake of glycine from the synaptic cleft into the presynapse (Brasnjo & Otis, 2003), where it can be used for SV (re)filling.

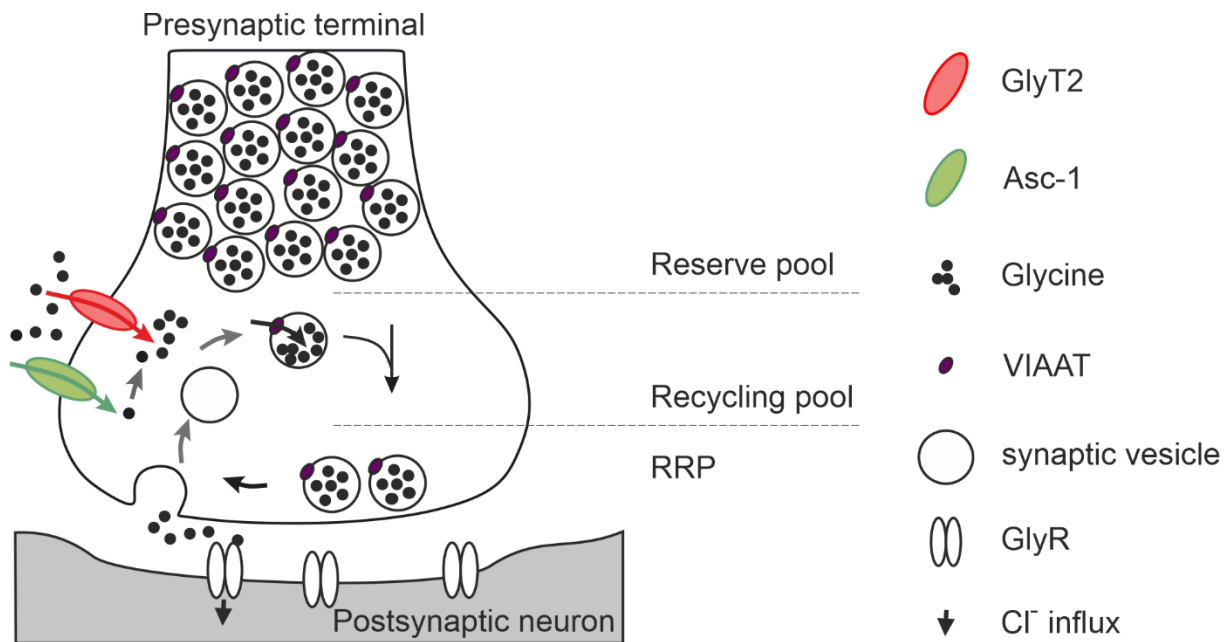


Figure 1.1 Glycinergic synaptic transmission. Schematic overview of inhibitory glycinergic transmission. The presynaptic axon terminal contains synaptic vesicles (SVs) that are filled with glycine (black dots). Two major glycine transporters are located at the presynaptic membrane: glycine transporter 2 (GlyT2; red) and alanine-cysteine-serine-1 transporter (Asc-1; green). When glycine is released from the readily releasable pool (RRP) upon SV exocytosis, it binds to glycine receptors (GlyRs). This opens the receptors, leading to an influx of chloride ions (Cl^-) and resulting in postsynaptic hyperpolarisation. Glycine is recycled by both transporters and (re)filled into SVs via the vesicular inhibitory amino acid transporter (VIAAT; purple). SV belongs then to the recycling pool, as indicated by gray arrows. The largest vesicle pool in the presynaptic terminal is the reserve pool. Modified from (Brill, 2018).

1.1.1 Glycine transporters at the axon terminal

One of these glycine transporters is the glycine transporter 2 (GlyT2). (Aubrey *et al.*, 2007; Rousseau *et al.*, 2008; Apostolides & Trussell, 2013). GlyT2 belongs to the *Slc6* family of sodium (Na^+) and Cl^- -dependent membrane transporters (Nelson, 1998; Bröer & Gether, 2012). GlyT2 transports one molecule of glycine into the presynaptic terminal together with three Na^+ ions and one Cl^- ion (Figure 1.2A; Supplisson & Roux, 2002). Another transporter, glycine transporter 1, located in astrocytes, decreases the glycine concentration in the synaptic cleft to ~ 100 nM (Stephan & Friauf, 2014; Vandenberg *et al.*, 2016). GlyT2 further decreases the extracellular glycine concentration to ~ 10 nM, while maintaining intracellular glycine levels between ~ 20 -40 mM (Supplisson & Roux, 2002). High levels of GlyT2 immunoreactivity are found in the caudal brain regions, especially in areas where glycinergic transmission is prominent (Jursky & Nelson, 1995; Zafra, Aragón, *et al.*, 1995). It is mainly localized to presynaptic terminals of inhibitory neurons (Zafra & Giménez, 2008; Eulenburg & Gomez, 2010). GlyT2 is a reliable marker protein for glycinergic neurons (Poyatos *et al.*, 1997; Zeilhofer *et al.*, 2005) and has been widely used for identifying glycinergic populations of neurons in the

brainstem and the spinal cord (Friauf *et al.*, 1999; Eulenburg *et al.*, 2005). GlyT2 knockout (GlyT2 KO) mice show severe neuromotor phenotypes such as tremors and hindfeet claspings (Gomez *et al.*, 2003). A neurological disorder characterized by exaggerated startle responses and generalized stiffness, called hyperekplexia (OMIM 149400), is caused by mutations in the GlyR subunits $\alpha 1$ (Shiang *et al.*, 1994) or β (Rees *et al.*, 2002), or in GlyT2 (*Slc6a5*; Eulenburg *et al.*, 2006; Rees *et al.*, 2006). Furthermore, Zhan *et al.*, 2021 reviewed additional genes involved in this disease. The involvement of GlyT2 in neuronal pathologies has been reviewed by Zafra *et al.*, 2017. Furthermore, GlyT2 KO mice showed alterations in breathing patterns (Hülsmann *et al.*, 2019). and die in the second postnatal week (Gomez *et al.*, 2003). In addition, intracellular glycine immunoreactivity is reduced in GlyT2 KO mice (Latal *et al.*, 2010). Impaired SV (re)filling and those alterations in glycinergic transmission have been reported, including reduced spontaneous inhibitory postsynaptic current amplitudes (sIPSC; Gomez *et al.*, 2003; Rousseau *et al.*, 2008; Aubrey, 2016), as well as reduced evoked inhibitory postsynaptic current amplitudes (eIPSC; Brill *et al.*, 2021). However, glycine loading into SVs is not completely abolished (Gomez *et al.*, 2003a; Latal *et al.*, 2010; Brill *et al.*, 2021). The residual transmission suggests the presence of an alternative glycine (re)uptake mechanism.

Transcriptomic data reveal a moderate expression level of an alternative glycine transporter, *Slc7a10*. The gene to the protein alanine-serine-cysteine-1 transporter (Asc-1; Brill *et al.*, 2021). While several plasma membrane transporters can accept glycine as a substrate, Asc-1 has a high glycine affinity (Fukasawa *et al.*, 2000). Asc-1 belongs to the *Slc7* family of amino acid transporters and is a Na⁺-independent antiporter that transports small neutral amino acids (AA; Figure 1.2B1-B2; Verrey *et al.*, 2004; Fotiadis *et al.*, 2013). In addition to glycine, it also mediates the transport of alanine, cysteine, L-serine, and D-serine (Fukasawa *et al.*, 2000; Nakauchi *et al.*, 2000; Rosenberg *et al.*, 2013; Sason *et al.*, 2017). Glycine is the preferred substrate of Asc-1, followed by D-serine and L-alanine (Li *et al.*, 2024). As an obligate AA antiporter, Asc-1 does not function as a unidirectional transporter. Instead its transport direction depends on the concentration gradient of the transported AA (Figure 1.2B1-B2; Fukasawa *et al.*, 2000; Errasti-Murugarren *et al.*, 2019; Mikou *et al.*, 2020; Li *et al.*, 2024). Asc-1 is widely expressed throughout the rodent brain (Helboe *et al.*, 2003), and like GlyT2, it is enriched in regions with strong glycinergic transmission (Ehmsen *et al.*, 2016). Asc-1 is found in both astrocytes (Ehmsen *et al.*, 2016) and neurons (Matsuo *et al.*, 2004). A functional role of neuronal Asc-1 is further supported by studies in the forebrain (Rutter *et al.*, 2007; Rosenberg *et al.*, 2013) and brainstem (Mesuret *et al.*, 2018). Interestingly, Asc-1 KO mice display a similar phenotype as GlyT2 KO, including motor deficits and hindlimb claspings, closely resembling hyperekplexia symptoms (Xie *et al.*, 2005; Safory *et al.*, 2015). Recent studies have identified *Slc7a10* as a possible gene for hyperekplexia in humans (Drehmann *et*

al., 2023). Asc-1 KO mice show reduced glycine levels in the brain and die during the first three postnatal weeks. Indicating the importance of Asc-1 in maintaining glycine homeostasis (Xie *et al.*, 2005; Safory *et al.*, 2015; Eulenburg & Hülsmann, 2022). A functional role in synaptic transmission has also been described. sIPSC frequency was reduced in hypoglossal neurons (Safory *et al.*, 2015) and in the spinal cord (Ehmsen *et al.*, 2016). Pharmacological studies have shown that blocking Asc-1 with a non-transportable inhibitor (Lu AE00527) does not affect sIPSCs, whereas the application of a transportable inhibitor (D-isoleucine) decreases sIPSCs amplitude and frequency (Mesuret *et al.*, 2018). For more detailed information on glycine transporters, see the review by Eulenburg & Hülsmann, 2022.

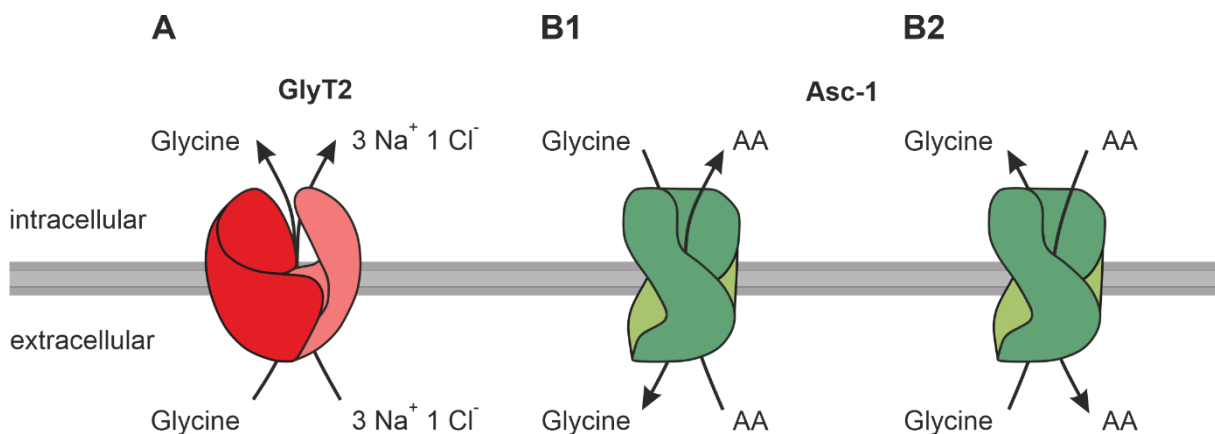


Figure 1.2 Glycine transporter. Schematic overview of presynaptic glycine transport mechanisms. **(A)** GlyT2 (red) operates with a stoichiometry of 3 Na⁺ and 1 Cl⁻ co-transported with one glycine molecule into the cytoplasm. **(B1)** Asc-1 (green), as described in the literature, functions as an antiporter that exchanges small amino acids (AA) from the extracellular space to export glycine from the intracellular side. **(B2)** Asc-1, as hypothesized in this thesis, may alternatively mediate glycine uptake into the presynapse by exchanging intracellular AA for extracellular glycine. Modified from Eulenburg & Hülsmann, 2022.

1.1.2 Mechanisms of SV (re)acidification and (re)filling

Upon (re)uptake the glycine concentration in the axon terminal is ~20-40 mM, thus facilitating glycine (re)fill into the SV lumen by the vesicular inhibitory amino acid transporter (VIAAT, also known as the vesicular γ -aminobutyric acid (GABA) transporter (VGAT); Supplisson & Roux, 2002). VIAAT/VGAT is located at the SV membrane (Martens *et al.*, 2008). It is a key component of glycinergic and GABAergic transmission and is commonly used as a marker for glycinergic and GABAergic neurons (McIntire *et al.*, 1997; Dumoulin *et al.*, 1999a; Zafra & Giménez, 2008). The inactivation of VIAAT is responsible for the lethality of the animals in the embryo stage (Wojcik *et al.*, 2006). A key requirement for efficient glycine (re)filling after SV endocytosis is the acidification of the SV lumen. This process is driven by the vacuolar H⁺-ATPase (V-ATPase) proton pump, which is located in the SV membrane (Figure 1.3A). The V-ATPase hydrolyzes ATP to transport protons (H⁺) from the cytosol into the SV lumen, thereby

establishing both a chemical and electrical gradient across the membrane (Moriyama et al., 1992; Edwards, 2007). VIAAT uses this electrochemical gradient to drive the import of glycine into SVs through an H⁺-exchange mechanism (Edwards, 2007; Anne & Gasnier, 2014). The lumen typically reaches a pH of ~6.4, which is essential for optimal VIAAT activity and efficient SV loading (Figure 1.3B; Egashira et al., 2016). Disrupting this acidification process, by inhibiting V-ATPase, impairs NT uptake and reduces spontaneous postsynaptic currents (PSC) amplitudes, frequency, or synaptic transmission (Zhou et al., 2000; Harrison & Jahr, 2003; Satake et al., 2012; Song et al., 2013; Arnold & Peters, 2025).

Another mechanism, besides V-ATPase, that could contribute to SV acidification, are the Na⁺/H⁺ exchanger (NHE; Figure 1.3). NHEs are a transporter family with thirteen identified isoforms (Pedersen & Counillon, 2019). One is NHE6 (encoded by *Slc9a6*), which is localized to intracellular membranes (Alexander et al., 2017) and has been found in SVs (Preobraschenski et al., 2014; Taoufiq et al., 2020). Functional studies indicate that NHE6 plays a role in regulating the luminal pH of endosomal and vesicular compartments (Ohgaki et al., 2011). Further transcriptomic data indicate that *Slc9a6* is expressed in inhibitory synapses in the auditory brainstem (unpublished data, Maraslioglu-Sperber, 2021), supporting a possible role for NHE6 in SV acidification and glycine (re)filling in glycinergic neurons.

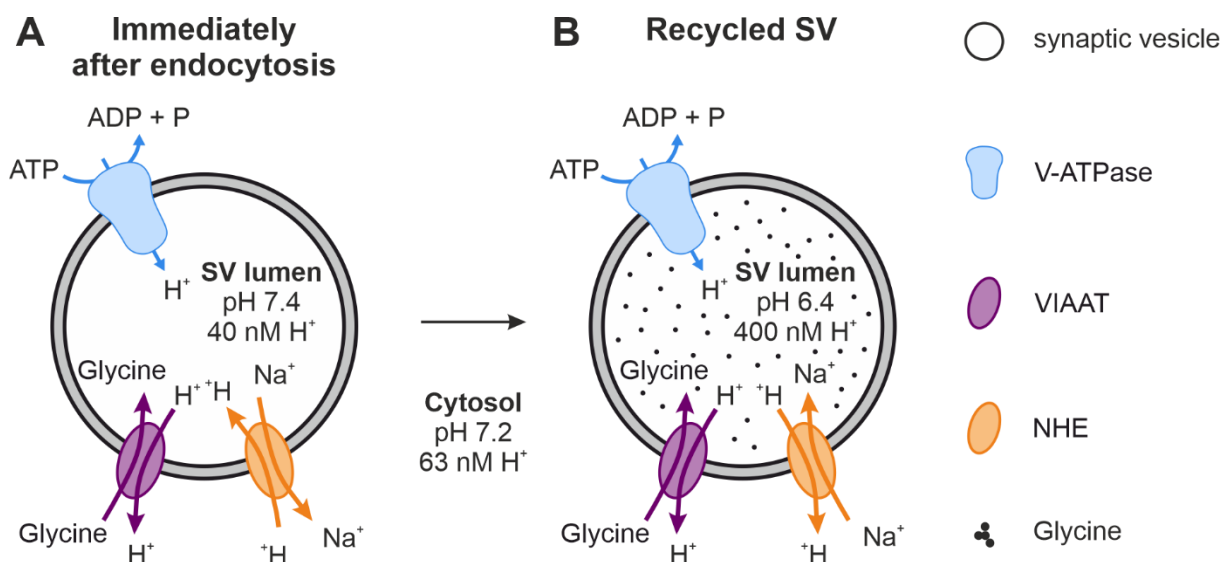


Figure 1.3 SV acidification. Schematic overview of the SV acidification mechanism. **(A)** Right after endocytosis, the lumen of a recycling SV contains extracellular fluid (~150 mM Na⁺; pH = 7.4, corresponding to ~40 nM H⁺) and is free of glycine (no black dots). Essential components for SV acidification and NT filling are the V-ATPase (blue) and the VIAAT (purple). The V-ATPase actively pumps H⁺ into the SV lumen, generating an electrochemical gradient that drives glycine uptake by VIAAT. To regulate the electrochemical gradient, an additional transport mechanism is involved, such as H⁺/Na⁺ exchangers (NHE, orange). NHE, as hypothesized in this thesis, works in reverse mode immediately after endocytosis by exchanging luminal Na⁺ for cytoplasmic H⁺. This supports H⁺-driven glycine antiport by VIAAT

and promotes efficient glycine loading. **(B)** Under physiological conditions, continuous H^+ -pumping by V-ATPase acidifies the SV lumen to pH a of ~ 6.4 (~ 400 nM H^+ ; Egashira *et al.*, 2016). This figure was created by Erika Pizzi (former postdoc, Animal Physiology, RPTU Kaiserslautern-Landau).

1.2 Inhibitory MNTB-LSO synapses in the auditory brainstem

The ascending auditory pathway consists of principal neurons that process acoustic information with high temporal precision. Sound is detected in the cochlea and transduced by the inner hair cells, which form synapses with the spiral ganglion neurons. These spiral ganglion neurons give rise to the auditory nerve fiber that innervates the cochlear nucleus (CN, Nayagam *et al.*, 2011). The spherical bushy and globular bushy cells are located in the anterior ventral CN, where they form large synapses, the endbulb of Held (Cant & Morest, 1979; Tolbert & Morest, 1982). The CN projects to the superior olivary complex (SOC), which is part of the central auditory system and consists of different nuclei (Reuss, 2000). The spherical bushy cells project ipsilateral excitatory input to the principal neurons in the lateral superior olive (LSO; Cant & Casseday, 1986). On the contralateral side, the globular bushy cells project excitatory input to principal neurons of the medial nucleus of the trapezoid body (MNTB), which form a large synapse called the Calyx of Held (Held, 1893). One prominent inhibitory synapse is formed by two nuclei of the SOC, the MNTB and LSO. MNTB principal neurons send inhibitory projections to the LSO (Figure 1.4; Spangler *et al.*, 1985; Tsuchitani, 1997; Yu & Goodrich, 2014; Friauf *et al.*, 2019). This MNTB-LSO synapse plays a key role in sound source localization. LSO principal neurons compute interaural level differences by integrating bilateral input (Tollin, 2003; Grothe *et al.*, 2010). A recent review summarizes the auditory brainstem stations and projections (Keine & Englitz, 2025).

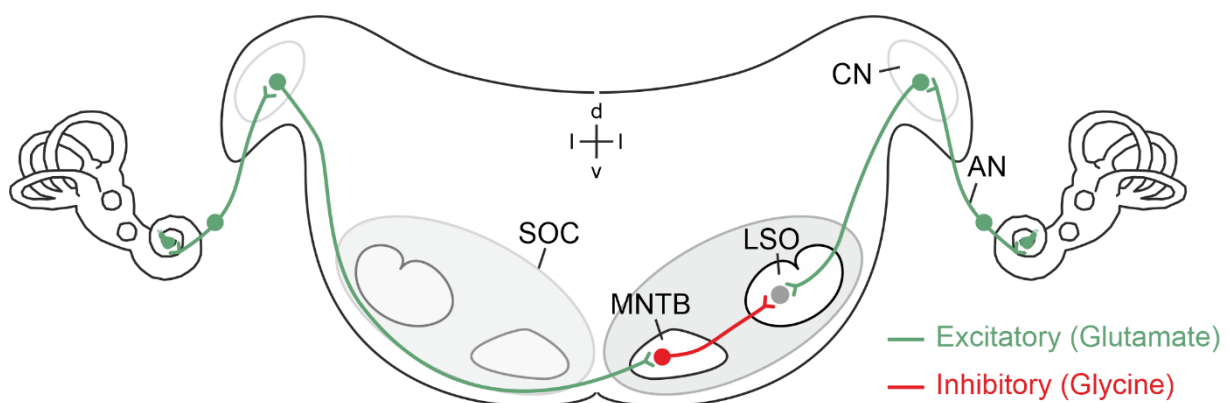


Figure 1.4 Sound source localization circuit in the auditory brainstem. Schematic overview of cochlear and auditory brainstem projection inputs to the LSO for processing interaural level differences. Excitatory glutamatergic projections and neurons are in green, inhibitory glycinergic projection and neuron in red, and the principal LSO neuron is gray. Abbreviations: AN, auditory nerve; CN, cochlear nucleus; d, dorsal; l, lateral; LSO, lateral superior olive; MNTB, medial nucleus of the trapezoid body; SOC, superior olivary complex; v, ventral.

1.3 Short-term plasticity and underlying mechanisms

Activity can change the strength of the synaptic transmission. This strength of synaptic transmission depends on previous synapse activity (Zucker & Regehr, 2002). Depending on the activity pattern, postsynaptic current amplitudes can either increase or decrease. The ability of a synapse to adapt in this way is referred to as synaptic plasticity. If these changes occur in a millisecond to the seconds range, they are classified as short-term plasticity (Zucker & Regehr, 2002; Citri & Malenka, 2008; Friauf *et al.*, 2015). Short-term plasticity can be divided into short-term depression, which is a decrease in postsynaptic current amplitude, and short-term facilitation, which is an increase in postsynaptic current amplitude.

Synaptic plasticity is influenced by multiple mechanisms (Atwood & Karunanithi, 2002; Ho *et al.*, 2011; Chen *et al.*, 2018). These mechanisms include the inactivation of presynaptic Ca^{2+} channels (Forsythe *et al.*, 1998; Xu *et al.*, 2007), the depletion of SVs from the RRP (Von Gersdorff & Matthews, 1997; Schneggenburger *et al.*, 1999; Wong *et al.*, 2003; Südhof, 2004), and changes in postsynaptic receptor sensitivity (Trussell *et al.*, 1993; Neher & Sakaba, 2001). Synaptic strength can be described by different parameters: the number of SVs in the RRP (N_{RRP}), the quantal size (q), defined as the postsynaptic current elicited by a single SV, and the SVs release probability (P_v). Synapses with low P_v typically facilitate during high-frequency activity, while those with high P_v tend to depress (Regehr & Abbott, 2004; Silva *et al.*, 2021). Short-term depression occurs when the RRP is depleted faster than it can be replenished, when q is reduced, or when P_v increases and leads to SV depletion.

1.4 Aims of the study

Glycine transporters enable glycine recycling and ensure reliable inhibitory transmission. GlyT2 is the primary transporter at presynaptic terminals, its role in glycinergic homeostasis and synaptic function is well understood. However, in GlyT2 KO mice, SV glycine (re)filling is not completely abolished, suggesting the existence of an alternative glycine uptake mechanism. One candidate is Asc-1. Although Asc-1 is classically described as an antiporter that facilitates glycine efflux, it may also provide glycine at axon terminals.

In addition, the V-ATPase generates a proton gradient necessary for VIAAT-mediated glycine (re)filling. Interestingly, synaptic transmission is not completely abolished after V-ATPase inhibition, indicating that another proton pump, potentially NHE, might acidify the SV to maintain VIAAT function.

Based on this, I hypothesize that:

1. Asc-1 recycles glycine at the axon terminals, thereby contributing to the synaptic strength at MNTB-LSO synapses.
2. NHE contributes to maintaining the proton gradient for VIAAT-mediated SV (re)filling.

To test these hypotheses, I addressed five different aims:

1. Is Asc-1 present at MNTB terminals in both WT and GlyT2 KO mice? This was determined by immunohistochemistry.
2. Does Asc-1 function as a glycine (re)uptake transporter and contribute to synaptic strength and reliability at MNTB-LSO synapses? This was determined through electrophysiological recordings upon Asc-1 inhibition.
3. Does Asc-1 sustain glycinergic transmission in the absence of GlyT2? This was determined through electrophysiological recordings upon GlyT2 inhibition or using GlyT2 KO mice.
4. Is NHE6 present at the MNTB terminals of mice? This was determined by immunohistochemistry.
5. What are the roles of V-ATPase and NHE in maintaining vesicle (re)filling and synaptic function at MNTB-LSO synapses? This was determined through electrophysiological recordings upon V-ATPase or NHE inhibition.

2 Material and Methods

2.1 Animals

All experiments were performed with mice (*mus musculus*) at postnatal day (P) 10-12 (P11), which is around the time of hearing onset (Mikaelian & Ruben, 1965). Both sexes were used. Either C57BL/6J or Sv129/OLA mice were used for experiments. In Sv129/OLA mice, the exon responsible for encoding the fourth transmembrane region was replaced with a PGK-neomycin resistance gene (Gomez et al., 2003). For the experiments shown in Figure 3.11-3.20 homozygous knock-out mice lacking the functional glycine transporter 2 (GlyT2 KO) isoform were used. GlyT2 KO mice have white fur because both the GlyT2 gene and the coat color genes are located on chromosome 7 (Simpson et al., 2001; Bogdanik et al., 2012; Hülsmann et al., 2019). From now on, GlyT2 KO will only be referred to as KO, as only one type of KO was used in this study, except for the discussion, because there are different KO types mentioned. For other experiments, C57BL/6J wildtype (WT) mice were used. Animal breeding and experiments were approved by the regional council of Rhineland-Palatinate according to the German Animal Welfare Act (TSchG §4, Absatz 3) and followed the guidelines for the protection and welfare of laboratory animals. All animals were housed at the animal facility of the University of Kaiserslautern under a 12 h light-dark cycle and with food and water *ad libitum*.

2.2 GlyT2 genotyping

Genotyping was performed using two separate polymerase chain reactions (PCRs) to detect either the WT or KO allele. Ear tissue samples were collected before the experiment to determine the genotype. After brainstem slice preparation, a second tissue sample was taken from the tail to confirm the initial genotyping results.

2.2.1 DNA isolation

To isolate DNA, tissue samples were digested in 200 µl of 25 mM NaOH (AppliChem, Darmstadt, Germany) and 0.2 mM EDTA (Carl Roth, Karlsruhe, Germany). Samples were incubated at 95°C for 1 h in a thermoshaker at 350 rpm. To stop the digestion process, 200 µl of 40 mM Tris-HCL was added. The mixture was centrifuged (10min, 13000 rpm), and 200 µl of the supernatant containing the DNA was collected for PCR.

2.2.2 PCR and DNA-Gelelectrophoresis

For PCR, the PCR reaction contained 7.9 μ l H₂O, 4 μ l 5x PCR buffer (Bioline), 5 pmol/ μ l of each primer (Figure 2.1A), 0.1 μ l Taq polymerase (Bioline) and 4 μ l DNA. Initial denaturation was performed at 94°C for 10 min. PCR cycles (WT: 35x; KO: 35x) were at 95°C for 30 s, followed by a primer-specific annealing phase of 30 s (WT: 57°C; KO: 50°C), and elongation at 72°C for 60 s. Final elongation was performed at 72°C for 10 min.

The PCR product was visualized on a 1.5% agarose gel (1.5 g agarose and 10 μ l ethidium bromide in 100 ml TAE buffer (10% EDTA pH 8.0; 5.7% acetic acid; 24.2% Tris)) and run in gel electrophoresis (30 min, 100 V; Figure 2.1B). 4 μ l HyperLadder™ 50bp (Bioline, Luckenwalde, Germany) was placed in the first pocket to identify bands. 6 μ l of ladder was added to each PCR product, and 12 μ l of each mix was applied to the gel. For each batch of DNA samples, a positive control, namely a DNA sample with an already confirmed genotype, and a negative control (DNA sample substituted by H₂O) were added. The genotype of the mice was determined via the bands (Figure 2.1B). WT (+/+) and KO (-/-) mice were used for experiments, and heterozygote (+/-) mice were used for breeding. Genotyping of the GlyT2 mice line was done by Jennifer Klein, Kornelia Ociepka (technical assistants; animal physiology group, RPTU Kaiserslautern), or me.

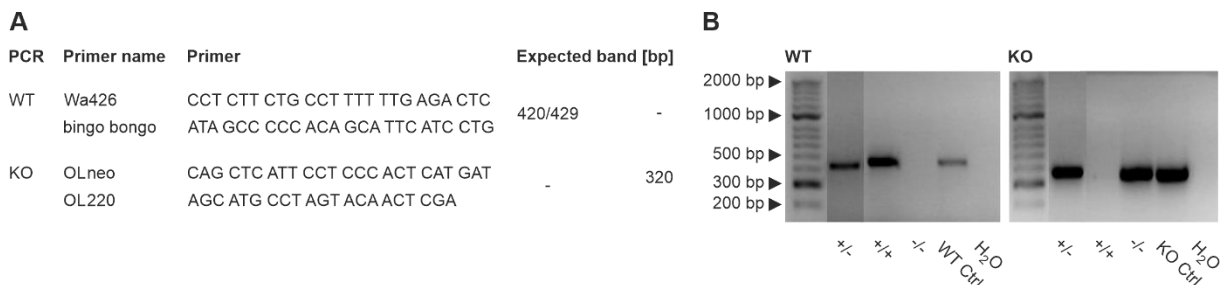


Figure 2.1 Genotyping of GlyT2 mice line. (A) PCR, primer names, primer sequence, and expected band sizes used for GlyT2 genotyping. **(B)** Agarose gel with PCR products upon testing different genotypes. WT band at 420/429 bp (left) and KO band at 320 bp (right).

2.3 Immunohistochemistry

The preparation of brainstem slices and immunohistochemistry were performed as previously described (Brill *et al.*, 2021). Lethally anesthetized mice (10% ketamine, 220 µg/g body weight plus 2% xylazine, 24 µg/g body weight, intraperitoneal) were transcardially perfused with 100 mM phosphate-buffered saline (PBS, pH 7.4) at room temperature (RT), followed by ice-cold 4% paraformaldehyde (PFA) for 20 min (Ecoline VC-360 pump, IsmaTec, Chicago, USA). Brains were removed from the skull, postfixed for 1 h in 4% PFA (4°C), and stored in 30% sucrose-PBS at 4°C overnight. Coronal brainstem slices were then cut at 30 µm with a sliding microtome (HM 400 R, MICROM) and transferred to 15% sucrose-PBS, followed by three rinses in PBS (30 min, RT). For Asc-1 and NHE6, a heat-induced epitope retrieval protocol was used before antibody treatment. For this, slices were immersed in 10 mM sodium citrate buffer (pH 6.0) plus 0.05% Tween 20 (Roth, Karlsruhe, Germany) and heated at 95°C for 20-40 min. They were then returned to RT, followed by three rinses in PBS (5 min, RT). Slides were applied free-floating at 4°C for 1 h in a blocking solution (0.3% Triton-X-100, 10% goat serum, and 2% bovine serum albumin (BSA) in PBS). Primary antibodies were applied free-floating at 4°C overnight in carrier solution (0.3% Triton-X-100, 1% goat serum, and 1% bovine serum albumin (BSA) in PBS). Used primary antibodies were against Asc-1 (1:1,000, host: rabbit, Origene formerly Acris Antibodies, Herford, Germany), GlyT2 (1:200, host: mouse, Synaptic Systems, Göttingen, Germany), NHE6 (1:200, host: rabbit, Sigma Aldrich, St. Louis, USA), and VIAAT (1:500, host: mouse, Synaptic Systems, Göttingen, Germany). VIAAT was used as a presynaptic marker instead of GlyT2 in KO mice, because of its localization in the SV membrane. After applying primary antibodies three rinses in PBS (30 min, RT) followed. Slices were then incubated in the dark for 2 h in a carrier solution, and secondary antibody (1:1,000; goat-anti-mouse, Alexa Fluor 488; goat-anti-rabbit, Alexa Fluor 647, Thermo Fisher Scientific, Waltham, USA). Followed by three rinses in PBS (30 min, RT). Slices were mounted on gelatin-coated glass slides and covered with mounting medium containing 2.5% DABCO (1,4-diazabicyclo[2.2.2]octane; Sigma-Aldrich, St. Louis, MO, USA). Images were acquired with a TCS SP5 II confocal microscope equipped with an HCX PL APO 63 × / 1.4 NA oil objective (Leica Microsystems, Wetzlar, Germany). Optical thickness was set to 1 µm with a resolution of 2048 x 2048 pixels with a pixel size of 120.19 nm. Overview images were acquired using a 20 x / 0.7 NA objective with a resolution of 2048 x 2048 pixels and a pixel size of 378.6 nm. Immunohistochemistry of WT and KO mice was done by Tina Kehrwald (technical assistant, animal physiology group, RPTU Kaiserslautern).

2.3.1 Quantification of co-distribution

To determine the co-distribution of Asc-1 with VIAAT or NHE6 to GlyT2-positive terminals, line scans were performed (Bolte & Cordelières, 2006). VIAAT and GlyT2 are established markers for inhibitory synapses (McIntire *et al.*, 1997; Poyatos *et al.*, 1997). Since experiments were also conducted in GlyT2 KO mice, VIAAT was used as the preferred presynaptic marker to reliably identify inhibitory terminals independent of GlyT2 expression. Line scans were manually drawn from the soma across membrane-associated VIAAT or GlyT2 signals, extending ~3-5 μM beyond the membrane into the extracellular space. Only points that were identifiable as part of a cell were included in the analysis. Per cell, four to eight intensity profiles were obtained. For WT Asc-1 and VIAAT co-distribution in WT, 128 intensity profiles from 39 cells of two P11 animals were analyzed. In KO, Asc-1 and VIAAT were measured in 31 cells from two P11 animals, again with 128 intensity profiles. For NHE and GlyT2 co-distribution, a total of 128 intensity profiles were collected from 16 cells of two P11 animals. The position of the maximum VIAAT or GlyT2 signal was set to zero. Negative scan distances represent intracellular and positive scan extracellular positions (Figure 2.2A). Images were processed in Fiji ImageJ 1.54 (Schindelin *et al.*, 2009) using a rolling ball background subtraction (radius20), followed by brightness adjustment. For further colocalization analysis, the Pearson correlation coefficient and Costes' test (P_{Costes} , 200 repetitions; Costes *et al.*, 2004) were calculated. Cells selected for line scans were outlined 1-2 μM from the soma in Fiji, and the Coloc2 plugin was used to assess the reliability of signal colocalization (Figure 2.2B). A P_{Costes} value of 1 indicates a >95% probability that true colocalization is present.

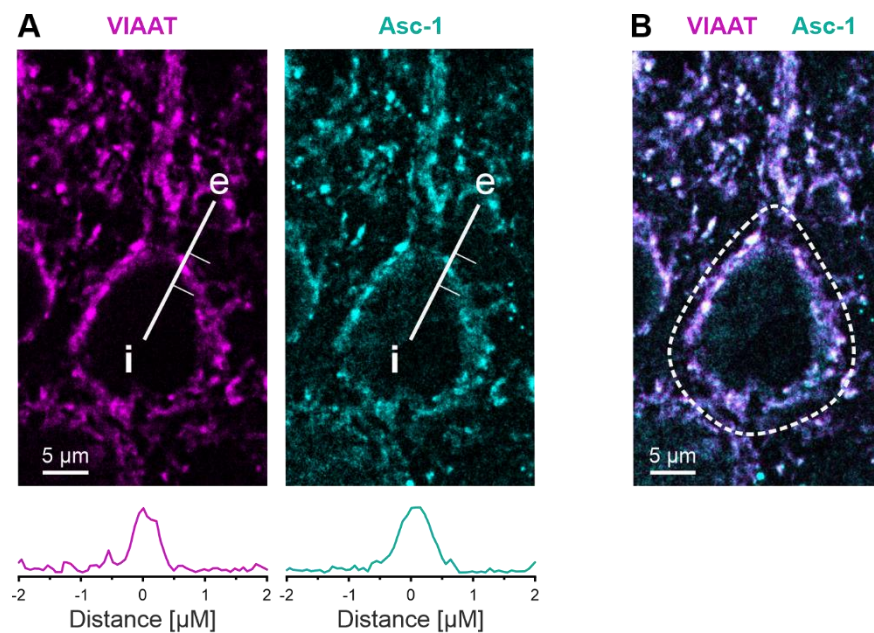


Figure 2.2 Colocalization analysis. Representative example for quantifying co-distribution. (A) Intensity profiles of VIAAT or Asc-1 immunosignals (bottom) were obtained by performing line scans (thick white line) in Fiji. Line scans were manually drawn across the soma from

intracellular to extracellular space, based on visible cell borders. Line scans were first placed in the VIAAT channel and then transferred to the corresponding Asc-1 channel. For NHE6 and GlyT2, line scans were drawn using the GlyT2 signal. Thin lines indicate the region shown in the intensity profiles (bottom). **(B)** For somatic co-distribution analysis, the soma was outlined at 1-2 μM from the membrane in the merged image (dotted white line).

2.4 Electrophysiological recordings

All experiments were performed in the Animal Physiology group at the Technical University of Kaiserslautern. In total, electrophysiological recordings from 160 neurons were analyzed for this thesis. For NT (re)uptake experiments, 108 neurons were included (79 WT and 29 KO). These recordings were performed by Jennifer Klein and me. For the SV (re)filling experiments, 52 neurons were included. Recordings were done by Erika Pizzi, Jennifer Klein, and me.

2.4.1 Preparation of acute brainstem slices

Stock solutions were used for up to seven days, and all working solutions for a maximum of three days. Solutions were stored at 4-6 °C. The pH was adjusted to 7.4 by continuously bubbling with carbogen (95% O₂/ 5% carbogen) for at least 20 min. For the preparation of brainstem slices from P11 mice, a glucose-based preparation solution was used, containing in 1 l (in mM): 2.5 KCl; 1.25 NaH₂PO₄; 2 sodium-pyruvate; 3 myo-inositol (all diluted from 5x stock); 25 NaHCO₃ (from 0.5 M stock); 260 D(+)-glucose H₂O; 1 MgCl₂ (from 1 M stock), and 2 CaCl₂ (from 2 M stock). Osmolarity was 325 ± 10 mOsmol/l. For recovery, storage, and perfusion during recordings, artificial cerebrospinal fluid (ACSF) was used, containing 1 l (in mM): 125 NaCl; 2.5 KCl; 1.25 NaH₂PO₄; 2 sodium-pyruvate; 3 myo-inositol; 0.44 ascorbic acid (all diluted from 10x stock); 25 NaHCO₃ (from 0.5 M stock); 10 D(+)-glucose H₂O; 1 MgCl₂ (from 1 M stock), and 2 CaCl₂ (from 2 M stock). Osmolarity was 290 ± 10 mOsmol/l. Patch pipettes were filled with an internal chloride solution (2mM Cl⁻), containing (in mM): 10 HEPES; 5 EGTA, 1 MgCl₂, 140 K-gluconate, 2 ATP-Na₂ and 0.3 GTP-Na₂. The internal solution was aliquoted and stored at -20 °C.

Coronal brainstem slices were prepared as previously described ([Hirtz et al., 2011](#)). P11 mice were weighed and decapitated without prior anesthesia. The brain was quickly removed and transferred to an ice-cold preparation solution. The brainstem was dissected and glued onto a magnetic plate, which was then mounted in a vibratome chamber (Leica VT 1200S, Leica, Wetzlar, Germany) filled with preparation solution. Brainstem slices of 270 μM thickness were cut at low speed (0.03 mm/s), transferred to ACSF at 37°C for ~30 min, and stored at RT and are continually carbogen-flushed.

2.4.2 Recording from LSO and MNTB neurons

Electrophysiology recordings were performed as previously described (Kramer et al., 2014; Müller et al., 2022). Experiments were blinded after slice preparation, as KO animals can be visually distinguished from WT littermates by their white fur (Hülsmann et al., 2019). To ensure blinding, the beakers were exchanged after slicing. All experiments were done blind, except for Bafi and EIPA recordings.

Whole-cell patch-clamp recordings were performed in brainstem slices from principal neurons of the MNTB and LSO at physiological temperature (36 ± 1 °C). Temperature control was maintained using a temperature controller VII (Luigs & Neumann; Ratingen; Germany). Recordings were performed in either voltage-clamp (VC) or current-clamp (CC) mode using an EPC10 patch-clamp amplifier, controlled by PatchMaster v2.73.5 software (HEKA Electronics, Lambrecht, Germany). Data were sampled at 50 kHz and low-pass filtered at 7.9 kHz. The liquid junction potential was set to 15.4 mV. Offset potentials were compensated online using the amplifier. For recordings, brainstem slices containing MNTB and LSO were transferred to the recording chamber and placed under an upright microscope (Eclipse E600FN; Nikon; Tokyo; Japan). Brainstem slices were continuously perfused with carbogen-flushed ASCF using a peristaltic pump-driven perfusion system (REGLO Analog; Ismatec; Wertheim; Germany). The microscope was equipped with infrared differential interference contrast optics, a 4x objective (CFI E Plan Achromat; Nikon; Tokyo; Japan), and a 60x water immersion objective (CFI Apo 60XW NIR; Nikon; Tokyo; Japan) and mounted on an air-damped table (Physic Instruments; Waldbronn; Germany) to minimize vibrations during recordings. To minimize electromagnetic interferences, the entire electrophysiology setup was surrounded by a Faraday cage. Brainstem slices were visualized using a CCD camera (CC995Mu, S/N 13756; Thorlabs, Newton, USA), controlled via µManager 2.00 (MMStudio 2.2, Open Imaging, Vale Lab, UCSF). Screenshots of brainstem slices and patched neurons were captured using the snipping tool (Windows desktop app). Patch and stimulation pipettes were positioned using a dual micromanipulator system equipped with a linear piezo drive (SMX 12 V; Sensapex; Oulu; Finland). Pipettes were pulled from borosilicate glass capillaries with filaments (GB150 (F-)-8P, Science Products, Hofheim am Taunus, Germany) using a horizontal puller (Flaming Brown Micropipette Puller P-1000; Sutter Instruments; Novato; USA). Patch pipette resistances ranged from 1.5 to 5 MΩ. After achieving whole-cell configuration, either in MNTB or LSO principal neurons, recordings were initiated using a combination of steps and continuous protocols in VC and CC mode. These protocols were used to assess cell quality and determine passive and active membrane properties (Figure 2.3B, D). Quality criteria include a resting membrane potential (V_{rest}) below -60 mV, the presence of a through hyperpolarization-activated inward current (IH), AP generation upon

depolarizing current injections, and a stable series resistance (R_s) below 15 M Ω , with a maximal fluctuation of ≤ 5 M Ω during the entire recording.

The location of the MNTB was identified at 4x magnification based on characteristic medial-ventral location in the brainstem (Figure 2.3A1; Paxinos & Franklin, 2001). MNTB principal neurons were identified at 60x magnification and selected based on soma size (20 μm ; Figure 2.3A2) and confirmed by their biophysical properties (I_H-sag, onset spike; Figure 2.3B; Morest, 1968; Hoffpauir et al., 2010; Sivaramakrishnan et al., 2018). The location of the LSO was identified at 4x magnification based on its characteristic bean shape (Figure 2.3C1). LSO principal neurons were identified at 60x magnification and selected by spindle-shaped somata (Figure 2.3C2) and their biophysical properties (I_H-sag, onset spike; Figure 2.3D; Sterenborg et al., 2010).

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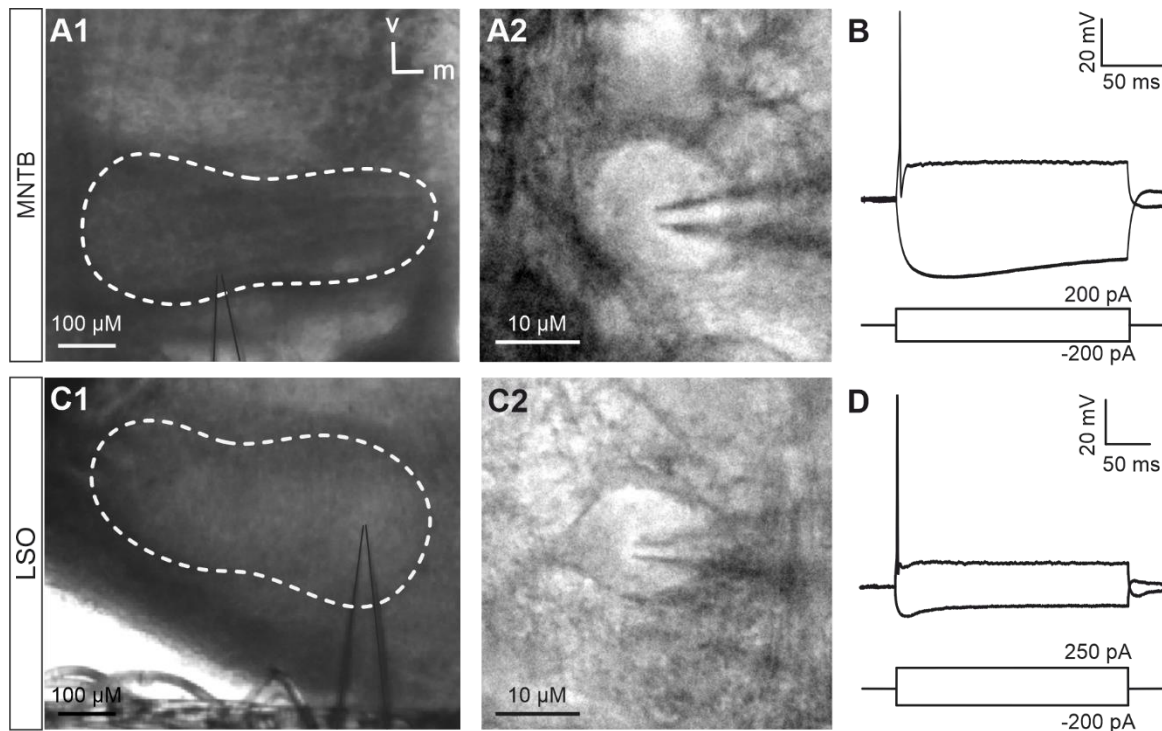


Figure 2.3 Identification and quality control check of MNTB and LSO principal neurons. (A1) Brainstem slice showing the MNTB (dotted line). A patch pipette indicates the position of the recorded neuron. Scale bar 100 μm . (A2) Higher magnification of the patched MNTB principal neuron. Scale bar 10 μm . (B) Representative voltage response to hyperpolarizing (-200 pA) and depolarizing (200 pA) current steps, showing characteristic I_H-sag and an onset spike AP. (C1-D) Same as A1-B but for LSO. Abbreviations: m, medial; v, ventral. Modified from (Hofmann, 2021).

2.4.3 Stimulation procedure

Orthodromic stimulation was performed in VC mode from LSO principal neurons, while antidromic stimulation was performed in CC from MNTB principal neurons. A bipolar stimulation pipette, filled with ACSF, was placed either at the lateral edge of the MNTB for orthodromic recordings (Figure 2.4A) or at the medial edge of the LSO for antidromic recording (Figure 2.4B), covering the full projection length of the MNTB axons. A copper wire was coiled around the stimulation pipette (+ pole), and another wire was inserted into the pipette (- pole). Stimulus pulses (100 μ s duration, up to 1.6 mA) rectangular pulses were applied using MC_stimulus II software and a programmable stimulator (STG4002; MultiChannel Systems; Reutlingen; Germany). The stimulation pipette was placed before establishing the whole-cell configuration. Depending on the configuration, stimulation evoked either postsynaptic currents (eIPSCs; orthodromic) or APs (antidromic). Stimulation intensity was increased until an eIPSC or AP was reliably evoked, and the stimulation protocol was then started at twice the threshold intensity. If no response could be detected, the stimulation pipette was repositioned.

Before each stimulation protocol, the recorded neuron underwent a quality control check (Figure 2.3). Once the criteria were reached, the corresponding stimulation protocol was applied. After each BL period and subsequent 60-s recovery period, a 6-s pause was introduced for data storage. All stimulation protocols (Protocol 1-Protocol 6) were used in orthodromic experiments (Figure 2.4A, C). Protocol 5 and Protocol 6 were additionally applied for antidromic stimulation (Figure 2.4B, C).

Protocol 1 consisted of a 60-s BL period at 0.2 Hz, followed by a 60-s challenge period at 10 Hz, and a 60-s recovery period at 0.1 Hz. All phases were initially recorded in ACSF (Ctrl). In total, the stimulation protocol lasted three minutes and included 618 stimulus pulses. After the wash-in of either 10 μ M or 50 μ M of the Asc-1 inhibitor BMS (see Section 2.4.4), the same protocol was repeated. This protocol was applied in both WT and KO recordings.

Protocol 2 was based on Protocol 1 but was repeated seven times. After the first round, either 50 μ M BMS or 0.1% dimethyl sulfoxide (DMSO) was continuously bath applied in separate experiments for both WT and KO. The stimulation protocol lasted 16 minutes in total and included 4,644 stimulus pulses.

Protocol 3 consisted of a 60-s BL period at 1 Hz, followed by a 50-Hz | 60-s challenge stimulation and a 1-Hz | 60-s recovery period. The challenge and recovery periods were repeated 10 times. In total, the protocol lasted 21 minutes and included 30,660 stimulus pulses. Recordings were performed either in ACSF (Ctrl), 2 μ M ALX, 50 μ M BMS, or a combination of 2 μ M ALX + 50 μ M BMS (see Section 2.4.4). The drugs were bath applied 10 min before the recording and maintained throughout the entire protocol.

Protocol 4 was an extension of Protocol 3 and included the application of 2 μM ALX + 50 μM BMS. After the end of Protocol 3, 1 Hz stimulation was continued for an additional 15 min while 100 nM Stry (see Section 2.4.4) was added to the drug cocktail (ALX + BMS + Stry). In total, the stimulation lasted 36 mins and included 31,560 stimulus pulses.

Protocol 5 consisted of a 60-s BL period at 1 Hz, followed by a 60-s stimulation challenge and a 60-s recovery period at 1 Hz. The challenge was applied at four different stimulation frequencies: 10, 50, 100, and 200 Hz. In total, the protocol lasted 9 mins and included 21,900 stimulus pulses. After a 10-min wash-in of 2 μM Bafi (see Section 2.4.4), the entire protocol was repeated. Subsequently, a new cell was patched, Bafi was continuously washed in, and the protocol was applied once more.

Protocol 6 consisted of a 60-s BL period at 1 Hz, followed by a 60-s stimulation challenge and a 60-s recovery period at 1 Hz. The challenge was applied at two stimulation frequencies: 50 and 100 Hz. In total, the protocol lasted 5 mins and included 9,180 stimulus pulses. After a 10-min wash-in of 100 μM EIPA, the protocol was repeated.

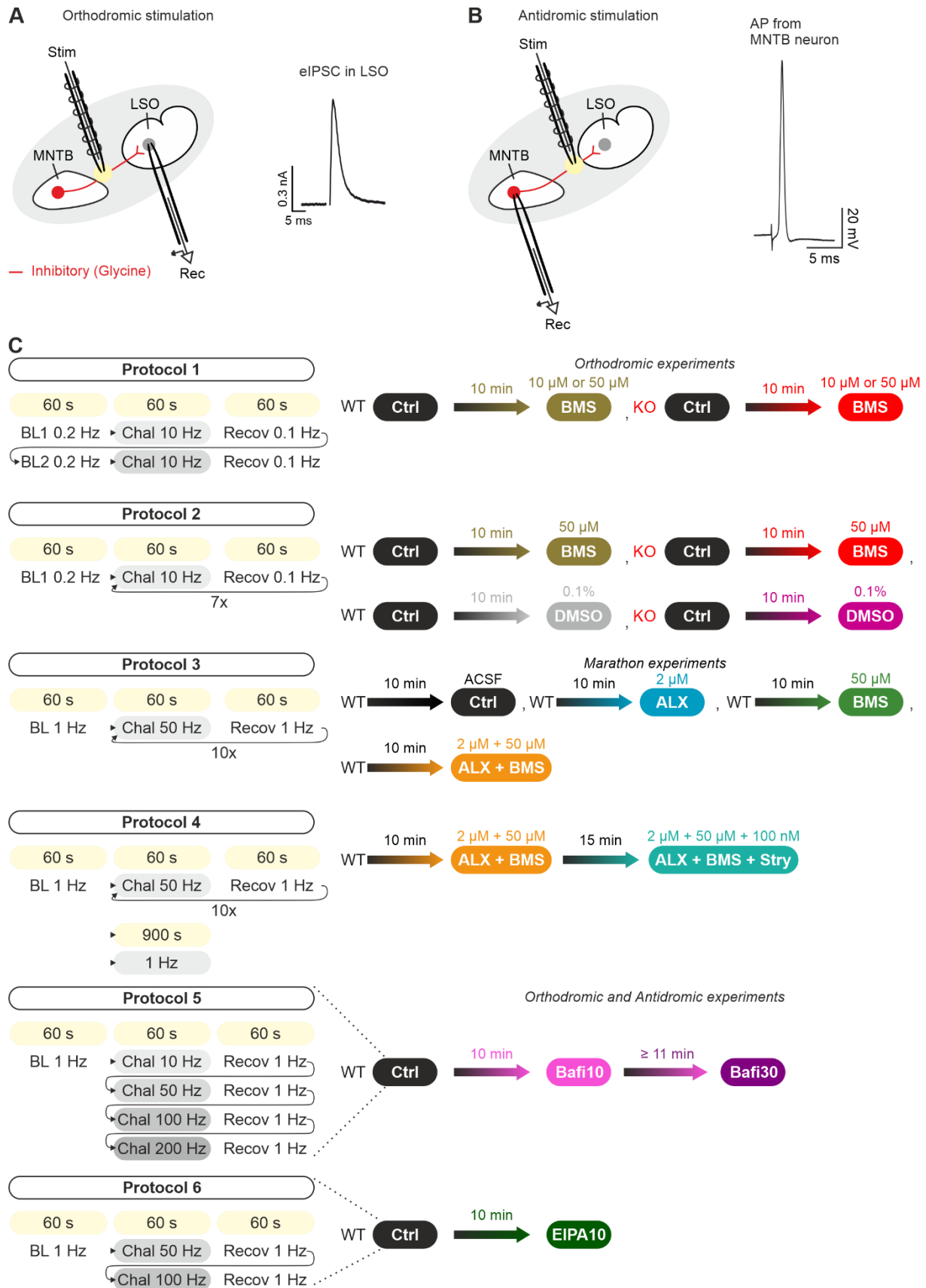


Figure 2.4 Overview of stimulation procedure and protocols. (A-B) Schematic overview of orthodromic and antidromic stimulation approaches. **(A)** Orthodromic stimulation: The stimulation pipette was placed at the lateral edge of the MNTB to stimulate glycinergic MNTB fibers. Whole-cell recordings were performed from a principal LSO neuron. A representative

evoked inhibitory postsynaptic current (eIPSC) is shown (right). **(B)** Antidromic stimulation: The stimulation pipette was placed at the medial edge of the LSO to stimulate the maximal length of MNTB axons. Recordings were obtained from a principal MNTB neuron. A representative antidromically evoked AP is shown (right). **(C)** Six different stimulation protocols were used across experiments. Protocols 1 and 2 were applied in both WT and KO. Protocols 3 to 6 were applied only in WT. Protocols 1-6 were used for orthodromic stimulation, whereas only Protocol 5 and Protocol 6 were applied in antidromic stimulation experiments. Panel C was created by Erika Pizzi (former postdoc, Animal Physiology, RPTU Kaiserslautern-Landau) and modified by myself.

2.4.4 Used pharmacy

In this thesis, I used various drugs to target distinct molecular components in specific experimental conditions. Below, all applied drugs are listed along with their molecular target, final concentrations, and solvents. All drugs were prepared as concentrated stock solutions, aliquoted, and stored at -20 °C. On the day of the experiment, stocks were freshly diluted in ACSF to the final working concentration.

ALX-1393 (ALX; Sigma-Aldrich, St. Louis, MO, USA) is a high-affinity and selective inhibitor of GlyT2 (Caulfield *et al.*, 2001; Mingorance-Le Meur *et al.*, 2013; Vandenberg *et al.*, 2016; Mostyn *et al.*, 2017; Benito-Muñoz *et al.*, 2021). ALX-1393 was dissolved in DMSO at a stock concentration of 2 mM. A final concentration of 2 μ M was used for all experiments. The IC₅₀ for GlyT2 is in the low nanomolar range (~10-100 nM).

Bafilomycin A1 (Bafi; Abcam, Cambridge, UK, or Thermo Fisher Scientific, Waltham, USA) is a V-ATPase antagonist (Bowman *et al.*, 1988; Crider *et al.*, 1994; Wang *et al.*, 2021). It was dissolved in ethanol (EtOH) to a stock concentration of 2 mM. A final concentration of 2 μ M was used for experiments. The IC₅₀ of Bafi is in the nanomolar (<10 nM) range.

Bristol-Myers Squibb, Compound 1 (BMS-466442, referred to as BMS; Tocris, Bristol, UK) is a non-transportable Asc-1 antagonist that blocks substrate binding and completely blocks transporter function (Brown *et al.*, 2014; Torrecillas *et al.*, 2019). BMS was dissolved in DMSO at a stock solution concentration of 50 mM. A final concentration of either 10 μ M or 50 μ M was used for experiments. The IC₅₀ of BMS is in the nanomolar (~10-400 nM) range.

6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX; Abcam, Cambridge, UK) is a competitive antagonist at AMPA- and kainate-type glutamate receptors (Honoré *et al.*, 1988). CNQX was dissolved in DMSO at a stock solution concentration of 10 mM. A final concentration of 10 μ M was used for experiments. The IC₅₀ for AMPA receptors is in the low micromolar (~0.2-2 μ M) range. CNQX was only used for experiments with KO animals to block excitatory currents.

Ethylisopropylamiloride (EIPA; Tocris, Bristol, UK) is a Na^+/H^+ exchanger antagonist (Alexander *et al.*, 2011; Li *et al.*, 2020). It was dissolved in DMSO to a stock concentration of 100 mM. A final concentration of 100 μM was used for experiments. The IC_{50} of EIPA is in the small micromolar ($\sim 1\text{-}10\ \mu\text{M}$) range.

Strychnine hydrochloride (Stry; Sigma-Aldrich, St. Louis, MO, USA) is a potent and selective competitive antagonist for GlyRs (Young & Snyder, 1973). Stry was dissolved in distilled water at a stock solution concentration of 20 mM. A final working concentration of 100 nM was used in experiments. The IC_{50} of Stry is in the small nanomolar ($\sim 1\text{-}10\ \text{nM}$) range.

2.5 Data analysis

2.5.1 EPSC and AP peak amplitudes

Data were initially imported and analyzed in IGOR Pro (versions 6.37 and 9.01; WaveMetrics Inc., Lake Oswego, OR, USA). Foot-to-peak eIPSC amplitudes (Figure 2.5A) were analyzed using current traces using custom-written routines in IGOR Pro written by Alexander Fischer (former PhD student, TU Kaiserslautern, Animal Physiology). eIPSC amplitudes were normalized to the mean 0.2 or 1 Hz BL amplitude recorded within each stimulation protocol (Figure 2.4C). AP fidelity was analyzed using voltage traces using custom-written routines in IGOR Pro written by Alexander Fischer (former PhD student, TU Kaiserslautern, Animal Physiology; Figure 2.5B). Further analysis was performed in Excel 2021 (version 2105, Microsoft) and GraphPad Prism 9 (GraphPad Software).

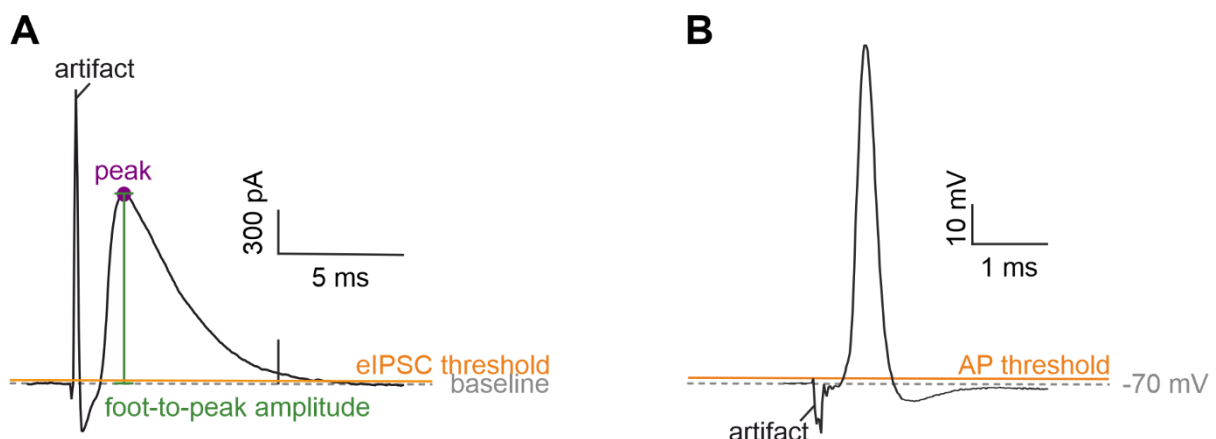


Figure 2.5 Analysis of eIPSCs and APs. (A) Representative eIPSC recorded from an LSO principal neuron. The food-to-peak amplitude is shown (green line), with the peak marked by a purple dot. An example detection threshold is indicated by the orange line; the gray dotted line represents the BL. **(B)** Representative AP recorded from an MNTB principal neuron. Events reaching a threshold of $\geq 1\ \text{mV}$ were detected as APs (orange line). The gray dotted line indicates resting membrane potential.

2.5.2 Analysis of synaptic performance

For Asc-1 experiments, a detection threshold, defined as 1x noise level, was applied to identify synaptic transmission failures. Noise values of individual eIPSCs amplitudes were determined as the 90% peak residual of a monoexponential decay fit. The mean noise level for each recording was calculated by averaging the noise values of eIPSCs amplitudes recorded during the BL period (Krächan, 2019). Failures were defined using an all-or-nothing approach, in which each event was classified as either success or failure. Events falling below the detection threshold were considered a failure (0% fidelity), while all the others were counted as successful responses (100% fidelity; Figure 2.5A). Failures were replaced with a value of zero. In the V-ATPase and NHE experiments, eIPSC amplitudes underwent zero-correction analysis, and all negative values were set to zero. AP failures were defined as peak amplitudes <1 mV. Steady-state eIPSC amplitudes and AP fidelity were determined from the last 10 seconds of the challenge or recovery period.

2.5.3 Analysis of synaptic release properties

sIPSCs were analyzed during 60-s stimulation at 0.2 Hz BL and 0.1 Hz recovery periods (Figure 3.9A1; Figure 3.19A1; Figure 3.27-3.29A1, A3) and 1-Hz | 60-s stimulation periods for BL and recovery periods (Figure 3.39A1-A6) and manually detected using Clampfit 10.2.0.14 (Molecular Devices, CA, USA). sIPSC peak amplitude histograms were generated and fitted with Gaussian curves (Figure 3.9A2; Figure 3.19A2; Figure 3.27-3.29A2, A4; Figure 3.39B1-B6). The quantal size (q) was determined by a Gaussian fit from 0 pA to the bin after the first local maximum (Krächan *et al.*, 2017), bin width was 5 pA or in NHE experiments 3 pA in some cases. The sIPSC frequency was calculated by dividing the number of events by the duration of a recovery period (*sIPSC frequency [Hz] = events/60 s*). The cumulative current evoked by the complete release of the RRP (I_{RRP}) was estimated by forward extrapolation of a linear fit through eIPSC₁₋₅ during 100 Hz stimulation (Elmqvist & Quastel, 1965). The RR_{s50-60} was assessed from the slope of a line fit through the last 10 s (s_{50-60}) of the cumulative vesicle curve in each challenge period. The number of SVs in the RRP (N_{RRP}) was calculated as I_{RRP}/q (Brill *et al.*, 2021). The quantal content was either determined as $m_{s50-60} = eIPSC_{s50-60}/q_{each\ trial}$ (Figure 3.30-3.32) or $m_1 = eIPSC_1/q_{Mean\ of\ BL-Recov4}$ (Figure 3.39 and Figure 3.41). The release probability (P_v) was calculated as $I_{RRP}/eIPSC_1$.

2.6 Data presentation

Data were visualized using Excel 2021 (version 2105, Microsoft) and GraphPad Prism 9 (GraphPad Software). Representative current or voltage traces were selected close to the mean. Data are presented as mean $\pm$ SEM. Time courses are simple smoothing averages over three (1 Hz) or nine (10, 50, 100, and 200 Hz) data points, with SEM indicated as lightly shaded areas. Bar graphs display means with individual data points shown as circles. Gray-shaded areas represent challenge periods. WT samples are shown in black, and KO in red.

2.7 Statistic

Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software). Details on the specific statistical tests used are provided in each table legend. The data were checked for normal distribution using the Kolmogorov-Smirnov test. A paired t-test was used for normally distributed, paired data; otherwise, the Wilcoxon test was applied. If the data values were normally distributed, they were compared using an unpaired two-tailed t-test. Otherwise, they were analyzed using the Mann-Whitney test. For multiple comparisons, one-way ANOVA with Bonferroni correction was used for normally distributed data (paired or unpaired). If data were not normally distributed, the Friedman test with Dunn's correction (paired) or the Kruskal-Wallis test with Dunn's correction (unpaired) was applied. *Post hoc* tests (Dunn's or Bonferroni's) were applied where appropriate ($\alpha_{\text{adjusted}} = \alpha/k$, where α is the significance level and k is the number of comparisons). For the V-ATPase and NHE experiments, *post hoc* corrections were applied manually, for all other comparisons, p-values in tables were adjusted p-values after correction.

3 Results

3.1 Role of Asc-1 in glycinergic transmission at MNTB-LSO synapses

GlyT2 KO (KO) mice do not exhibit a complete blockade of synaptic transmission (Brill *et al.*, 2021), I therefore investigated whether an additional glycine transporter can facilitate the (re)uptake of glycine from the synaptic cleft to (re)fill SVs in the presynaptic MNTB terminal in WT. One potential candidate is the alanine-serine-cysteine transporter 1 (Asc-1; Figure 1.2). Pharmacological and genetic studies have shown that Asc-1 contributes to synaptic transmission by regulating presynaptic glycine availability, which influences spontaneous release (see Section 1.1).

3.1.1 Asc-1 localization in presynaptic axon terminals of MNTB-LSO synapses

Using immunohistochemistry and confocal microscopy, the Asc-1 signal in the SOC was analyzed. My hypothesis was guided by mRNA sequencing results from laser-dissected tissues, which revealed high expression of *Slc7a10*, the gene encoding Asc-1, in the MNTB of P11 $\pm$ 1 mice (Brill *et al.*, 2021), as well as Asc-1 expression in the neurons (Helboe *et al.*, 2003; Ehmsen *et al.*, 2016). These results suggested that Asc-1 is an interesting candidate for an additional glycine transporter at MNTB-LSO synapses. Asc-1 labeling (cyan) was observed in the WT MNTB and LSO (Figure 3.1A1). At the cellular level, LSO neurons were surrounded by puncta (Figure 3.1A2-A4). To determine whether Asc-1 was present in glycinergic neurons, its co-labeling with VIAAT was analyzed in the SOC. VIAAT is a marker for inhibitory synapses (McIntire *et al.*, 1997; Sagné *et al.*, 1997; Dumoulin *et al.*, 1999b; Wojcik *et al.*, 2006). Previous studies have shown VIAAT immunoreactivity in the SOC and LSO of mice (Hirtz *et al.*, 2012; Ebberts *et al.*, 2017; Brill *et al.*, 2021). My results confirmed these previous findings (Figure 3.1B1-B4). Co-distribution of Asc-1 and VIAAT (magenta) was observed in both MNTB and LSO (Figure 3.1C1). Double-labeled puncta surrounded the LSO neurons (Figure 3.1C2-C4). A close-up of the white frame in Figure 3.1C2 highlighted the co-distribution of Asc-1 and VIAAT as white puncta (Figure 3.1C3-C4), which were attributed to glycinergic MNTB terminals. Intensity profiles of Asc-1 and VIAAT were analyzed to assess co-distribution. Both profiles overlapped, indicating the co-distribution of both proteins for a representative LSO neuron (Figure 3.1D1) and average data (Figure 3.1D2). Further quantification using Pearson's coefficient (Figure 3.1D3; 0.65 ± 0.02 , $n = 39$) and the P_{Costes} coefficient (Figure 3.1D4; 1.00 ± 0.00 , $n = 39$) confirmed co-distribution. Taken together, these results demonstrated close proximity of Asc-1 and VIAAT in glycinergic MNTB axon terminals in WT.

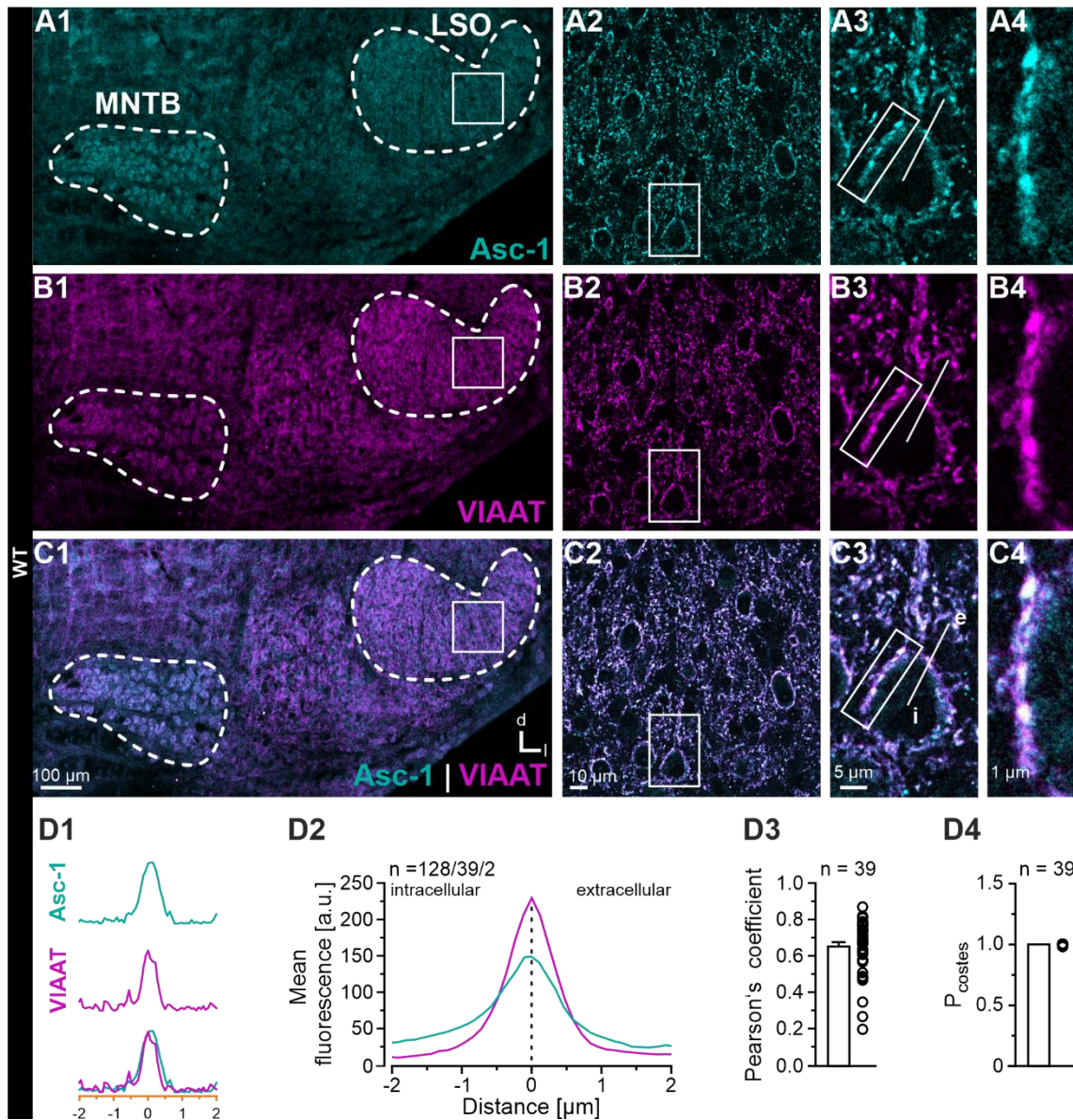


Figure 3.1 Asc-1 is abundant in the MNTB and LSO and co-distributes with VIAAT in the LSO in WT. (A-C) Immunohistochemical analysis of Asc-1 and VIAAT. **(A1)** Labeling for Asc-1 (cyan) in the SOC. Highlighted are MNTB and LSO. **(A2)** Zoom-in of LSO framed in A1. **(A3)** Close-up of a representative LSO in the framed area in A2. The white line showed the intensity profile shown in D1. **(A4)** Close-up of the framed area in A3. **(B)** Same as A, but for VIAAT labeling (magenta). **(B1-B4).** **(C)** Same as A, but for Asc-1 and VIAAT double labeling **(C1-C4).** **(D1)** Quantification of Asc-1 and VIAAT co-distribution. Individual intensity profile for Asc-1 and VIAAT (orange lines in A3-C3). **(D2)** Average intensity profiles for Asc-1 and VIAAT. Before averaging, each profile was aligned to the peak of the VIAAT signal. For more details, see Material and Methods. Negative μ m values show intracellular (i) and positive μ m values extracellular (e) signals of the LSO neuron, indicated in C3. n, number of profiles/cells/animals. **(D3)** Pearson's coefficient and **(D3)** corresponding P_{Costes} values. Optical thickness: 1 μ m. Coronal brainstem slice at P11 WT.

3.1.2 Contribution of Asc-1 to synaptic transmission at MNTB-LSO synapses

To determine whether Asc-1 contributed to inhibitory synaptic transmission at MNTB-LSO synapses, I examined synaptic transmission in the LSO under MNTB fiber stimulation in P11±1 WT mice. In the first set of electrophysiology recordings, the selective Asc-1 antagonist BMS-466442 (BMS) was applied to brainstem slices. BMS is a non-transportable antagonist. It inhibits substrate binding and completely blocks transporter function (Brown *et al.*, 2014; Torrecillas *et al.*, 2019). MNTB fibers were stimulated using Protocol 1 (Figure 2.4C), which consisted of an initial 60-s period at 0.2 Hz to establish the baseline (BL) eIPSC amplitudes, followed by a 10-Hz | 60-s stimulation challenge (Chal) and a 0.1-Hz | 60-s recovery (Recov) period; all were recorded in ACSF (Ctrl). In total, Protocol 1 lasts three minutes and includes 618 stimulus pulses. Subsequently, the Asc-1 inhibitor BMS (10 μ M) was bath applied under 0.1-Hz | 10-min stimulation, followed by a second application of Protocol 1 (BMS condition). eIPSC amplitudes of a representative neuron under Ctrl and BMS conditions were shown in Figure 3.2A1-A2 at different time points during the recording. The observed synaptic depression and recovery dynamics are consistent with previous studies (Kramer *et al.*, 2014; Brill *et al.*, 2019; Krächan, 2019). Under Ctrl conditions, the mean BL eIPSC amplitude was 1,852 pA. During the 10-Hz challenge, peak amplitudes decreased to 995 pA in the last 10 s and returned to BL (1,966 pA) during the recovery period. After the application of 10 μ M BMS for 10 minutes, mean eIPSC amplitudes remained stable (2,251 pA). Under BMS conditions, mean BL amplitudes reached 2,021 pA. Synaptic depression during BMS resulted in amplitudes of 1,153 pA in the last 10 s, which returned to BL (2,034 pA) during the recovery phase (Figure 3.2A3). Thus, these single-cell results provide some evidence that Asc-1 inhibition was incomplete or that Asc-1 does not contribute to synaptic transmission at MNTB-LSO synapses.

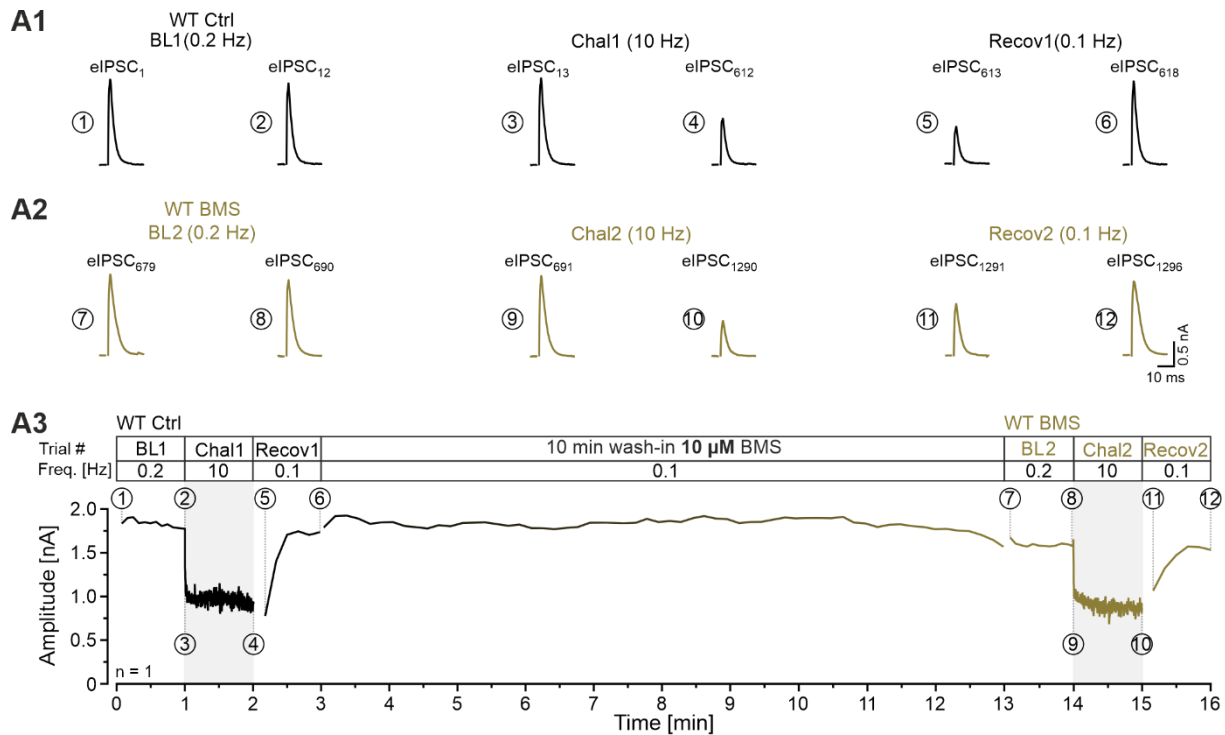


Figure 3.2 Reliable transmission under *Asc-1* inhibition with 10 μ M BMS in WT. (A1) Representative eIPSC amplitudes for WT Ctrl (ACSF, black), showing during BL1 eIPSC₁ and eIPSC₁₂, during Chal1 eIPSC₁₃ and eIPSC₆₁₂, and Recov1 eIPSC₆₁₃ and eIPSC₆₁₈. Numbers in circles in A1 show eIPSC in Protocol 1 in A3. **(A2)** Same as A1, but for WT BMS (olive). **(A3)** Time course of absolute peak eIPSC amplitudes obtained with Protocol 1 (see Figure 2.4C) from a P11 LSO neuron. 0.2-Hz | 60-s BL1, 10-Hz | 60-s challenge (Chal1), and 1-Hz | 60-s recovery (Recov1) for WT Ctrl. Followed by 0.1-Hz | 10-min 10 μ M BMS wash-in and BL2, Chal2, and Recov2 for WT BMS. Trace is color-coded from black to olive. Challenge periods are marked with gray areas. Time courses are simple moving averages. The total number of stimulus pulses is 1296.

Time courses of all recorded cells were shown in Figure 3.3A. The mean BL time courses were comparable between conditions (Ctrl: 1,597 pA; BMS: 1,739 pA, corresponding to 100% and 109%, respectively; Figure 3.3B1). There was no significant difference between them (Figure 3.3B2; Table 3.1). Time courses synaptic depression showed no difference between Ctrl and BMS conditions. Amplitudes decreased to 870 pA (Ctrl) and 887 pA (BMS), corresponding to 54% and 56% of BL1 values during the last 10 s (s_{50-60} ; Figure 3.3C1). Statistical analysis showed no significant difference (Figure 3.3C2; Table 3.1). Mean amplitudes returned to BL1 levels, showing no significant difference between Ctrl and BMS (1,634 pA vs. 1,637 pA, corresponding to 102% and 103%; Figure 3.3D1-D2; Table 3.1). Overall, the results showed no significant differences in BL, synaptic depression, or recovery between Ctrl and BMS, confirming that synaptic transmission was unaffected after a 10-min wash-in with 10 μ M BMS.

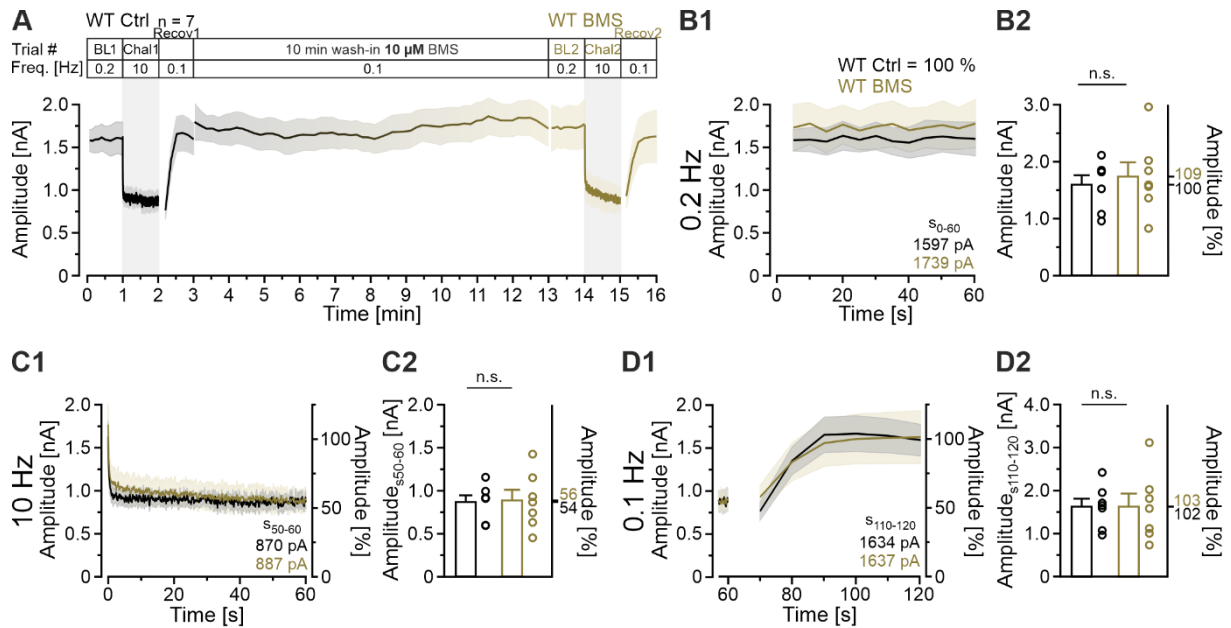


Figure 3.3 Unchanged transmission under Asc-1 inhibition with 10 μ M BMS in WT. (A) Time course of mean eIPSC peak amplitudes obtained with Protocol 1 (see Figure 2.4A1) for WT Ctrl (black) and WT BMS (olive). Trace is color-coded from black to olive. Challenge periods are marked with gray areas. (B1) BL1 and BL2 traces (WT Ctrl vs. WT BMS), WT Ctrl was set to 100%. Mean values are shown at the bottom right. (B2) Statistics for BL analysis. (C1) Time course of absolute and normalized mean eIPSC peak amplitudes during 10-Hz | 60-s challenge periods. The bottom right numbers indicate the mean amplitudes at s_{50-60} . (C2) Statistics for challenge analysis at s_{50-60} . Mean normalized values are shown on the right y-axis. (D1) Same as C1, but during 0.1-Hz | 60-s recovery periods. (D2) Same as C2, but for recovery analysis at $s_{110-120}$. Note the different amplitude scaling in B1-D2. Values are shown as mean $\pm$ SEM. Time courses are simple moving averages (SEM lightly shaded). $n = 7$, same neurons. See Table 3.1 for details, including statistics.

Previous results had shown no effect of Asc-1 during transmission (Figures 3.1-3.2). Therefore, a higher concentration of BMS (50 μ M) was applied, using again Protocol 1 (Figure 2.4C). Like the results observed with 10 μ M BMS, eIPSC amplitudes followed comparable time courses throughout the entire recording period (Figure 3.4A). Mean BL amplitudes were similar between conditions (Ctrl: 1,918 pA, BMS: 1,567 pA, corresponding to 100% and 82%, Figure 3.4B1) and did not differ significantly (Figure 3.4B2; Table 3.1). Synaptic depression between the Ctrl and BMS conditions was not statistically different. The amplitudes decreased to 966 pA (Ctrl) and 848 pA (BMS), corresponding to 50% and 44% during s_{50-60} (Figure 3.4C1-C2; Table 3.1). During the recovery period, mean amplitudes returned to 83% (Ctrl) and 76% (BMS), corresponding to 1,787 pA and 1,488 pA, respectively (Figure-3.4D1-D2; Table 3.1). In summary, increasing the BMS concentration to 50 μ M did not significantly reduce eIPSC amplitudes during BL, challenge, or recovery periods. This indicates that Asc-1 does not contribute to synaptic transmission under these stimulation conditions.

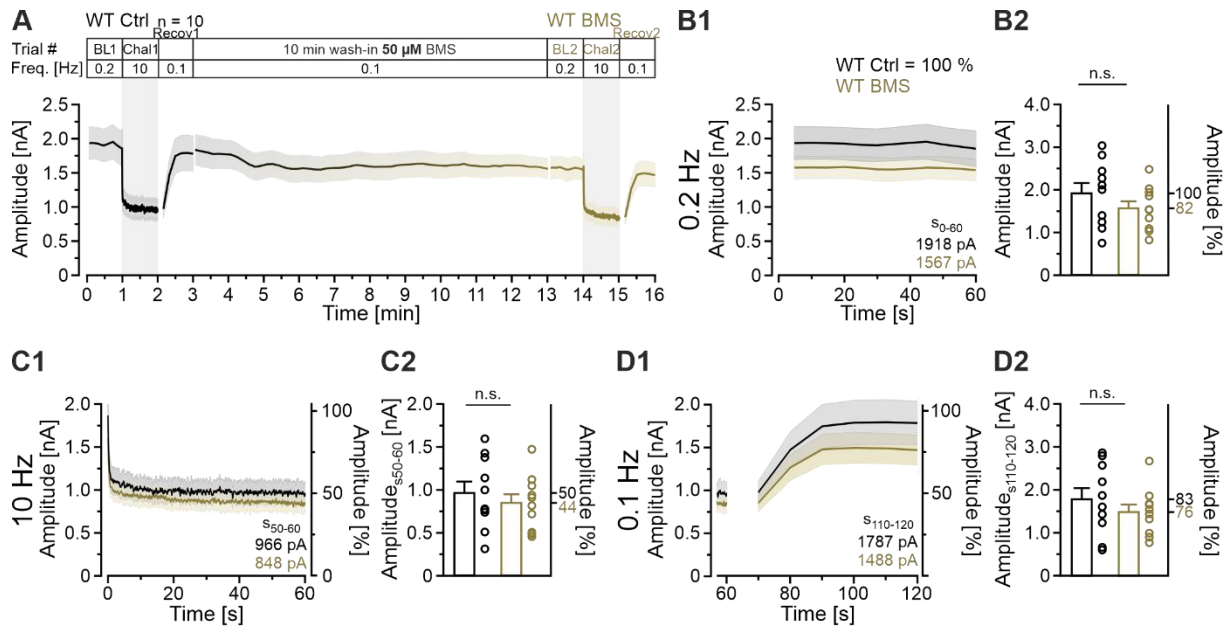


Figure 3.4 Unchanged transmission under Asc-1 inhibition with a higher concentration of BMS (50 μ M) in WT. (A) Time course of mean eIPSC peak amplitudes obtained with Protocol 1 (see Figure 2.4C) for WT Ctrl (black) and WT BMS (olive). Trace is color-coded from black to olive. Challenge periods are marked with gray areas. (B1) BL1 and BL2 traces (WT Ctrl vs. WT BMS), WT Ctrl was set to 100%. Mean values are shown at the bottom right. (B2) Statistics for BL analysis. (C1) Time course of absolute and normalized mean eIPSC peak amplitudes during 10-Hz | 60-s challenge periods. The bottom right numbers indicate the mean amplitudes at s_{50-60} . (C2) Statistics for challenge analysis at s_{50-60} . Mean normalized values are shown on the right y-axis. (D1) Same as C1, but during 0.1-Hz | 60-s recovery periods. (D2) Same as C2, but for recovery analysis at $s_{110-120}$. Note the different amplitude scaling in B1-D2. Values are shown as mean $\pm$ SEM. Time courses are simple moving averages (SEM lightly shaded). $n = 7$, same neurons. See Table 3.1 for details, including statistics.

Table 3.1: Mean eIPSC amplitudes during 10-Hz challenge | 0.1-Hz recovery with 10 and 50 μ M BMS in WT.

10 μ M and 50 μ M BMS			Ctrl (ACSF) $n = 7$		BMS $n = 7$		Ctrl vs. BMS	
	Stimulation freq. [Hz]	Amplitudes [pA]	Mean	SEM	Mean	SEM	p-value	Figure
10 μ M BMS	0.2	BL	1597	163	1739	249	0.685	3.3B2
	10	Chal s_{50-60}	870	78	887	123	0.914	3.3C2
	0.1	Recov $s_{110-120}$	1634	189	1637	305	0.995	3.3D2
			Ctrl (ACSF) $n = 10$		BMS $n = 10$		Ctrl vs. BMS	
	Stimulation freq. [Hz]	Amplitudes [pA]	Mean	SEM	Mean	SEM	p-value	Figure
50 μ M BMS	0.2	BL	1918	242	1567	163	0.071	3.4B2
	10	Chal s_{50-60}	966	133	848	102	0.312	3.4C2
	0.1	Recov $s_{110-120}$	1787	261	1488	175	0.180	3.4D2

Data are presented as mean $\pm$ SEM. Ctrl vs. BMS: Paired two-tailed t-test.

Although no statistically significant difference in amplitudes was observed, there is a trend toward decreased amplitudes. Synaptic fidelity was examined to determine whether MNTB-LSO synapses transmit SVs without failures. In 10 μM BMS Ctrl and 10 Hz challenge, synaptic transmission remained failure-free, with a mean fidelity of 100% across all challenge periods (Figure 3.5A1; Table 3.2), and the results are statistically indistinguishable at s_{50-60} (Figure 3.5A2). Experiments comparing Ctrl and 50 μM BMS also showed that synaptic transmission remained failure-free, with a mean fidelity of 100% (Figure 3.5B1-B2; Table 3.2). Synaptic transmission at MNTB-LSO synapses remained failure-free under both 10 and 50 μM BMS, showing no impact of Asc-1 inhibition.

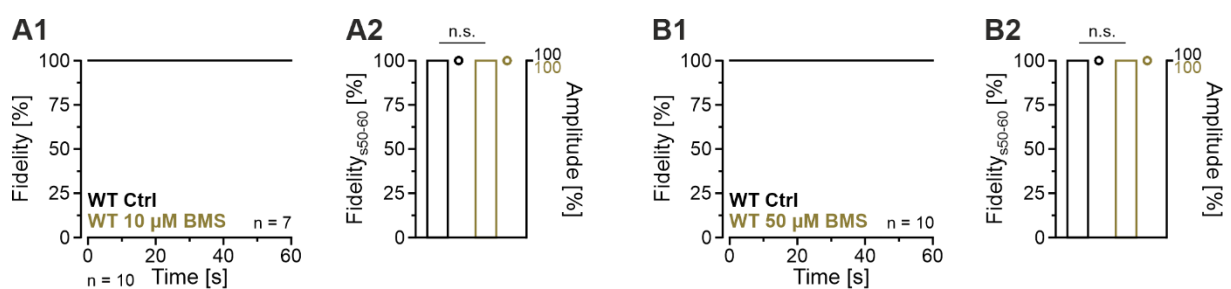


Figure 3.5 Unchanged fidelity under Asc-1 inhibition with 10 or 50 μM BMS in WT. (A) Fidelity rate of eIPSC amplitudes for WT Ctrl (black) and 10 μM WT BMS (olive). (A1) Time course of mean fidelity during 10-Hz | 60-s challenge periods. (A2) Statistics for fidelity analysis at s_{50-60} of challenge periods. Mean values are shown on the right y-axis. (B) Same as A, but for 50 μM BMS. Values are shown as mean $\pm$ SEM. $n = 7$ neurons. See Table 3.2 for details, including statistics.

Table 3.2: eIPSC Fidelity $_{s50-60}$ during 10-Hz challenge | 0.1-Hz recovery with 10 and 50 μM BMS in WT.

10 μM and 50 μM BMS			Ctrl (ACSF) $n = 7$		BMS $n = 7$		Ctrl vs. BMS	
	Stimulation freq. [Hz]	Fidelity [%]	Mean	SEM	Mean	SEM	p-value	Figure
10 μM BMS	10	s_{50-60}	100	0	100	0	-	3.5A2
	Stimulation freq. [Hz]	Fidelity [%]	Ctrl (ACSF) $n = 10$		BMS $n = 10$		Ctrl vs. BMS	
50 μM BMS	10	s_{50-60}	100	0	100	0	-	3.5B2

Data are presented as mean $\pm$ SEM. Ctrl vs. BMS: Wilcoxon test. Each row has zero difference and cannot calculate a paired test.

3.1.3 Repetitive stimulation of MNTB fibers, combined with Asc-1 inhibition, led to a reduction in eIPSC amplitudes

Because 10-Hz stimulation of MNTB fibers under 10 μ M or 50 μ M BMS did not result in changed in eIPSC amplitudes, I hypothesized that the stimulation Protocol 1 may have been too mild to reveal the functional contribution of Asc-1. In addition, the system retains its primary glycine uptake mechanism via GlyT2. To further explore the potential contribution of Asc-1 to glycinergic transmission at MNTB-LSO synapses, a stronger stimulation protocol was used (Protocol 2; Figure 2.4C). Similar to Protocol 1, Protocol 2 began with a 60-s BL period at 0.2 Hz (BL1), followed by a 10-Hz | 60-s challenge (Chal1) and a 1-Hz | 60-s recovery period (Recov1), all recorded under Ctrl condition. After the Recov1, 50 μ M BMS was bath-applied, and at the same time the Chal and Recov sequence was repeated five times (Chal2-Chal6, Recov2-Recov6). Finally, another BL (BL2), followed by a seventh challenge (Chal7) and recovery (Recov7) was recorded under continued BMS application. In total, Protocol 2 lasted 16 minutes and included 4,266 stimulus pulses (Figure 3.6A). BL eIPSC amplitudes were significantly reduced between Ctrl and BMS, with mean values of 871 pA (Ctrl) and 540 pA (BMS), corresponding to 100% vs. 62% (Figure 3.6A; Table 3.3). The time courses across all challenge periods showed a decrease in eIPSC amplitudes (Figure 3.6B1). During steady-state (s_{50-60}), four out of six comparisons were not significant. At the end of the protocol, Chal1 vs. Chal6 (50% vs. 37%) and Chal1 vs. Chal7 (50% vs. 33%) were significantly different (Figure 3.6B2; Table 3.4). eIPSC amplitudes decreased by 17% over the challenge periods. Similar results were found for Recov periods, eIPSC amplitudes were reduced during the protocol (Figure 3.6C1). Quantitative analysis showed no significance in five out of seven comparisons. Recov5 and Recov7 were significantly reduced compared to BL1, with values of 100% vs. 68% (BL1 vs. Recov5) and 100% vs. 62% (BL1 vs. Recov7, Figure 3.6C2; Table 3.4). Taken together, reduced eIPSC amplitudes in BL and at the end of Chal, and Recov periods demonstrate that Asc-1 inhibition with 50 μ M BMS under repetitive stimulation reduces the synaptic strength, but the effects accumulate over time. This suggests a decrease in glycine availability in the presynaptic MNTB terminal for SV (re)filling.

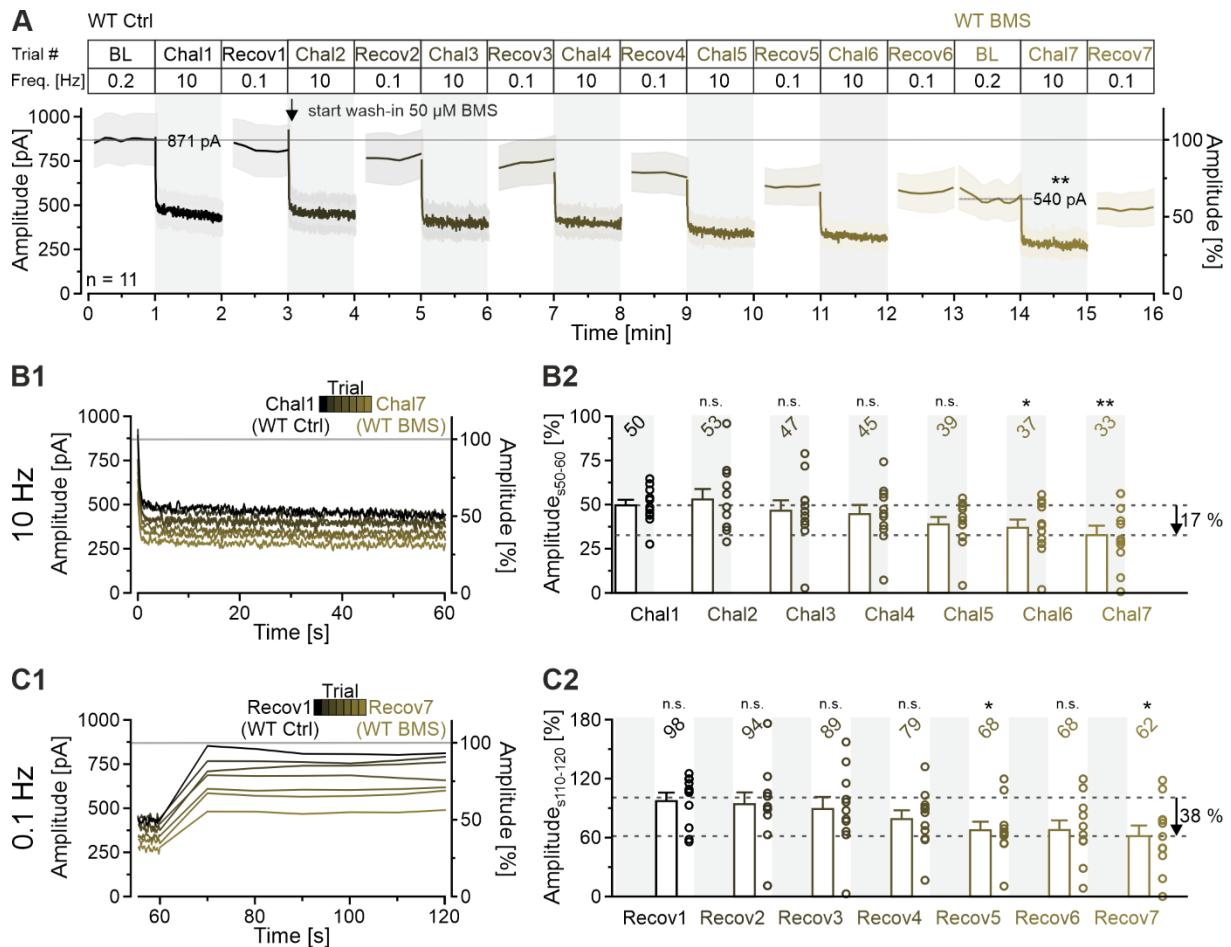


Figure 3.6 Decreased eIPSC amplitudes at the late phase upon repetitive stimulation under Asc-1 inhibition with 50 μ M BMS in WT. (A) Time course of absolute and normalized eIPSC peak amplitudes for WT Ctrl (black) and WT BMS (olive) were obtained with Protocol 2 (see Figure 2.4C). Trials are color-coded from black to olive. BL mean absolute values are written in the figure. Gray horizontal lines show normalization (BL1 = 100%). The arrow marks the start of the BMS wash-in. (B) Overlay of time course of absolute and normalized mean eIPSC peak amplitudes during 10-Hz | 60-s challenge periods (B1). (B2) Statistics of s_{50-60} during challenge periods. Mean value above bars. (C) Same as B, but for $s_{110-120}$ 0.1-Hz | 60-s recovery periods. Gray areas highlight the challenge periods. Values are shown as mean $\pm$ SEM. Time courses are simple moving averages (SEM lightly shaded). $n = 11$, same neurons. See Tables 3.3 and 3.4 for details, including statistics.

To determine whether the decrease in eIPSC amplitudes was due to Asc-1 inhibition rather than a time-dependent effect, I performed sham control experiments. These followed the same structure as Protocol 2 (Figure 2.4C), only the solvent for BMS, 0.1% DMSO, was washed in (Figure 3.7A).

BL eIPSC amplitudes were similar between conditions, with mean values of 1,004 pA (Ctrl) and 1,104 pA (DMSO), corresponding to 100% and 110% (Figure 3.7A; Table 3.3). The time course of eIPSC amplitudes remained stable during overall challenge periods (Chal1-Chal7; Figure 3.7B1). At steady-state (s_{50-60}), the synaptic performance during DMSO-treated

challenge periods (Chal2-Chal7) was statistically indistinguishable from Ctrl (Chal1), indicating no significant time-dependent rundown (Figure 3.7B2; Table 3.4). Similar to the Chal periods, the Recov phases (Recov1-Recov7) returned to the BL1 levels, or even slightly exceeded them (Figure 3.7C1). Quantification showed no significant differences between any of the recovery periods and BL1, displaying full recovery throughout the protocol (Figure 3.7C2; Table 3.4). Comparisons between BMS and DMSO conditions during challenge periods showed a significant difference at Chal7 (Table 3.4). For the recovery periods, significant differences were observed from Recov5 on (Table 3.4), supporting that the effects of Asc-1 inhibition occurred over time. Taken together, the data showed no effect of DMSO on synaptic transmission, suggesting that the reduction in synaptic strength is drug specific.

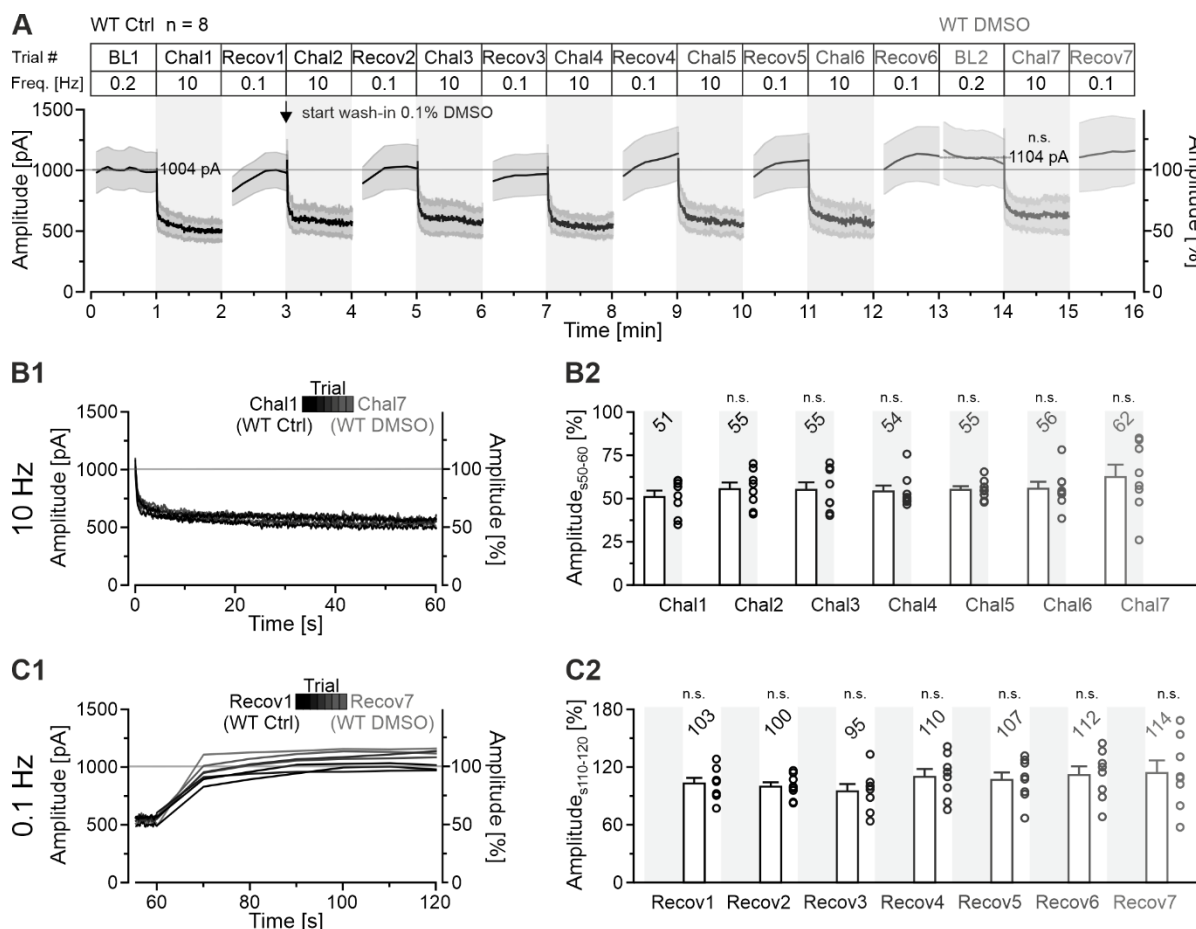


Figure 3.7 Unchanged eIPSC amplitudes during repetitive stimulation under 0.1% DMSO, the solvent for BMS in WT. (A) Time course of absolute and normalized eIPSC peak amplitudes for WT Ctrl (black) and WT DMSO (gray) were obtained with Protocol 2 (see Figure 2.4C). Trials are color-coded from black to gray. BL mean absolute values are written in the figure. Gray horizontal lines show normalization (BL1 = 100%). The arrow marks the start of the DMSO wash-in. **(B)** Overlay of time course of absolute and normalized mean eIPSC peak amplitudes during 10-Hz | 60-s challenges **(B1)**. **(B2)** Statistics of s_{50-60} during challenge periods. Mean value above bars. **(C)** Same as B, but for $s_{110-120}$ 0.1-Hz | 60-s recovery periods. Gray areas highlight the challenge periods. Values are shown as mean $\pm$ SEM. Time courses are simple moving averages (SEM lightly shaded). $n = 8$, same neurons. See Tables 3.3 and 3.4 for details, including statistics.

Table 3.3: BL eIPSC amplitudes with 50 μ M BMS and 0.1% DMSO in WT.

50 μ M BMS and 0.1% DMSO	Ctrl (ACSF) n = 11		BMS n = 11		Ctrl vs. BMS	
	Mean	SEM	Mean	SEM	p-value	Figure
BL [pA]	871	139	540	106	0.004	3.6A
	Ctrl (ACSF) n = 8		DMSO n = 8		Ctrl vs. DMSO	
BL [pA]	1004	164	1104	222	0.461	3.7A

Data are presented as mean $\pm$ SEM. BL: Ctrl vs. BMS: Paired t-test; Ctrl vs. DMSO: Wilcoxon test. Significant P-values are marked in green.

Table 3.4: eIPSC amplitudes_{s50-60} during 7x 10-Hz challenge | 0.1-Hz recovery with 50 μ M BMS and 0.1% DMSO in WT.

50 μ M BMS and 0.1% DMSO		Chal1	Chal2	Chal3	Chal4	Chal5	Chal6	Chal7
Stimulation freq. [Hz]		10						
50 μ M BMS	Mean [%]	50	53	47	45	39	37	33
	SEM	3	6	6	5	4	5	5
	n	11	11	11	11	11	11	11
	p-value		>0.999	>0.999	>0.999	0.143	0.048	0.003
	Figure	3.6B2						
0.1% DMSO	Mean [%]	51	56	55	54	55	56	62
	SEM	4	4	4	3	2	4	7
	n	8	8	8	8	8	8	8
	p-value		>0.999	>0.999	>0.999	>0.999	>0.999	0.631
	Figure	3.7B2						
DMSO vs. BMS	p-value	>0.999	>0.999	>0.999	>0.999	0.071	0.053	0.004
		Recov1	Recov2	Recov3	Recov4	Recov5	Recov6	Recov7
Stimulation freq. [Hz]		0.1						
50 μ M BMS	Mean [%]	98	94	89	79	68	68	62
	SEM	8	12	12	9	9	10	11
	n	11	11	11	11	11	11	11
	p-value	>0.999	>0.999	>0.999	0.973	0.037	0.104	0.049
	Figure	3.6C2						
0.1% DMSO	Mean [%]	103	100	95	110	107	112	114
	SEM	6	4	7	8	8	9	13
	n	8	8	8	8	8	8	8
	p-value	>0.999	>0.999	>0.999	>0.999	>0.999	>0.999	>0.999
	Figure	3.7C2						
DMSO vs. BMS	p-value	>0.999	>0.999	>0.999	0.170	0.028	0.020	0.016

Data are presented as mean $\pm$ SEM. BMS: One-way ANOVA (Chal) with Bonferroni correction, Friedmann test with Dunn's correction (Recov), DMSO: Friedmann test with Dunn's correction (Chal + Recov). DMSO vs. BMS: Kruskal-Wallis test with Dunn's correction (Chal + Recov). P-values are adjusted values after correction. Significant P-values are marked in green.

Since eIPSC amplitudes decreased under harsher stimulation (7x 10-Hz challenge | 1-Hz recovery) and blockade of Asc-1, the synaptic fidelity was examined of MNTB-LSO synapses. During repetitive stimulation, transmission showed few synaptic failures with fidelity values of 100, 100, 96, 99, 98, 97, and 92% for Chal1-Chal7, respectively (Figure 3.8A1). A close-up of the fidelity time courses during the last 10 s (Fidelity_{s50-60}) is shown in the inset, with trials color-coded from black (Ctrl) to olive (BMS). Despite the occurrence of synaptic failures, quantification of Fidelity_{s50-60} showed no significant differences between Chal2-Chal7 and Chal1 (Figure 3.8A2; Table 3.5). This suggests that MNTB-LSO synapses maintain reliable transmission even under harsher stimulation and Asc-1 inhibition.

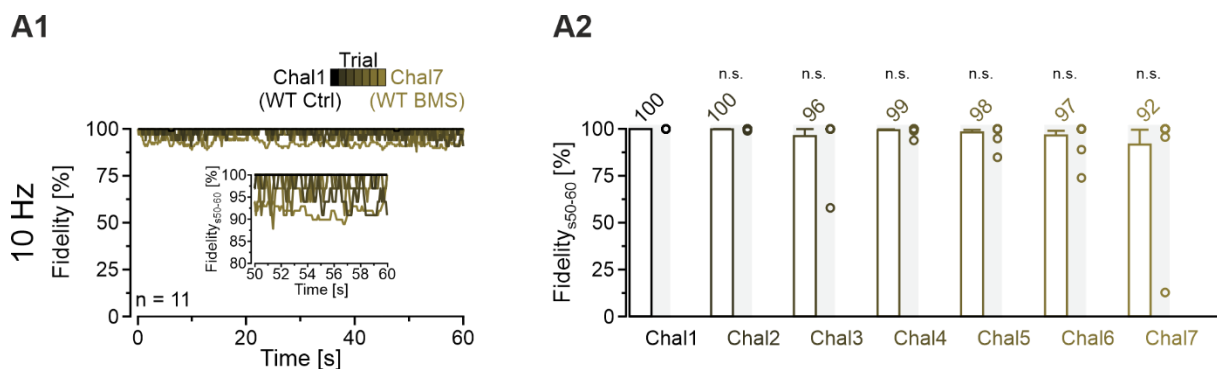


Figure 3.8 Unchanged fidelity during repetitive stimulation under Asc-1 inhibition with 50 μ M BMS in WT. (A) Fidelity rate of eIPSC amplitudes for WT Ctrl (black) and WT BMS (olive). **(A1)** Time course of mean fidelity. Trials are color-coded from black to olive. The inset shows a close-up of the last 10 s. **(A2)** Statistics for fidelity analysis at s₅₀₋₆₀ of challenge periods (Chal1-Chal7). Gray areas highlight the challenge periods. Mean value above bars. Values are shown as mean $\pm$ SEM. n = 11, same neurons. See Table 3.5 for details, including statistics.

Table 3.5: eIPSC Fidelity_{s50-60} during 7x 10-Hz challenge with 50 μ M BMS in WT.

50 μ M BMS	Chal1	Chal2	Chal3	Chal4	Chal5	Chal6	Chal7
Stimulation freq. [Hz]	10						
Mean [%]	100	100	96	99	98	97	92
SEM	0	0	4	1	1	2	8
n	11	11	11	11	11	11	11
p-value		>0.999	>0.999	>0.999	>0.999	>0.999	>0.999
Figure	3.9A2						

Data are presented as mean $\pm$ SEM. Friedmann test with Dunn's correction. P-values are adjusted values after correction.

3.1.4 Asc-1 inhibition and repetitive stimulation does not alter q

With Protocol 2 and inhibition of Asc-1, eIPSC amplitudes were reduced, suggesting a decrease in glycine availability at the presynaptic MNTB terminal. This reduction may affect SV (re)filling, so there should be a reduction in quantal size (q) or the frequency of spontaneous IPSCs (sIPSCs). To test these hypotheses, I determined q and sIPSC frequency during BL1 and all recovery periods (Recov1-Recov7). Example current traces for both conditions during the BL1 (Ctrl, black) and the Recov7 (BMS, olive) periods are shown in Figure 3.9A1. A histogram of the peak sIPSC amplitudes was plotted, and q was determined using a Gaussian fit for each condition (Figure 3.9A2). The cumulative probability of sIPSC amplitudes showed no difference between Ctrl and BMS (Figure 3.9B). A total of 4,596 (Ctrl) and 4,332 (BMS) sIPSC amplitudes from 11 cells were analyzed. The mean sIPSC amplitudes were not significantly different between Ctrl and BMS (25 vs. 24 pA, Table 3.6). These results show that Asc-1 blockade does not change the distribution of sIPSC amplitudes. Similar to these results, no significant changes were observed in q in all comparisons between Recov1-7 and BL1 (Figure 3.9C; Table 3.6). The average q values were 20 pA for BL; 21 pA for Recov1; 22 pA for Recov2; 23 pA for Recov3; 21 pA for Recov4; 21 pA for Recov5; 20 pA for Recov6; and 21 pA for Recov7. The sIPSC frequency was calculated by dividing the number of events by the duration of a recovery period (*sIPSC frequency [events/s] = events/60 s*). Similarly, no significant differences in sIPSC frequency were found between Recov1-7 compared to BL (Figure 3.9D; Table 3.6). The mean sIPSC frequencies were 7, 8, 8, 7, 8, 7, 7, and 6 events/s for BL-Recov7, respectively. Taken together, the unchanged spontaneous release suggests that MNTB axon terminals maintain an RRP of SVs despite the Asc-1 blockade. This suggests that the glycine loading of SVs is not abolished.

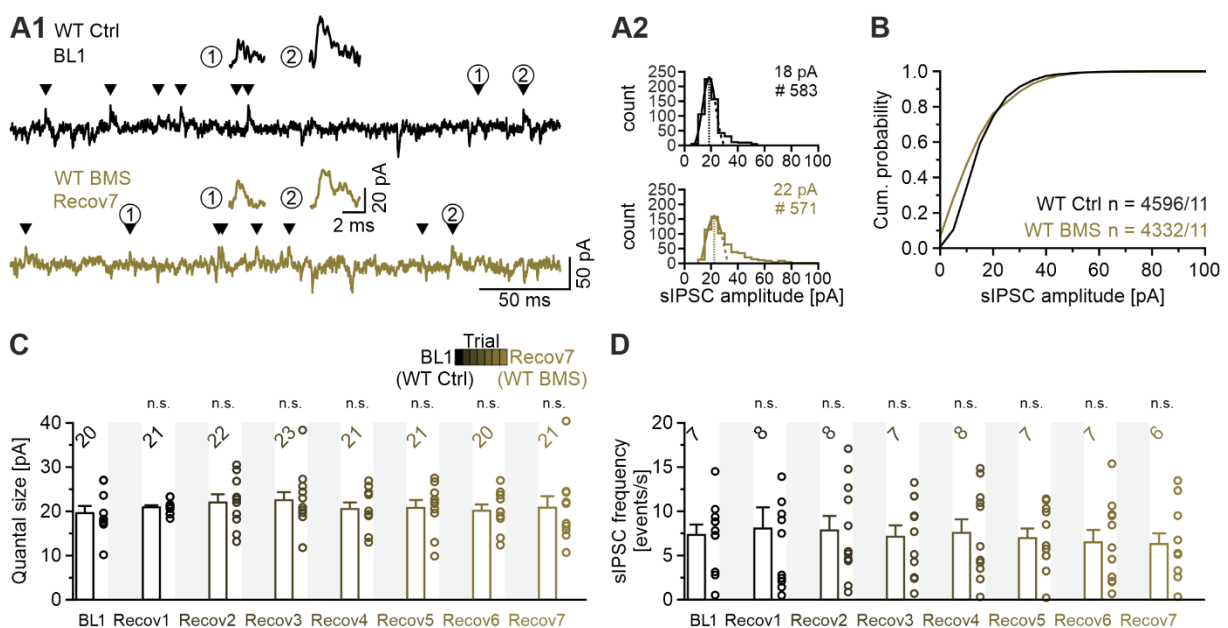


Figure 3.9 Unchanged spontaneous release during repetitive stimulation under 50 μ M BMS in WT. (A) Representative sIPSC current traces for WT Ctrl (black) and WT BMS (olive). **(A1)** Arrows mark sIPSC amplitudes during BL1 (WT Ctrl) and Recov7 (WT BMS). Numbers over arrows mark example sIPSCs, which are shown over traces. **(A2)** Estimation of q . Histograms of sIPSC amplitudes to current traces in A1 during BL1 and Recov7 with Gaussian fit from 0 pA to the bin after the first local maximum (dotted curves; Krächan et al., 2017). Numbers in panels are q and the number of sIPSCs. **(B)** Cumulative probability of sIPSC amplitudes for WT Ctrl (black) and WT BMS (olive) **(C)** Statistics for q during BL1-Recov7. Mean values above bars. Trials are color-coded from black to olive. **(D)** Same as C, but for sIPSC frequency. Gray areas highlight the challenge periods. Values are shown as mean $\pm$ SEM. $n = 11$, same neurons. See Table 3.6 for details, including statistics.

Table 3.6: sIPSC properties during 7x 0.1-Hz recovery with 50 μ M BMS in WT.

50 μ M BMS		Ctrl (ACSF) $n = 11$		BMS $n = 11$		Ctrl vs. BMS		
		Mean	SEM	Mean	SEM	p-value	Figure	
sIPSC amplitudes [pA]		25	1	24	3	0.540	3.10B	
	BL	Recov1	Recov2	Recov3	Recov4	Recov5	Recov6	Recov7
Stimulation freq. [Hz]	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1
q	Mean [pA]	20	21	22	23	21	21	21
	SEM	1.6	0.5	1.8	1.8	1.5	1.8	2.6
	p-value		>0.999	>0.999	0.555	>0.999	>0.999	>0.999
	Figure	3.10C						
sIPSC freq.	Mean [Hz]	7	8	8	7	8	7	6
	SEM	1.2	2.4	1.6	1.3	1.5	1.1	1.4
	p-value		>0.999	>0.999	>0.999	>0.999	>0.999	>0.999
	Figure	3.10D						

Data are presented as mean $\pm$ SEM. sIPSC amplitudes: Paired t-test. q and sIPSC freq.: One-way ANOVA with Bonferroni correction. P-values are adjusted values after correction.

3.1.5 Asc-1 inhibition and repetitive stimulation reduce m_{s50-60} and RR_{s50-60}

Blockade of Asc-1 transport activity and harsher stimulation did not cause changes in q , consistent with a stable RRP of SVs. To test whether there is an impaired SV replenishment, changes in the SV availability were analyzed with Protocol 2. Two quantitative parameters were analyzed: quantal content (m), the number of SVs released at the event, and replenishment rate (RR), as the number of SVs are recruited per second to the RRP to maintain transmission. First, m was calculated for all challenge periods (Chal1-Chal7), using $m = eIPSC/q$. Individual trials are color-coded from black to olive (Figure 3.10A1). Quantification of m values during steady-state (m_{s50-60}) showed that Chal1 was not significantly different from Chal2-Chal6, indicating stable m over these periods. However, m_{s50-60} of Chal7 was significantly lower than Chal1, suggesting an Asc-1 inhibition effect at the end of Protocol 2 (Figure 3.10A2; Table 3.7). To assess transmission capacity over the full 60-s challenge

periods, the cumulative number of SVs was plotted (Müller *et al.*, 2022). Under Ctrl conditions, synapses released a total of 81,660 SVs. In contrast, during challenge periods Chal2-Chal7, cumulative SV release progressively decreased, with synapses releasing 13,174, 13,422, 11,981, 12,710, 10,878, 10,673, and 8,822 SVs, respectively (Figure 3.10B1). To quantify the number of SVs recruited during the steady-state phase (s_{50-60}) of the challenge periods, the RR was analyzed. No statistically significant differences were observed in the first five comparisons (Chal2-Chal6 vs. Chal1). However, a significantly reduced $RR_{s_{50-60}}$ was observed when comparing Ctrl vs. Chal7 (Figure 3.10B2; Table 3.7). Taken together, as with the effect on eIPSC amplitudes, the decrease in m and RR accumulated over time. At the end of the stimulation protocol, there is a reduced number of released SVs, indicating that there are fewer SVs available due to Asc-1 inhibition and repetitive stimulation.

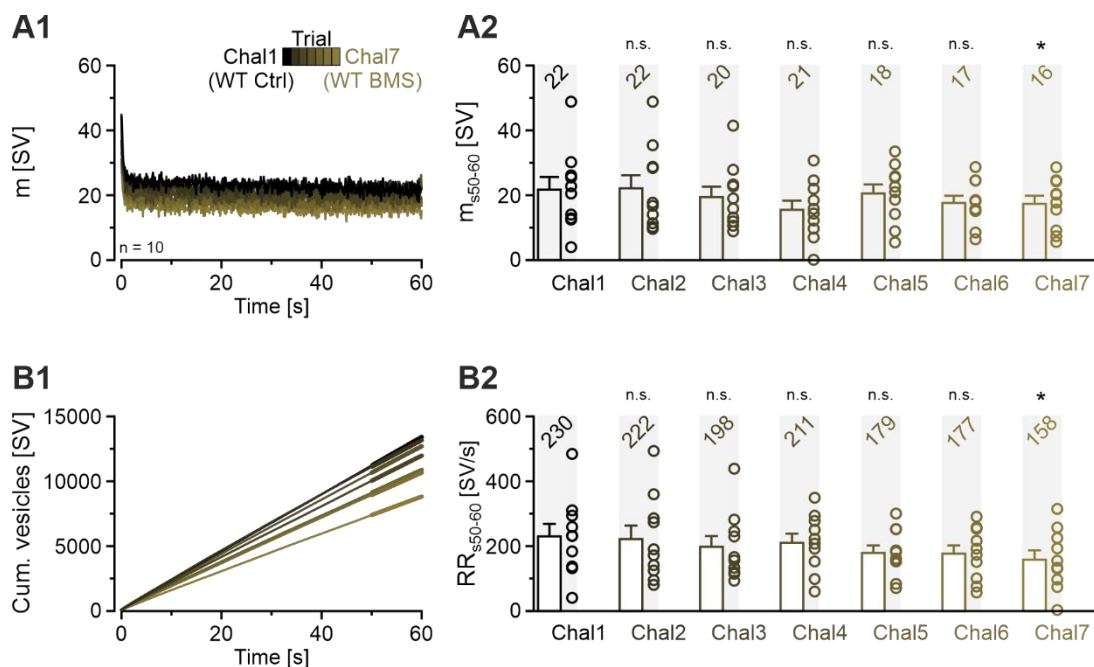


Figure 3.10 Reduced number of released SVs at the late phase of repetitive stimulation under 50 μ M BMS in WT. (A) Quantification of quantal content (m). (A1) Time course of m during 10-Hz | 60-s challenge periods (Chal1-Chal7) for WT Ctrl (black) and WT BMS (olive). Trials are color-coded from black to olive. (A2) Statistics for m at s_{50-60} of challenge periods (Chal1-Chal7). Mean values above bars. (B) Time course of mean cumulative released synaptic vesicles. Thicker lines at s_{50-60} mark the period in which RR was quantified (B1). (B2) same as A2, but for RR. Gray areas highlight the challenge periods. Values are shown as mean $\pm$ SEM. $n = 10$, same neurons. See Table 3.7 for details, including statistics.

Table 3.7: m_{s50-60} and RR_{s50-60} during 7x 10-Hz challenge with 50 μ M BMS in WT.

50 μ M BMS		Chal1	Chal2	Chal3	Chal4	Chal5	Chal6	Chal7
Stimulation freq. [Hz]		10						
m_{s50-60}	Mean [SV]	22	22	20	21	18	17	16
	SEM	4	4	3	3	2	2	3
	n	10	10	10	10	10	10	10
	p-value		>0.999	>0.999	>0.999	0.264	0.202	0.018
	Figure	3.11A2						
RR_{s50-60}	Mean [SV/s]	230	222	198	211	179	177	158
	SEM	38	41	33	28	23	25	29
	n	10	10	10	10	10	10	10
	p-value		>0.999	0.648	>0.999	0.077	0.056	0.004
	Figure	3.11B2						

Data are presented as mean $\pm$ SEM. m and RR: One-way ANOVA with Bonferroni correction. P-values are adjusted values after correction. Significant P-values are marked in green.

3.2 Role of Asc-1 in glycinergic transmission at MNTB-LSO synapses in KO

Having analyzed whether Asc-1 contributes to transmission in WT mice, the next step was to investigate whether Asc-1 contributes to the residual glycinergic transmission at MNTB-LSO synapses in KO mice. KO mice still maintain glycinergic transmission under 50-Hz | 60-Hz stimulation (Brill *et al.*, 2021), despite the loss of GlyT2. This raises the question of the glycine source. In WT MNTB-LSO synapses, Asc-1 inhibition affected transmission under harsher stimulation (Protocol 2) and in the presence of 50 μ M BMS, with GlyT2 remaining the primary (re)uptake mechanism. Therefore, I tested whether Asc-1 could compensate for the absence of GlyT2 in KO mice under the same stimulation conditions as described for WT (see Section 3.1).

3.2.1 Asc-1 localization in presynaptic axon terminals of MNTB-LSO synapses in KO

Immunohistochemistry was used to examine Asc-1 expression in the SOC of KO P11 $\pm$ 1 mice. Like the method used in WT mice, to check the expression of Asc-1 in KO to understand the synaptic organization in the absence of GlyT2 and to check if there are differences in the expression pattern in the genotypes. Asc-1 immunoreactivity (cyan) was detected in both the MNTB and LSO nuclei of the SOC (Figure 3.11A1). In the LSO, neurons were surrounded by cyan puncta, showing presynaptic localization (Figure 3.11A2-A4). Similarly, VIAAT labeling (magenta) was observed in both the MNTB and LSO (Figure 3.11B1), with VIAAT puncta surrounding the LSO neurons (Figure 3.11B2-B4). The labeling of VIAAT immunoreactivity was comparable to that seen in WT (Figure 3.11B1-B4). In both nuclei, MNTB and LSO co-distribution was observed, with double-labeled puncta surrounding LSO neurons (Figure 3.11 C1-C4). This indicates that Asc-1 is expressed in KO MNTB terminals. Intensity

profiles of Asc-1 and VIAAT from an individual line scan are overlapping (Figure 3.11D1) as well as in averaged data (Figure 3.11D2). Pearson's coefficient (0.63 ± 0.02 , $n = 31$; Figure 3.11D3) and the P_{Costes} coefficient (1.00 ± 0.00 , $n = 31$; Figure 3.11D4) confirmed the co-distribution. Taken together, there is no change in the VIAAT labeling pattern compared to WT, indicating that SV filling is maintained despite the loss of GlyT2. Furthermore, Asc-1 is expressed in glycinergic MNTB axon terminals in KO. This suggests that Asc-1 serves as the glycine source that maintains residual glycinergic transmission in KO.

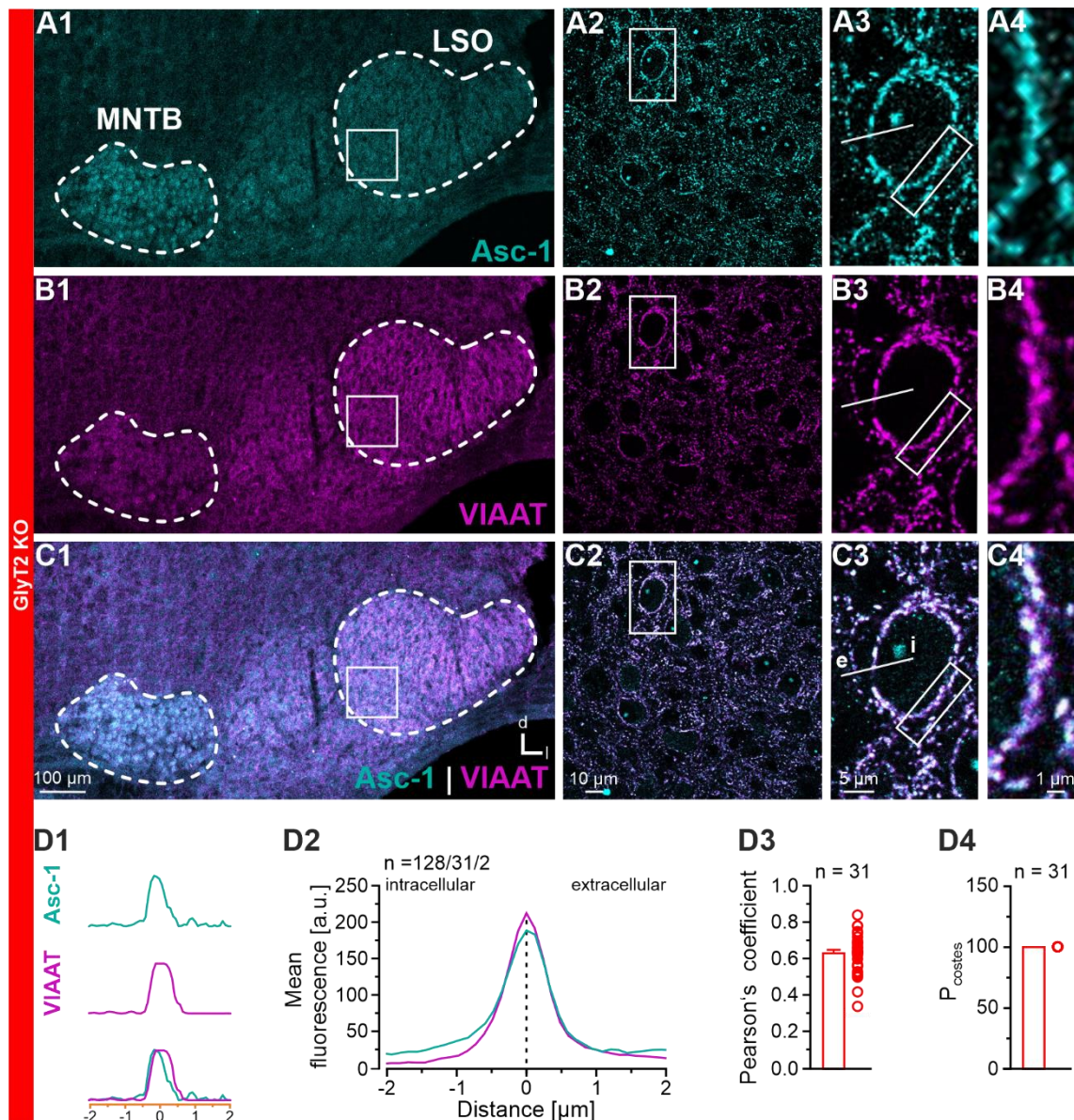


Figure 3.11 Asc-1 is abundant in the MNTB and LSO, where it is co-distributed with VIAAT in the LSO in KO. (A-C) Immunohistochemical analysis of Asc-1 and VIAAT. **(A1)** Labeling for Asc-1 (cyan) in the SOC. Highlighted are MNTB and LSO. **(A2)** Zoom-in of LSO framed in A1. **(A3)** Close-up of a representative LSO in the framed area in A2. The white line showed the intensity profile shown in D1. **(A4)** Close-up of the framed area in A3. **(B)** Same

as A, but for VIAAT labeling (magenta). **(B1-B4)**. **(C)** Same as A, but for Asc-1 and VIAAT double labeling **(C1-C4)**. **(D1)** Quantification of Asc-1 and VIAAT co-distribution. Individual intensity profile for Asc-1 and VIAAT (orange lines in A3-C3). **(D2)** Average intensity profiles for Asc-1 and VIAAT. Before averaging, each profile was aligned to the peak of the VIAAT signal. For more details, see Material and Methods. Negative μm values show intracellular (i) and positive μm values extracellular (e) signals of the LSO neuron, indicated in C3. n, number of profiles/cells/animals. **(D3)** Pearson's coefficient and **(D3)** corresponding P_{Costes} values. Optical thickness: 1 μm . Coronal brainstem slice at P11 WT. Abbreviations: Asc-1, alanine-serine-cysteine transporter 1; a.u., arbitrary units; d, dorsal; l, lateral; LSO, lateral superior olive; MNTB, medial nucleus of the trapezoid body; VIAAT, vesicular inhibitors amino acid transporter.

3.2.2 Contribution of Asc-1 to synaptic transmission at MNTB-LSO synapses in KO

To test whether Asc-1 serves as the glycine source that maintains residual glycinergic transmission in P11 $\pm$ 1 KO mice, electrophysiological recordings were performed with Protocol 1 (Figure 2.4C) under 10 μM BMS application. 10 μM CNQX was added to block excitatory currents. To avoid rapid synaptic fatigue, as previously observed with stronger stimulation paradigms (50-Hz | 60-s challenge followed by 1-Hz | 60-s recovery; Brill *et al.*, 2021), a milder stimulation protocol (10-Hz | 60-s challenge followed by 0.1-Hz | 60-s recovery) was applied. It was shown that KO eIPSC amplitudes can recover to BL levels ($\sim$ 100%) following recovery at 0.1-Hz stimulation (Kurz, 2019). The eIPSC amplitudes of a single KO neuron at different times during the stimulation followed the synaptic depression and recovery dynamics under both Ctrl (dark red; Figure 3.12A1) and BMS (light red; Figure 3.12A2). During Ctrl, the mean BL1 eIPSC amplitudes were 65 pA. They decreased to 45 pA during the steady-state phase and returned to 61 pA. During the wash-in period, the eIPSC amplitudes remained around 61 pA. For BMS, the mean eIPSC amplitudes were 61 pA during the BL2 phase, dropping to 52 pA at steady-state and recovering to 62 pA around the BL2 level (Figure 3.12A3). Thus, these single-cell results provide some evidence that Asc-1 does not contribute to synaptic transmission at MNTB-LSO synapses in KO.

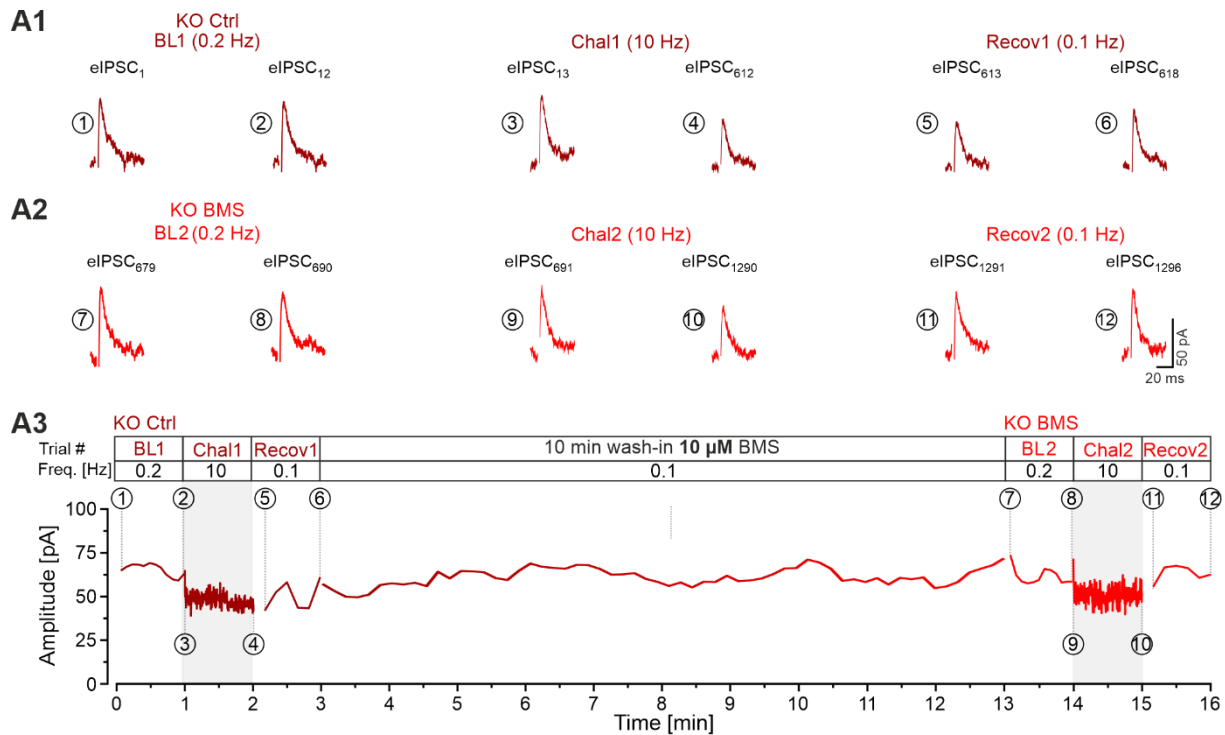


Figure 3.12 Reliable transmission under Asc-1 inhibition with 10 μ M BMS in KO. (A1) Representative eIPSC amplitudes for KO Ctrl (ACSF, dark red), showing during BL1 eIPSC₁ and eIPSC₁₂, during Chal1 eIPSC₁₃ and eIPSC₆₁₂, and Recov1 eIPSC₆₁₃ and eIPSC₆₁₈. Numbers in circles in A1 show eIPSC in Protocol 1 in A3. **(A2)** Same as A1, but for KO BMS (light red). **(A3)** Time course of absolute peak eIPSC amplitudes obtained with Protocol 1 (see Figure 2.4C) from a P11 LSO neuron. 0.2-Hz | 60-s BL1, 10-Hz | 60-s challenge (Chal1), and 1-Hz | 60-s recovery (Recov1) for KO Ctrl. Followed by 0.1-Hz | 10-min 10 μ M BMS wash-in and BL2, Chal2, and Recov2 for KO BMS. The trace is color-coded from dark to light red. Challenge periods are marked with gray areas. Time courses are simple moving averages. The total number of stimulus pulses is 1296.

Averaged data from all KO neurons showed similar time courses to the representative neuron (Figure 3.13A). The mean BL time courses were comparable between conditions (Ctrl: 91 pA; BMS: 97 pA, corresponding to 100% and 107%, respectively; Figure 3.13B1). There is no significant difference between KO Ctrl and BMS (Figure 3.13B2; Table 3.8). The time courses of synaptic depression were indistinguishable for both conditions. Amplitudes decreased to 41 pA (Ctrl) and 43 pA (BMS), corresponding to 45% and 47% of BL1 values during steady-state (Figure 3.13C1). Quantification showed no significant difference (Figure 3.13C2; Table 3.8). Mean eIPSC amplitudes were able to recover back to BL1 levels, 80 pA for Ctrl and 89 pA for BMS (Figure 3.13D1). Steady-state recovery values showed no significant difference (Ctrl: 88% vs. BMS: 98%; Figure 3.13D2; Table 3.8). Taken together, there were no significant differences in BL, synaptic depression, or recovery between Ctrl and BMS, confirming that synaptic transmission was unaffected after a 10-min wash-in with 10 μ M BMS in KO.

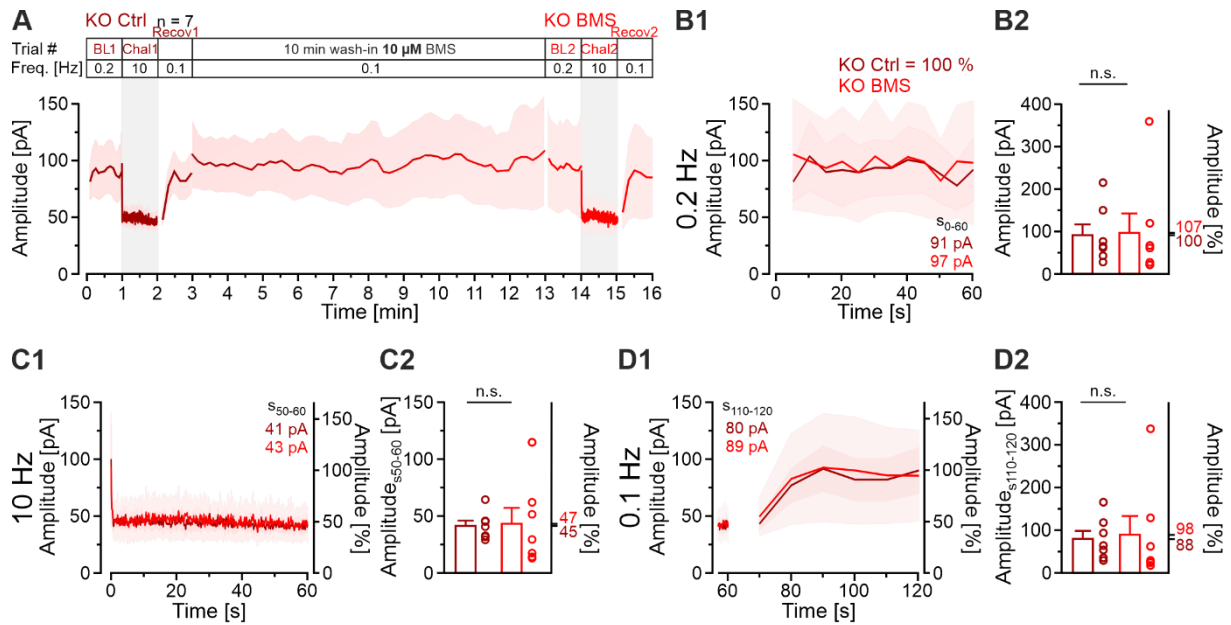


Figure 3.13 Unchanged transmission under Asc-1 inhibition with 10 μ M in KO. (A) Time course of mean eIPSC peak amplitudes for KO Ctrl (dark red) and KO BMS (light red) using Protocol 1 (see Figure 2.4C). The trace is color-coded from dark to light red. Challenge periods are marked with gray areas. (B1) BL1 and BL2 traces (KO Ctrl vs. KO BMS), KO Ctrl was set to 100%. Mean values are shown at the bottom right. (B2) Statistics for BL analysis. (C1) Time course of absolute and normalized mean eIPSC peak amplitudes during 10-Hz | 60-s challenge periods. The top right numbers indicate the mean amplitudes at s_{50-60} . (C2) Statistics for challenge analysis at s_{50-60} . Mean normalized values are shown on the right y-axis. (D1) Same as C1, but during 0.1-Hz | 60-s recovery periods. The top left numbers indicate the mean amplitudes at $s_{110-120}$. (D2) Same as C2, but for recovery analysis at $s_{110-120}$. Note the different amplitude scaling in B1-D2. Values are shown as mean $\pm$ SEM. Time courses are simple moving averages (SEM lightly shaded). $n = 7$, same neurons. See Table 3.8 for details, including statistics.

Table 3.8: eIPSC amplitudes during 10-Hz challenge | 0.1-recovery with 10 μ M BMS in KO.

10 μ M and 50 μ M BMS			Ctrl (ACSF) $n = 7$		BMS $n = 7$		Ctrl vs. BMS	Figure
	Stimulation freq. [Hz]	Amplitude [pA]	Mean	SEM	Mean	SEM	p-value	
10 μ M BMS	0.2	BL [pA]	91	25	97	46	0.812	3.14B2
	10	Chal s_{50-60} [pA]	41	5	43	14	0.856	3.14C2
	0.1	Recov $s_{110-120}$ [pA]	80	19	89	44	>0.999	3.14D2

Data are presented as mean $\pm$ SEM. Ctrl vs. BMS: Paired two-tailed t-test. If bold Wilcoxon test.

Inhibition of Asc-1 with 10 μM BMS and Protocol 1 (Figure 2.4C) revealed no significant changes in the eIPSC amplitudes of KO mice. Therefore, the concentration of BMS was increased to 50 μM . 10 μM CNQX was added to block excitatory currents. With this higher concentration, eIPSC amplitudes decreased with time (Figure 3.14A). Time courses of mean BL periods showed reduced values for BMS compared to Ctrl (Ctrl: 85 pA; BMS: 63 pA, corresponding to 100% and 74%, respectively; Figure 3.14B1). However, this reduction did not reach statistical significance (Figure 3.14B2; Table 3.9). Time courses of challenge periods displayed a different synaptic depression kinetic between Ctrl and BMS, whereas they were similar at steady-state (Figure 3.14C1). Quantification of the first eIPSC₁ amplitude (eIPSC₁; Ctrl: 82 pA; BMS: 57 pA) and the mean amplitude of the first five seconds (s_{1-5} ; Ctrl: 31 pA; BMS: 26 pA) showed no significant differences (Figure 3.14C1; Table 3.9). At steady-state, mean eIPSC amplitudes decreased to 31 pA (Ctrl) and 26 pA (BMS), corresponding to 36% and 31%, respectively (Figure 3.14C2; Table 3.9). During the recovery period, the mean amplitudes returned toward BL values to 74 pA (Ctrl) and 61 pA (BMS), corresponding to 87% and 72%, respectively (Figure 3.14D1-D2; Table 3.9). In summary, the application of 50 μM BMS resulted in a non-significant reduction of eIPSC amplitudes throughout the protocol. With a more pronounced, still no significant difference during the initial five seconds of the challenge period. These results suggest that Asc-1 inhibition with 50 μM BMS does not significantly impair synaptic transmission under the given stimulation conditions in mice.

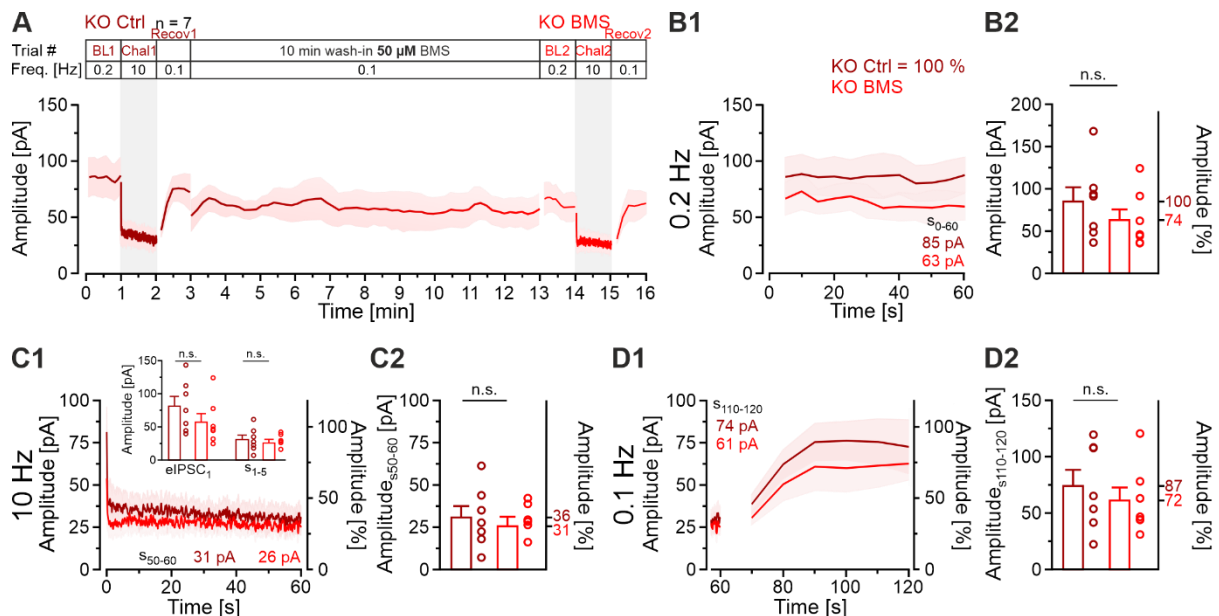


Figure 3.14 Unchanged transmission under Asc-1 inhibition with a higher concentration of BMS (50 μM) in KO. (A) Time course of mean eIPSC peak amplitudes for KO Ctrl (dark red) and KO BMS (light red) using Protocol 1 (see Figure 2.4C). The trace is color-coded from dark to light red. Challenge periods are marked with gray areas. **(B1)** BL1 and BL2 traces (KO Ctrl vs. KO BMS), KO Ctrl was set to 100%. Mean values are shown at the bottom right. **(B2)**

Statistics for BL analysis. **(C1)** Time course of absolute and normalized mean eIPSC peak amplitudes during 10-Hz | 60-s challenge periods. The bottom numbers indicate the mean amplitudes at s_{50-60} . Insert shows quantification of eIPSC₁ and mean s_{1-5} eIPSC amplitudes. **(C2)** Statistics for challenge analysis at s_{50-60} . Mean normalized values are shown on the right y-axis. **(D1)** Same as C1, but during 0.1-Hz | 60-s recovery periods. The top left numbers indicate the mean amplitudes at $s_{110-120}$. **(D2)** Same as C2, but for recovery analysis at $s_{110-120}$. Note the different amplitude scaling in B1-D2. Values are shown as mean $\pm$ SEM. Time courses are simple moving averages (SEM lightly shaded). $n = 7$, same neurons. See Table 3.9 for details, including statistics.

Table 3.9: eIPSC amplitudes during 10-Hz challenge | 0.1-recovery with 50 μ M BMS in KO.

10 μ M and 50 μ M BMS			Ctrl (ACSF) $n = 7$		BMS $n = 7$		Ctrl vs. BMS	
	Stimulation freq. [Hz]	Amplitude [pA]	Mean	SEM	Mean	SEM	p-value	Figure
50 μ M BMS	0.2	BL	85	17	63	13	0.188	3.15B2
	10	Chal eIPSC ₁	82	15	57	13	0.060	3.15C1
	10	Chal _{s1-5}	31	7	26	6	0.268	3.15C1
	10	Chal _{s50-60}	31	7	26	6	0.268	3.15C2
	0.1	Recov _{s110-120}	74	14	61	11	0.307	3.15D2

Data are presented as mean $\pm$ SEM. Ctrl vs. BMS: Paired two-tailed t-test. If bold Wilcoxon test.

Because synaptic failures in KO mice have previously been reported (Brill *et al.*, 2021), synaptic fidelity was tested to assess if MNTB-LSO synapses were capable of sustaining SV release without failures. At 10 μ M BMS, synaptic transmission remained failure-free in KO Ctrl, whereas failures were observed in KO BMS, most pronounced during the first 10 s of the challenge period (Figure 3.15A1; Table 3.10). Quantification of Fidelity_{s50-60} revealed no significant difference between conditions (Ctrl: 100%; BMS: 98%; Figure 3.15A2). At 50 μ M BMS, synaptic failures occurred throughout the 60-s challenge period in both conditions (Figure 3.15B1), strongest observed during the initial 10 s, consistent with the time course of eIPSC amplitudes (Figure 3.14C1). This suggests that, within the first 10 s under 50 μ M BMS, presynaptic glycine availability may be insufficient to support full synaptic vesicle refilling at MNTB terminals. Nevertheless, a comparison of Fidelity_{s50-60} values again showed no significant difference between Ctrl (87%) and BMS (83%; Figure 3.15B2; Table 3.10). Overall, 10 μ M BMS had minimal effects on synaptic fidelity, and 50 μ M BMS induced an increase in synaptic failures. Despite this, overall synaptic fidelity remained high and differences between conditions did not reach statistical significance. These results suggest that Asc-1 inhibition may impair presynaptic glycine recycling during the first 10 s but is still able to maintain high-fidelity transmission at MNTB-LSO synapses.

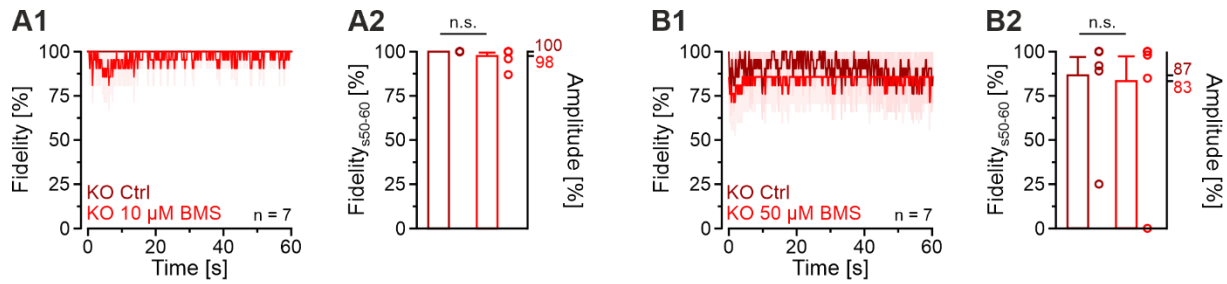


Figure 3.15 Unchanged fidelity under Asc-1 inhibition with 10 or 50 μ M BMS in KO. (A) Fidelity rate of eIPSC amplitudes for KO Ctrl (dark red) and 10 μ M KO BMS (light red). (A1) Time course of mean fidelity during 10-Hz | 60-s challenge periods. (A2) Statistics for fidelity analysis at s₅₀₋₆₀ of challenge periods. Mean values are shown on the right y-axis. (B) Same as A, but for 50 μ M BMS. Values are shown as mean $\pm$ SEM. n = 7 neurons. See Table 3.10 for details, including statistics.

Table 3.10: eIPSC Fidelity_[50-60] during 10-Hz challenge with 10 and 50 μ M BMS in KO.

10 μ M and 50 μ M BMS			Ctrl (ACSF) n = 7		BMS n = 7		Ctrl vs. BMS	
	Stimulation freq. [Hz]	Fidelity [%]	Mean	SEM	Mean	SEM	p-value	Figure
10 μ M BMS	10	s ₅₀₋₆₀	100	0	98	2	0.500	3.16A2
50 μ M BMS			87	10	83	14	0.750	3.16B2

Data are presented as mean $\pm$ SEM. Ctrl vs. BMS: Wilcoxon test.

3.2.3 Repetitive stimulation of MNTB fibers, combined with Asc-1 inhibition, led to a reduction in eIPSC amplitudes in KO

As Protocol 1 showed no significant differences in eIPSC amplitudes between Ctrl and BMS, the experiments were repeated using Protocol 2 (Figure 2.4C) in KO mice. Since KO mice lack functional GlyT2 (Gomez *et al.*, 2003a), it was assumed that Asc-1 maintains the residual glycinergic transmission. However, inhibition of Asc-1 with 10 or 50 μ M BMS had no significant effect on glycinergic transmission. Therefore, it was suggested that by increasing synaptic activity, preloaded SVs still present in the MNTB terminal were depleted (Brill *et al.*, 2021). If Asc-1 is the main source of glycine in KO mice, eIPSC amplitudes should decrease after SV depletion because no glycine would be available to refill presynaptic SVs at MNTB terminals.

Mean absolute and normalized time courses of Protocol 2 in KO mice showed a progressive decrease in eIPSC amplitudes starting from the onset of 50 μ M BMS wash-in combined with stimulation (Figure 3.16A). 10 μ M CNQX was added to block excitatory currents. BL values were significantly reduced between Ctrl (106 pA) and BMS (13 pA), corresponding to 100% and 12%, respectively (Figure 3.16A, Table 3.11). Overlay of all challenge periods confirmed

a reduction in eIPSC amplitudes (Figure 3.16B1). Steady-state values showed a significant difference from Chal3 onward compared to Chal1 (36% vs. 18%), as well as for the following periods: Chal1 vs. Chal4 (36% vs. 15%); Chal1 vs. Chal5 (36% vs. 11%); Chal1 vs. Chal6 (36% vs. 9%), and Chal1 vs. Chal7 (36% vs. 10%; Figure 3.16B2, Table 3.12). Over the entire recording, the total eIPSC amplitude level decreased by 26% during challenge periods. An overlay of eIPSC amplitudes during recovery periods showed a similar pattern to the challenge periods. Time courses continued to decrease throughout the stimulation (Figure 3.16C1). Quantification of s_{50-60} values showed impaired eIPSC amplitudes from Recov2 (67%) and Recov3 (45%) and a significant decrease compared to BL1 from Recov4 (26%) onward (Recov5: 29%; Recov6: 29%, and Recov7: 23%, Figure 3.16C2, Table 3.12). Overall, eIPSC amplitudes decreased by 75% during recovery periods. Taken together, the results showed a 4-fold reduction in eIPSC amplitudes from Ctrl (Chal1) to BMS (Chal7) over both challenge and recovery periods. This suggests that Asc-1 inhibition with 50 μ M BMS under repetitive stimulation affects synaptic strength in KO. These results suggest that Asc-1 serves as the main glycine source for maintaining glycinergic transmission in the absence of GlyT2.

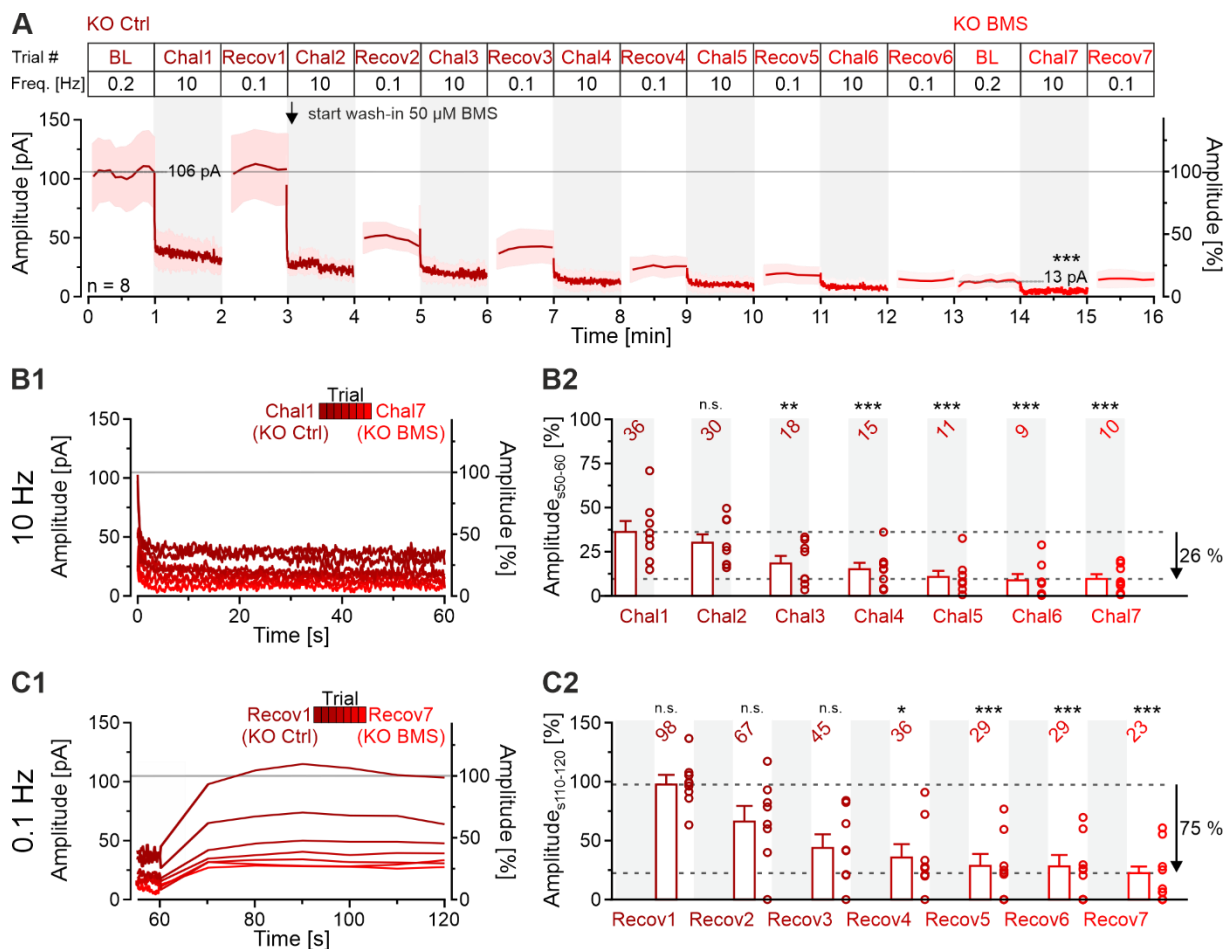


Figure 3.16 Decreased eIPSC amplitudes upon repetitive stimulation under Asc-1 inhibition with 50 μ M BMS in KO. (A) Time course of absolute and normalized eIPSC peak amplitudes for KO Ctrl (dark red) and KO BMS (light red) were obtained with Protocol 2 (see Figure 2.4C). Trials are color-coded from dark to light red. BL mean absolute values are written in the figure. Gray horizontal lines show normalization (BL1 = 100%). The arrow marks the start of the BMS wash-in. (B) Overlay of time course of absolute and normalized mean eIPSC peak amplitudes during 10-Hz | 60-s challenge periods (B1). (B2) Statistics of s_{50-60} during challenge periods. Mean value above bars. (C) Same as B, but for $s_{110-120}$ 0.1-Hz | 60-s recovery periods. Gray areas highlight the challenge periods. Values are shown as mean $\pm$ SEM. Time courses are simple moving averages (SEM lightly shaded). $n = 8$, same neurons. See Tables 3.11 and 3.12 for details, including statistics.

To test if the eIPSC amplitudes decrease because of an effect of inhibition of Asc-1 with BMS and not because of a time-dependent rundown, control experiments with Protocol 2 (Figure 2.4C) and the solvent for BMS (0.1% DMSO) were performed in KO mice (Figure 3.17A). The control experiments showed that there was no time dependence. Synaptic strength was statistically indistinguishable from Ctrl (ACSF) for BL (Figure 3.17A; Table 3.11), all challenge periods (Figure 3.17B1-B2; Table 3.12), and for recovery periods (Figure 3.17C1-C2; Table 3.12). Comparisons between BMS and DMSO conditions during challenge periods showed a significant difference from Chal4 onward. For the recovery periods, significant differences were observed from Recov5 ongoing (Table 3.12), supporting that the effects of Asc-1 inhibition accumulated over time. Taken together, the data show no effect of DMSO on synaptic transmission, suggesting that the reduction in synaptic strength is drug (BMS) specific rather than solvent or time-dependent.

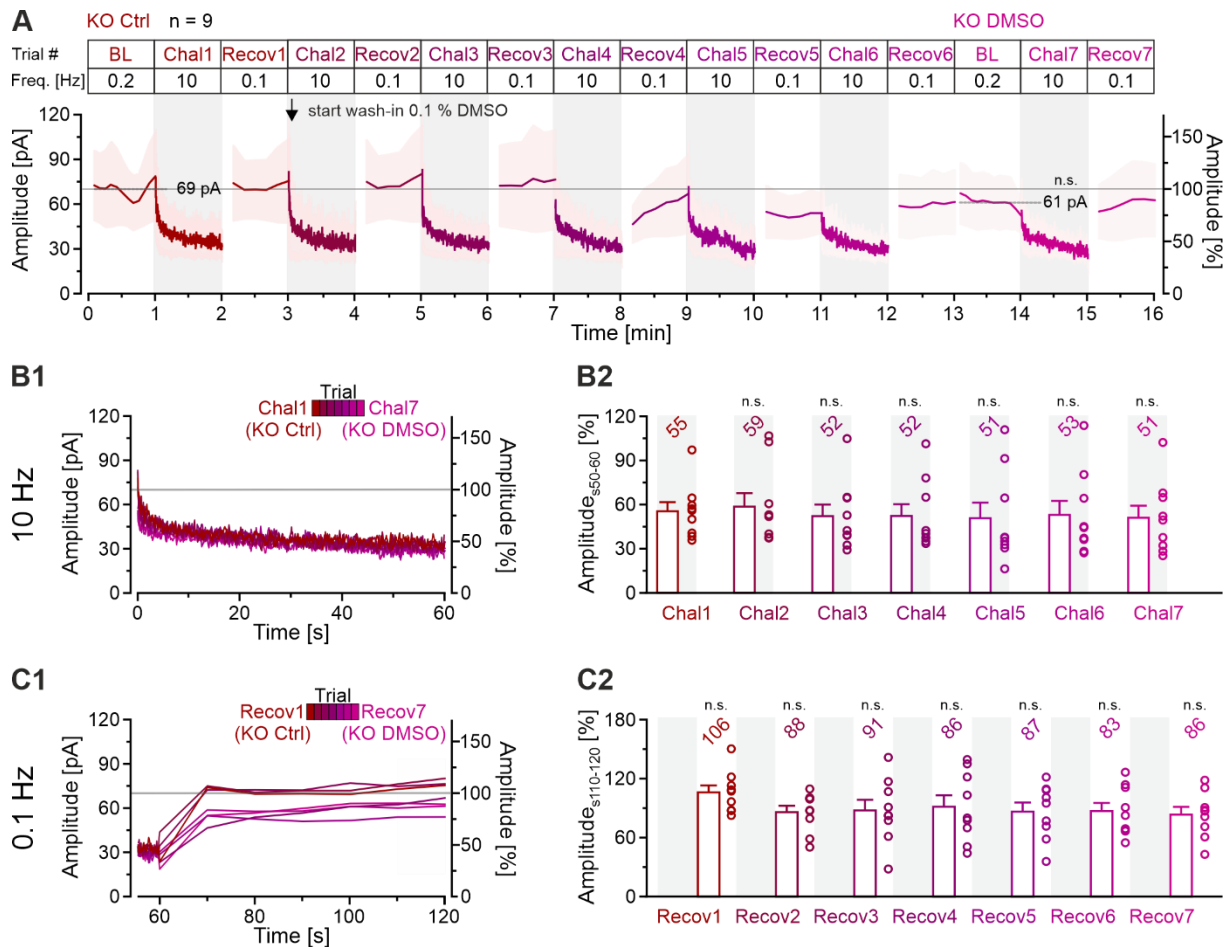


Figure 3.17 Unchanged eIPSC amplitudes during repetitive stimulation under 0.1% DMSO, the solvent for BMS in KO. (A) Time course of absolute and normalized eIPSC peak amplitudes for KO Ctrl (dark red) and KO DMSO (magenta) were obtained with Protocol 2 (see Figure 2.4C). Trials are color-coded from dark red to magenta. BL mean absolute values are written in the figure. Gray horizontal lines show normalization (BL1 = 100%). The arrow marks the start of the DMSO wash-in. **(B)** Overlay of time course of absolute and normalized mean eIPSC peak amplitudes during 10-Hz | 60-s challenges **(B1)**. **(B2)** Statistics of s_{50-60} during challenge periods. Mean value above bars. **(C)** Same as B, but for $s_{110-120}$ 0.1-Hz | 60-s recovery periods. Gray areas highlight the challenge periods. Values are shown as mean $\pm$ SEM. Time courses are simple moving averages (SEM lightly shaded). n = 9 cells, same neurons. See Tables 3.11 and 3.12 for details, including statistics.

Table 3.11: BL eIPSC amplitudes with 50 μ M BMS and 0.1% DMSO in KO.

50 μ M BMS and 0.1% DMSO	Ctrl (ACSF) n = 8		BMS n = 8		Ctrl vs. BMS	Figure
	Mean	SEM	Mean	SEM	p-value	
BL [pA]	106	27	13	5	0.001	3.16A
50 μ M BMS and 0.1% DMSO	Ctrl (ACSF) n = 9		DMSO n = 9		Ctrl vs. DMSO	Figure
	Mean	SEM	Mean	SEM	p-value	
BL [pA]	69	23	63	22	0.301	3.17A

Data are presented as mean $\pm$ SEM. BL: Ctrl vs. BMS and Ctrl vs. DMSO: Wilcoxon test in bold. Significant P-values are marked in green.

Table 3.12: eIPSC amplitudes_{s50-60} during 10-Hz challenge | 0.1-Hz recovery with 50 μ M BMS and 0.1% DMSO in KO.

50 μ M BMS and 0.1% DMSO		Chal1	Chal2	Chal3	Chal4	Chal5	Chal6	Chal7
Stimulation freq. [Hz]		10						
50 μ M BMS	Mean [%]	36	30	18	15	11	9	10
	SEM	6	5	4	4	3	3	3
	n	8	8	8	8	8	8	8
	p-value		>0.999	0.004	<0.001	<0.001	<0.001	<0.001
	Figure	3.16B2						
0.1% DMSO	Mean [%]	55	59	52	52	51	53	51
	SEM	6	9	8	8	11	9	8
	n	9	9	9	9	9	9	9
	p-value		>0.999	>0.999	>0.999	>0.999	>0.999	>0.999
	Figure	3.17B2						
DMSO vs. BMS	p-value	0.444	0.320	0.013	0.008	0.033	0.002	<0.001
		Recov1	Recov2	Recov3	Recov4	Recov5	Recov6	Recov7
Stimulation freq. [Hz]		0.1						
50 μ M BMS	Mean [%]	98	67	45	36	29	29	23
	SEM	7	13	11	11	9	9	8
	n	8	8	8	8	8	8	8
	p-value	>0.999	0.796	0.115	0.035	<0.001	<0.001	<0.001
	Figure	3.16C2						
0.1% DMSO	Mean [%]	106	88	91	86	87	83	86
	SEM	7	11	12	9	8	8	7
	n	9	9	9	9	9	9	9
	p-value	>0.999	>0.999	>0.999	>0.999	>0.999	0.583	>0.999
	Figure	3.17C2						
DMSO vs. BMS	p-value	>0.999	0.771	0.109	0.067	0.009	0.004	0.001

Data are presented as mean $\pm$ SEM. BMS: One-way ANOVA (Chal) with Bonferroni correction, Friedmann test with Dunn's correction (Recov). DMSO: Friedmann test with Dunn's correction (Chal + Recov). DMSO vs. BMS: Kruskal-Wallis test with Dunn's correction (Chal + Recov). P-values are adjusted values after correction. Significant P-values are marked in green.

Under repetitive stimulation and inhibition of Asc-1, eIPSC amplitudes decreased. Therefore, eIPSC amplitude fidelity was determined. eIPSC amplitude failures indicate reduced glycine availability. In KO, the overlay of synaptic fidelity time courses showed failures under Ctrl (ACSF) that increased during BMS wash-in (Figure 3.18A1). Quantification of Fidelity_{s50-60} showed a significant decrease from Chal1 starting at Chal6 (Chal1: 84%, Chal2: 85%, Chal3: 55%, Chal4: 56%, Chal5: 50%, Chal6: 45%, and Chal7: 35%, respectively; Figure 3.18A2; Table 3.13). Although fidelity begins to decline at Chal3-Chal5 (~50%), this reduction is not statistically significant. A significant difference in Fidelity_{s50-60} appeared at the final challenge

periods. This suggests depletion of presynaptic glycine and possibly fewer filled or reduced number of SV.

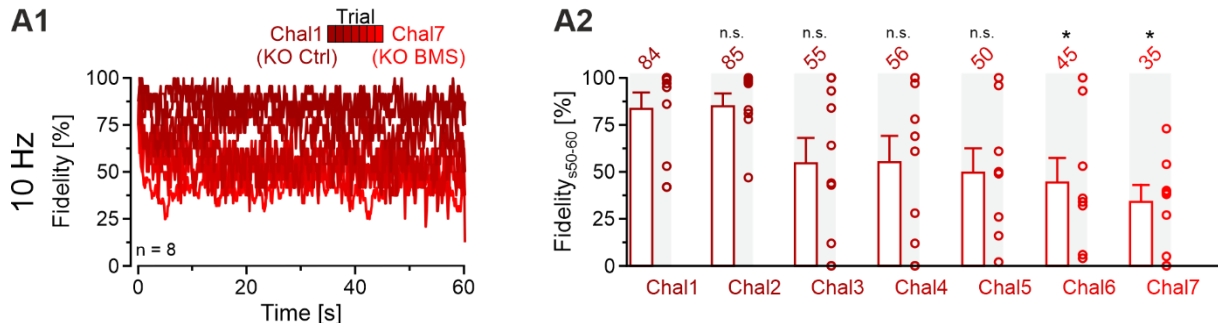


Figure 3.18 Reduced Fidelity_{s50-60} at the late phase of repetitive stimulation under 50 μ M BMS in KO. (A) Fidelity rate of eIPSC amplitudes for KO Ctrl (dark red) and KO BMS (light red). **(A1)** Time course of mean fidelity. Trials are color-coded from dark to light red. **(A2)** Statistics for fidelity analysis at s₅₀₋₆₀ of challenge periods (Chal1-Chal7). Gray areas highlight the challenge periods. Mean value above bars. Values are shown as mean $\pm$ SEM. n = 11, same neurons. See Table 3.13 for details, including statistics.

Table 3.13: eIPSC Fidelity_{s50-60} with 50 μ M BMS in KO during 7x 10-Hz challenge.

50 μ M BMS	Chal1	Chal2	Chal3	Chal4	Chal5	Chal6	Chal7
Stimulation freq. [Hz]	10						
Mean [%]	84	85	55	56	50	45	35
SEM	8	6	13	13	13	13	8
n	8	8	8	8	8	8	8
p-value		>0.999	0.384	0.795	0.065	0.033	0.019
Figure	3.18A2						

Data are presented as mean $\pm$ SEM. Friedmann test with Dunn's correction. P-values are adjusted values after correction. Significant P-values are marked in green.

3.2.4 Asc-1 inhibition and repetitive stimulation did not lead to alterations in spontaneous release in KO

Reduced eIPSC amplitudes and fidelity suggest a depletion of presynaptic glycine and possibly less filled or empty SVs. This should be evidenced by a reduction in q or the frequency of sIPSCs. These parameters were determined during BL1 and all recovery periods (Recov1-Recov7). Representative current traces for BL1 (KO Ctrl, dark red) and the Recov7 (KO BMS, light red) periods are shown in Figure 3.19A1. A histogram of sIPSC peak amplitudes was plotted, and q was determined using a Gaussian fit for each condition (Figure 3.19A2). Cumulative probability plots confirmed no difference in sIPSC amplitudes (Figure 3.19B). In total, 640 (Ctrl) and 506 (BMS) sIPSC events from 8 cells were analyzed. Mean sIPSC amplitudes were not significantly different between KO Ctrl and BMS (14 vs.

15 pA, Table 3.14). Asc-1 blockade did not alter the distribution of sIPSC amplitudes in KO. q remained stable across all recovery periods compared to BL1 (BL1: 14 pA; Recov1: 14 pA; Recov2: 15 pA; Recov3: 15 pA; Recov4: 14 pA; Recov5: 13 pA; Recov6: 13 pA, and Recov7: 13 pA; Figure 3.19C, Table 3.14). sIPSC frequency also showed no change during the protocol (BL1-Recov7: 1 Hz; Figure 3.19D, Table 3.14). Taken together, spontaneous release remained unchanged despite reduced eIPSC amplitudes and fidelity. This suggests that glycine loading of SVs is not abolished in MNTB axon terminals despite the Asc-1 blockade in KO.

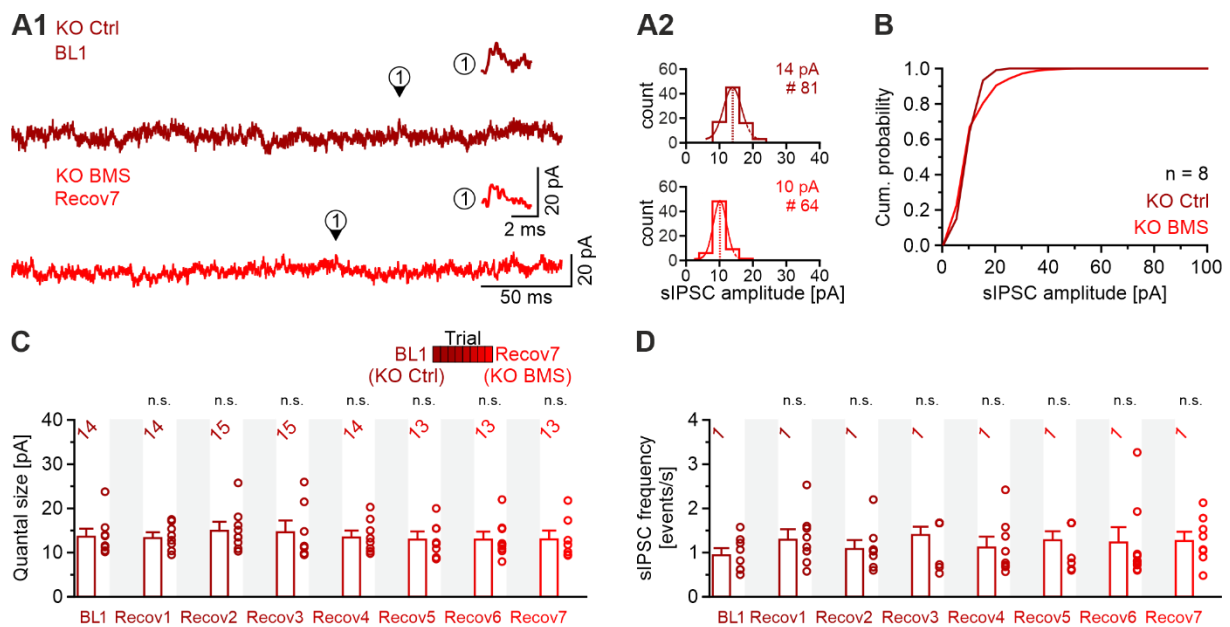


Figure 3.19 Unchanged spontaneous release in KO during repetitive stimulation under 50 μ M BMS (A) Representative sIPSC current traces for KO Ctrl (dark red) and KO BMS (light red). (A1) Arrow marks sIPSC amplitudes during BL1 (KO Ctrl) and Recov7 (KO BMS). Numbers over arrows mark example sIPSCs, which are shown over traces. (A2) Estimation of q . Histograms of sIPSC amplitudes to current traces in A1 during BL1 and Recov7 with Gaussian fit from 0 pA to the bin after the first local maximum (dotted curves; Krächan et al., 2017). Numbers in panels are q and the number of sIPSCs. (B) Cumulative probability of sIPSC amplitudes for KO Ctrl (dark red) and KO BMS (light red) (C) Statistics for q during BL1-Recov7. Mean values above bars. Trials are color-coded from dark to light red. (D) Same as C, but for sIPSC frequency. Gray areas highlight the challenge periods. Values are shown as mean $\pm$ SEM. $n = 11$, same neurons. See Table 3.14 for details, including statistics.

Table 3.14 sIPSC properties during 7x 0.1-Hz recovery with 50 μ M BMS in KO.

50 μ M BMS			Ctrl (ACSF) n = 8		BMS n = 8		Ctrl vs. BMS		
			Mean	SEM	Mean	SEM	p-value		Figure
sIPSC amplitudes [pA]			14	1	15	2	0.440		3.19B
		BL	Recov1	Recov2	Recov3	Recov4	Recov5	Recov6	Recov7
Stimulation freq. [Hz]		0.2	0.1						
q	Mean [pA]	14	14	15	15	14	13	13	13
	SEM	2	1	2	2	1	2	2	2
	p-value		>0.999	>0.999	>0.999	>0.999	>0.999	>0.999	>0.999
	Figure	3.19C							
sIPSC	Mean [Hz]	1.0	1.3	1.1	1.4	1.1	1.3	1.3	1.3
	SEM	0.1	0.2	0.2	0.2	0.2	0.2	0.3	0.2
	p-value		0.972	>0.999	0.197	>0.999	>0.999	>0.999	0.645
	Figure	3.19D							

Data are presented as mean $\pm$ SEM. sIPSC amplitudes: Paired t-test. q: One-way ANOVA with Bonferroni correction. sIPSC freq.: Friedman test with Dunn's correction. P-values are adjusted values after correction.

3.2.5 Asc-1 inhibition and repetitive stimulation reduce m_{s50-60} and RR_{s50-60} in KO

Spontaneous release remained unchanged during Asc-1 blockade and repetitive stimulation, suggesting that glycine loading of SVs in MNTB axon terminals is not abolished in KO. To test whether reduced eIPSC amplitudes and fidelity were due to decreased SV availability, m and RR were analyzed. Overlay of time courses of m showed a decline in the number of SVs released per event over challenge periods (Figure 3.20A1). Quantification of m_{s50-60} showed a decreased SV availability from Chal5 onward (Chal1 vs. Chal5, 2 vs. 1 SV). Mean m values were Chal1: 2 SVs; Chal2: 2 SVs; Chal3: 1 SV; Chal4: 1 SV; Chal5: 1 SV, Chal6: 1 SV, and Chal 7: 0.5 SVs (Figure 3.20A2; Table 3.15). Cumulative SV release decreased progressively across challenge periods (Chal1: 1,699 SVs; Chal2: 1,147 SVs; Chal3: 767 SVs; Chal4: 635 SVs; Chal5: 565 SVs, Chal6: 417 SVs, and Chal7: 212 SVs; Figure 3.20B1), resulting in a total release of 5,442 SVs. To assess the number of SVs recruited during the steady-state phase, RR_{s50-60} were analyzed. A significant reduction was observed from the Chal5 onward, with RR_{s50-60} decreasing from 22 to 3 SVs (Figure 3.20B2; Table 3.15). Taken together, the reduction in the number of released SVs and the RR indicates that with Asc-1 blockade and repetitive stimulation, fewer SVs are available in KO.

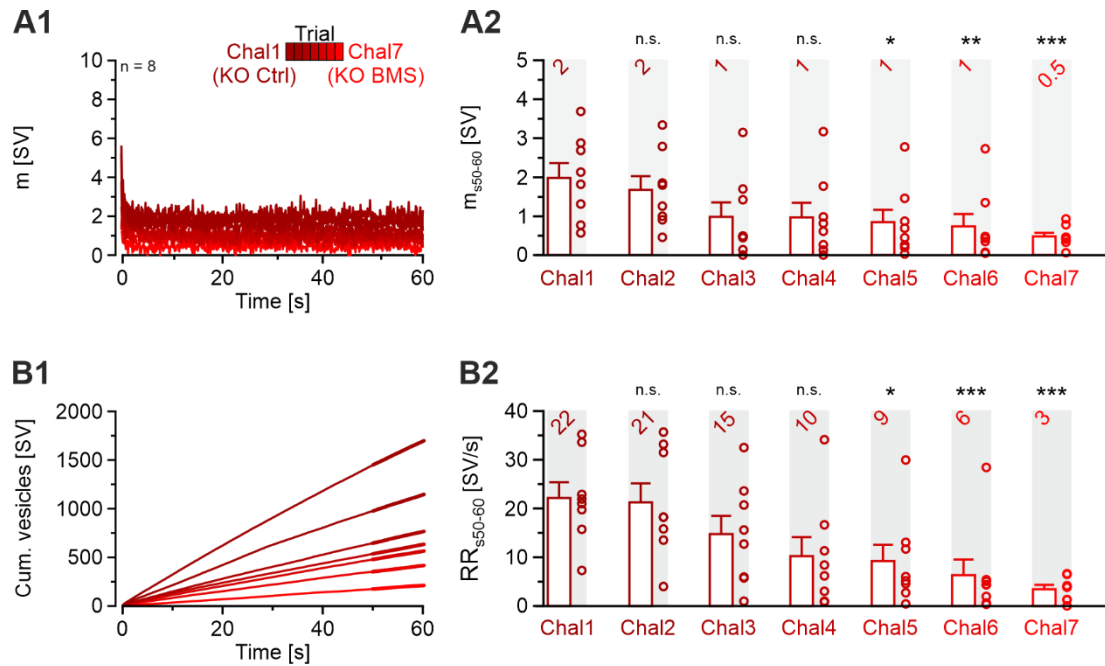


Figure 3.20 Reduced number of released SVs at the late phase of repetitive stimulation under 50 μ M BMS in KO. (A) Quantification of quantal content (m). (A1) Time course of m during 10-Hz | 60-s challenge periods (Chal1-Chal7) for KO Ctrl (dark red) and KO BMS (light red). Trials are color-coded from dark to light red. (A2) Statistics for m at s₅₀₋₆₀ of challenge periods (Chal1-Chal7). Mean values above bars. (B) Time course of mean cumulative released synaptic vesicles. Thicker lines at s₅₀₋₆₀ mark the period in which RR was quantified (B1). (B2) same as A2, but for RR. Gray areas highlight the challenge periods. Values are shown as mean $\pm$ SEM. n = 11, same neurons. See Table 3.15 for details, including statistics.

Table 3.15: m_{s50-60} and RR_{s50-60} during 7x 10-Hz challenge with 50 μ M BMS in KO.

50 μ M BMS		Chal1	Chal2	Chal3	Chal4	Chal5	Chal6	Chal7
Stimulation freq. [Hz]		10						
m _{s50-60}	Mean [SV]	2	2	1	1	1	1	0.5
	SEM	0.4	0.3	0.4	0.4	0.3	0.3	0.1
	n	8	8	8	8	8	8	8
	p-value		>0.999	0.077	0.257	0.016	0.003	<0.001
	Figure	3.20A2						
RR _{s50-60}	Mean [SV/s]	22	21	15	10	9	6	3
	SEM	3	4	4	4	3	3	1
	n	8	8	8	8	8	8	8
	p-value		>0.999	0.384	0.091	0.016	<0.001	<0.001
	Figure	3.20B2						

Data are presented as mean $\pm$ SEM. m and RR: Friedman test with Dunn's correction. P-values are adjusted values after correction. Significant P-values are marked in green.

3.3 Impact of GlyT2 and Asc-1 on sustained transmission at MNTB-LSO synapses

Next, synaptic transmission was examined under very prolonged stimulation to further challenge the synapses, assess the contribution of glycine transporters under high activity, and determine whether the synapses were pushed to their limits. MNTB fibers were stimulated using Protocol 3 (Figure 2.4C), so-called “marathon experiments,” which consist of an initial 60-s period at 1 Hz to establish the BL eIPSC amplitudes. This is followed by a 50-Hz | 60-s challenge and a 1-Hz | 60-s recovery period, repeated ten times (Trial 1-10). In total, Protocol 3 lasts 21 minutes and includes 30,660 stimulus pulses (Figure 3.21A). Before the stimulation protocol, either ACSF, 2 μ M ALX, 50 μ M BMS, or a cocktail of both drugs (2 μ M ALX + 50 μ M BMS) was applied to the bath and during the whole stimulation.

3.3.1 Synaptic transmission declines over time during very prolonged stimulation under GlyT2, Asc-1, or combined inhibition

During Ctrl experiments (ACSF), synaptic transmission was, on average, resistant to very prolonged stimulation (Figure 3.21A). Trials were color-coded from black to gray. Steady-state (s_{50-60}) eIPSC amplitudes were not significantly different during challenge periods and decreased to 21-27% (Figure 3.21B, Table 3.16). The same results were seen for recovery periods. During all ten trials, eIPSC amplitudes $_{s_{50-60}}$ were able to recover back to ~100% and did not differ significantly (Figure 3.21C, Table 3.16). Despite very prolonged stimulation, synaptic transmission at MNTB-LSO synapses remained stable under Ctrl, with consistent eIPSC amplitude depression and full recovery throughout all ten trials.

Loss of GlyT2 function by 2 μ M ALX had no significant effect on eIPSC amplitudes across all challenge periods compared to Chal1, with eIPSC amplitudes depressed between 13-25% (Figure 3.21B; Table 3.16). Trials were color-coded from dark to light blue. During recovery, ALX showed a significant effect from Recov8 onward, with eIPSC amplitudes recovered from depression to ~60% compared to BL (Figure 3.21C; Table 3.16). Comparing Ctrl with ALX, they start around the same BL level 906 pA (Ctrl) and 1084 pA (ALX; Figure 21A; Table 3.16). ALX compared to Ctrl showed a significant difference at Chal10 (27% vs. 13%) and from Recov8 onward (Recov8: 106% vs. 57%; Recov9: 106% vs. 63%; and Recov10: 103% vs. 62%). The reduction of eIPSC amplitudes during recovery periods under GlyT2 blockade at the end of the stimulation protocol suggests the role of GlyT2 in maintaining synaptic strength during prolonged activity at MNTB-LSO synapses, but it's limited.

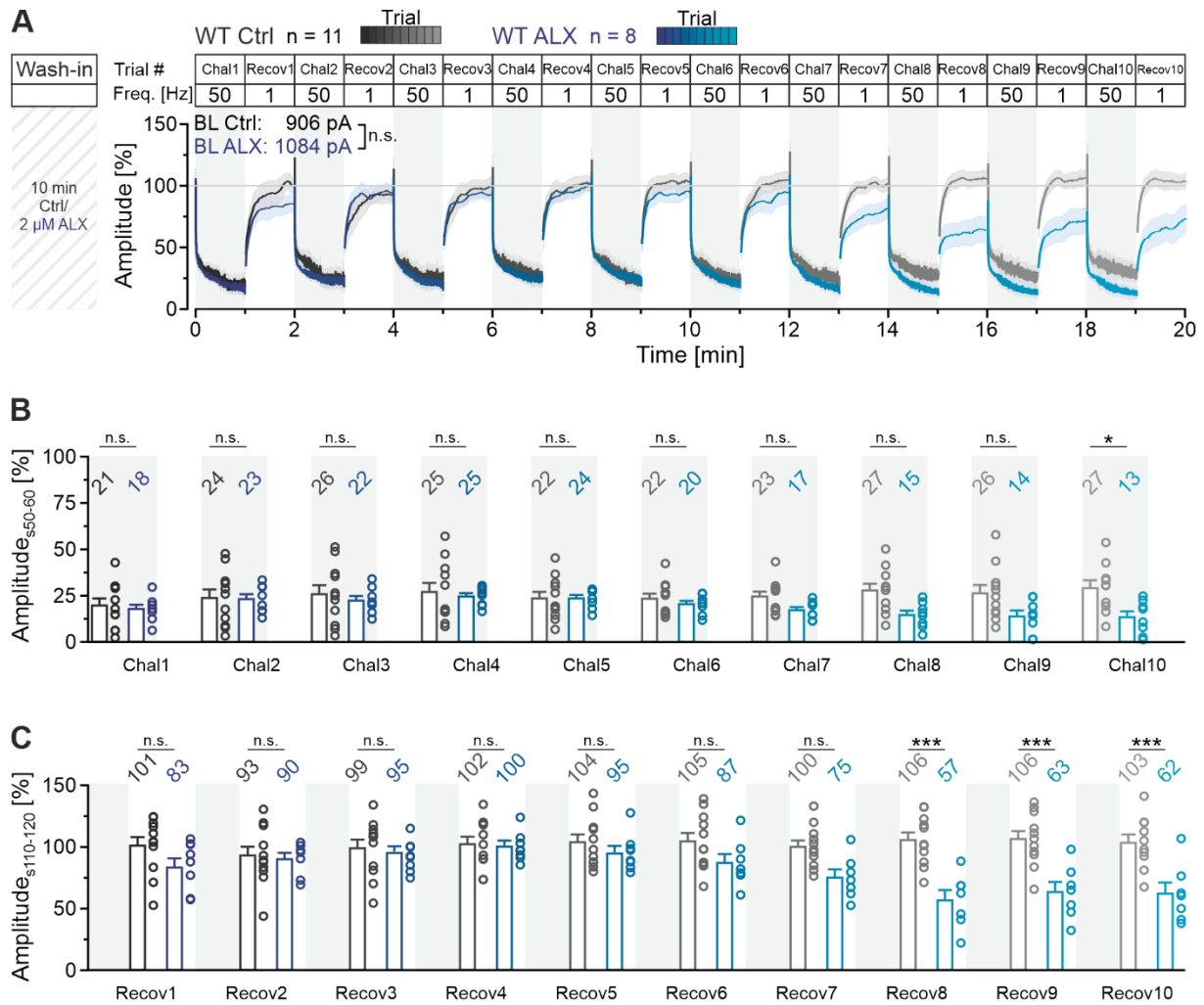


Figure 3.21 Impaired challenge and recovery periods upon GlyT2 inhibition in WT towards the end of marathon experiments showed. (A) Time course of normalized mean eIPSC peak amplitudes for Ctrl (black) and ALX experiments (blue) were obtained with Protocol 3 (see Figure 2.4C). Trials 1-10 are color-coded from dark to light. The diagonally striped region indicates perfusion periods of ACSF or ALX for 10 mins before recording. Numbers in the panel represent mean BL from Ctrl or ALX. The horizontal line shows normalization (WT Ctrl or ALX BL = 100 %). **(B)** Statistics for normalized challenge analysis at s₅₀₋₆₀ of all 10 trials for Ctrl and ALX experiments. Mean normalized values above bars. **(C)** Same as B, but for recovery analysis at s₁₁₀₋₁₂₀. Note the different amplitude scaling in A-C. Values are shown as mean $\pm$ SEM. Time courses are simple moving averages (SEM lightly shaded). Gray areas highlight the challenge periods. WT Ctrl, n = 11, and WT ALX, n = 8. See Table 3.16 for details, including statistics.

Loss of Asc-1 function by 50 μ M BMS showed no significant differences in eIPSC amplitudes across all challenge periods compared to Chal1, with eIPSC amplitudes decreased to 17-11% (Figure 3.22A, B; Table 3.16). Trials were color-coded from dark to light green. During recovery, eIPSC amplitudes recovered to 85-63% in Recov1-Recov7. From Recov8 onward, eIPSC amplitudes were significantly reduced compared to BL and recovered only to ~60% (Figure 3.22A, C; Table 3.16).

Comparing Ctrl and BMS conditions, BL levels showed no significant difference between BMS and Ctrl, 746 pA and 906 pA, respectively. Under BMS, time courses were overall reduced compared to Ctrl (Figure 3.22A; Table 3.16). However, across all ten challenge periods, no significant differences were observed between Ctrl and BMS (Figure 3.22B; Table 3.16). In the first four recovery periods, eIPSC amplitudes were also not significantly different between groups. From Recov5 onward, eIPSC amplitudes in BMS were significantly reduced compared to Ctrl (100-106% vs. 60-64%; Figure 3.22C; Table 3.16). Although BL and challenge periods remained unaffected, from the middle of stimulation recovery phases were impaired, indicating a role of Asc-1 in sustaining synaptic transmission during prolonged activity at MNTB-LSO synapses.

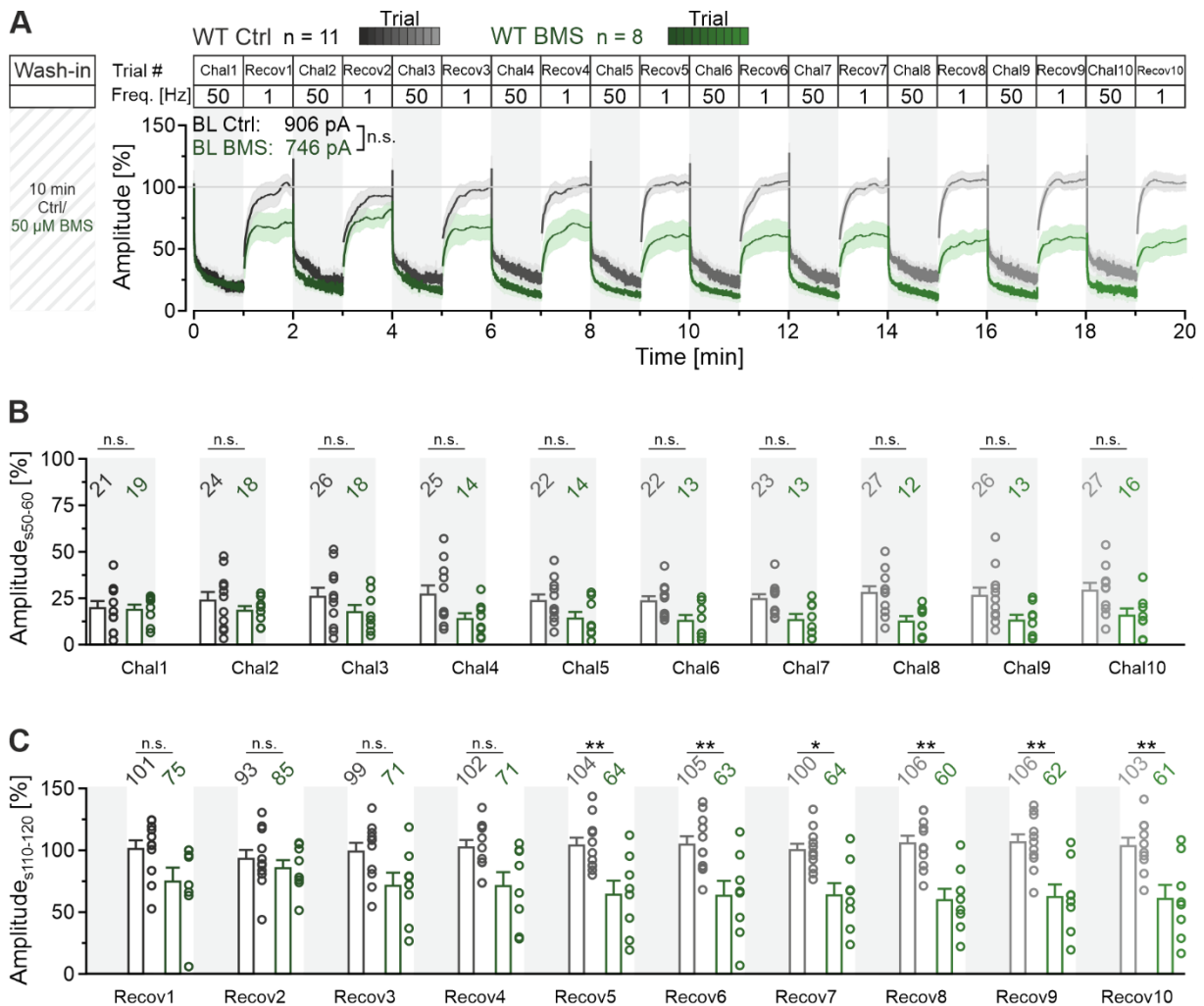


Figure 3.22 Impaired recovery periods upon Asc-1 inhibition in WT from the mid-marathon experiments. (A) Time course of normalized mean eIPSC peak amplitudes for Ctrl (black) and BMS experiments (green) were obtained with Protocol 3 (see Figure 2.4C). Trials 1-10 are color-coded from dark to light. The diagonally striped region indicates perfusion periods of ACSF or BMS for 10 mins before recording. Numbers in the panel represent mean BL from Ctrl and BMS. The horizontal line shows normalization (WT Ctrl or BMS BL = 100 %).

(B) Statistics for normalized challenge analysis at s_{50-60} of all 10 trials for Ctrl and BMS experiments. Mean normalized values above bars. **(C)** Same as B, but for recovery analysis at $s_{110-120}$. Note the different amplitude scaling in A-C. Values are shown as mean $\pm$ SEM. Time courses are simple moving averages (SEM lightly shaded). Gray areas highlight the challenge periods. WT Ctrl, $n = 11$ and WT BMS, $n = 8$. See Table 3.16 for details, including statistics.

The combined loss of GlyT2 and Asc-1 function by a cocktail with 2 μ M ALX + 50 μ M BMS (ALX + BMS) showed no significant differences in eIPSC amplitudes across all challenge periods compared to Chal1, with eIPSC amplitudes decreased to 17% to 8%, they decreased 9% overall ten trials (Figure 3.23A, B; Table 3.16). Trials were color-coded from brown to orange. During recovery, eIPSC amplitudes recovered to 95% to 80% in Recov1-Recov4. From Recov5 onward, eIPSC amplitudes were significantly reduced compared to BL and recovered only from 75% to 54%, which showed a decrease from 46% for Recov10 to BL (Figure 3.23A, C; Table 3.16).

Comparing Ctrl and ALX + BMS conditions, BL levels were not significantly different (Ctrl: 906 pA; ALX + BMS: 630 pA). Under ALX + BMS, time courses were overall reduced compared to Ctrl (Figure 3.23A; Table 3.16). However, in the first seven challenge periods, no significant differences were observed between Ctrl and BMS. From Chal8 onward the comparison was significant (Figure 3.23B; Table 3.16). Comparing the end of the marathon experiments (trial 10), the eIPSC amplitudes decreased to 30% of the Ctrl condition. The first four recovery periods showed that eIPSC amplitudes were not significantly different between groups, equal to Asc-1 blockade alone. From Recov5 onward, eIPSC amplitudes in BMS were significantly reduced compared to Ctrl (100-106% vs. 75-54%; Figure 3.23C; Table 3.16). These results suggest that both transporters are necessary for maintaining synaptic strength during prolonged activity, especially in the later phases.

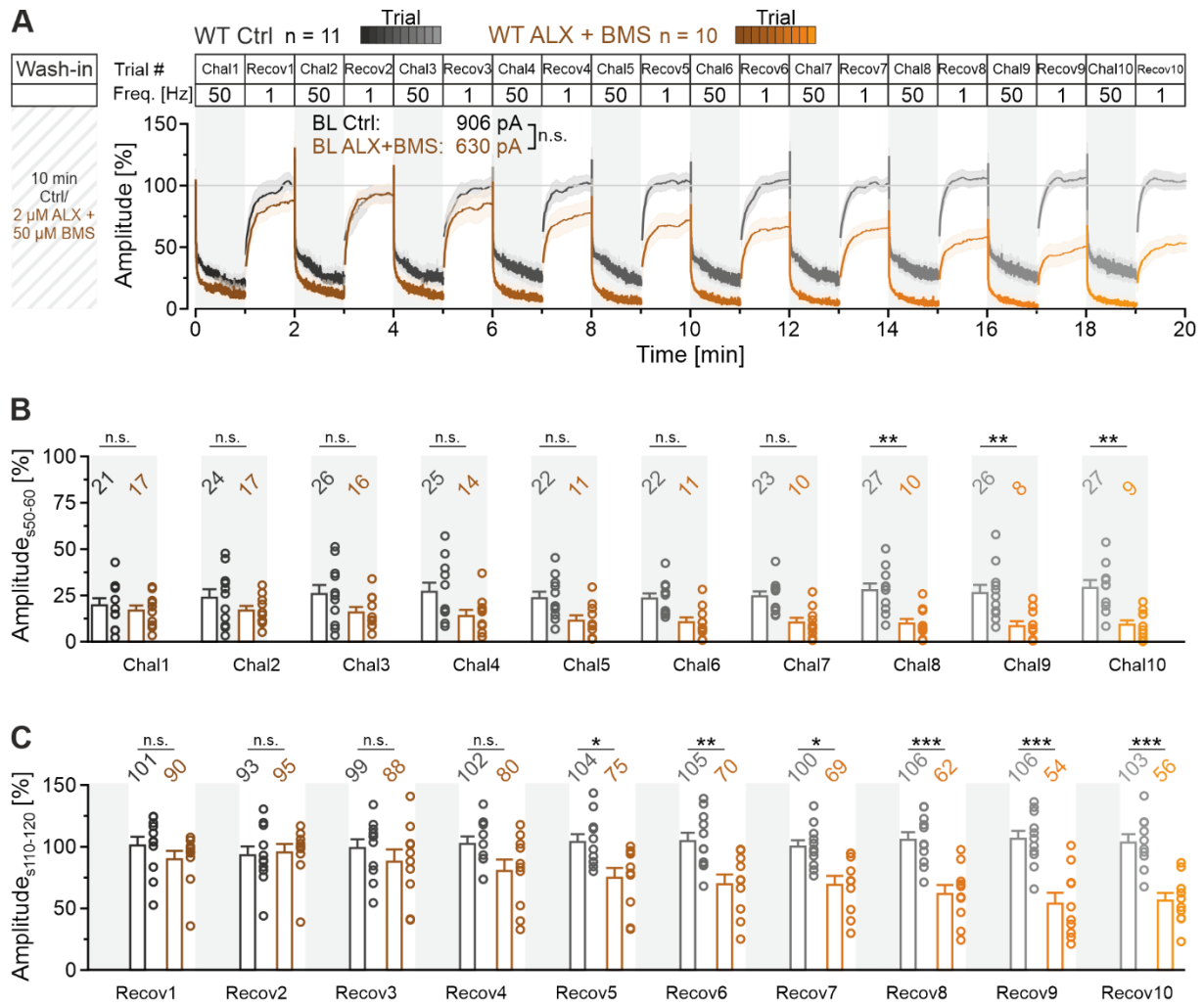


Figure 3.23 Impaired late challenge and mid-recovery periods under combined GlyT2 and Asc-1 inhibition in WT. (A) Time course of normalized mean eIPSC peak amplitudes for Ctrl (black) and ALX + BMS experiments (brown) were obtained with Protocol 3 (see Figure 2.4C). Trials 1-10 are color-coded from dark to light. The diagonally striped region indicates perfusion periods of ACSF or ALX + BMS for 10 mins before recording. Numbers in the panel represent mean BL from Ctrl and ALX + BMS. The horizontal line shows normalization (WT Ctrl or ALX + BMS BL = 100 %). **(B)** Statistics for normalized challenge analysis at s_{50-60} of all 10 trials for Ctrl and ALX + BMS experiments. Mean normalized values above bars. **(C)** Same as B, but for recovery analysis at $s_{110-120}$. Note the different amplitude scaling in A-C. Values are shown as mean $\pm$ SEM. Time courses are simple moving averages (SEM lightly shaded). Gray areas highlight the challenge periods. WT Ctrl, n = 11 and WT ALX + BMS, n = 10. See Table 3.16 for details, including statistics.

Table 3.16 Absolute BL and normalized eIPSC amplitudes_{s50-60} during marathon experiments for Ctrl, ALX, BMS, and ALX + BMS WT neurons.

	Ctrl n = 11			ALX n = 8			BMS n = 8			ALX + BMS n = 10			Ctrl vs. ALX	Ctrl vs. BMS	Ctrl vs. ALX + BMS
	Mean	SEM	Figure	Mean	SEM	Figure	Mean	SEM	Figure	Mean	SEM	Figure	p-value	p-value	p-value
BL [pA]	906	143	3.21	1084	221	3.21	746	149	3.22	630	144	3.23	0.488	0.451	0.152
	Mean	SEM	p-value	Mean	SEM	p-value	Mean	SEM	p-value	Mean	SEM	p-value	p-value	p-value	p-value
Chal1 [%]	21	4		18	2		19	3		17	3		>0.999	>0.999	>0.999
Chal2 [%]	24	4	>0.999	23	3	0.169	19	3	>0.999	17	3	>0.999	>0.999	>0.999	>0.999
Chal3 [%]	26	5	>0.999	22	2	>0.999	18	4	>0.999	16	3	>0.999	>0.999	>0.999	0.357
Chal4 [%]	25	5	>0.999	25	2	0.215	14	3	>0.999	14	3	>0.999	>0.999	0.435	0.272
Chal5 [%]	22	3	>0.999	24	2	>0.999	14	4	>0.999	11	3	>0.999	>0.999	>0.999	0.365
Chal6 [%]	22	2	>0.999	20	2	>0.999	13	3	>0.999	11	3	>0.999	>0.999	0.905	0.209
Chal7 [%]	23	3	>0.999	17	2	>0.999	13	3	>0.999	10	3	>0.999	>0.999	0.692	0.099
Chal8 [%]	27	4	>0.999	15	2	>0.999	12	3	>0.999	10	3	>0.999	0.084	0.073	0.005
Chal9 [%]	26	5	>0.999	14	3	>0.999	13	3	>0.999	8	3	>0.999	0.322	0.177	0.005
Chal10 [%]	27	4	>0.999	13	3	>0.999	16	4	>0.999	9	2	>0.999	0.043	0.471	0.006
Recov1 [%]	101	7	>0.999	83	8	0.906	75	11	>0.999	90	7	>0.999	0.717	0.252	>0.999
Recov2 [%]	93	7	>0.999	90	5	>0.999	85	7	>0.999	95	7	>0.999	>0.999	>0.999	>0.999
Recov3 [%]	99	7	>0.999	95	4	>0.999	71	11	>0.999	88	10	>0.999	>0.999	0.193	>0.999
Recov4 [%]	102	6	>0.999	100	5	>0.999	71	11	>0.999	80	9	>0.999	>0.999	0.083	0.263
Recov5 [%]	104	6	>0.999	95	6	>0.999	64	11	>0.999	75	8	0.104	>0.999	0.008	0.035
Recov6 [%]	105	7	>0.999	87	7	>0.999	63	12	>0.999	70	8	0.037	0.783	0.005	0.005
Recov7 [%]	100	5	>0.999	75	7	0.061	64	10	>0.999	69	7	0.007	0.121	0.020	0.018
Recov8 [%]	106	6	>0.999	57	8	0.017	60	9	>0.999	62	7	<0.001	<0.001	0.001	<0.001
Recov9 [%]	106	6	>0.999	63	8	0.007	62	10	>0.999	54	9	<0.001	<0.001	0.002	<0.001
Recov10 [%]	103	6	>0.999	62	9	0.004	61	11	>0.999	56	6	<0.001	<0.001	0.004	<0.001

Data are presented as mean ± SEM. BL Ctrl vs. ALX and Ctrl vs. BMS: Unpaired t-test. Ctrl vs. ALX + BMS: Mann-Whitney test. Ctrl, ALX, BMS, and ALX + BMS (Chal): One-way ANOVA with Bonferroni correction. Each Chal was tested against Chal1. Ctrl, ALX, BMS, and ALX + BMS (Recov): Friedman test with Dunn's correction. Each Recov was tested against BL. Ctrl vs. ALX (Chal): Kruskal-Wallis test with Dunn's correction. Ctrl vs. ALX (Recov): One-way ANOVA with Bonferroni correction. Ctrl vs. BMS and Ctrl vs. ALX + BMS (Chal + Recov): One-way ANOVA with Bonferroni correction. P-values are adjusted values after correction. Significant P-values are marked in green.

Due to a reduction in eIPSC amplitudes with GlyT2, Asc-1, and with combined GlyT2 and Asc-1 inhibition, the fidelity of eIPSC amplitudes was determined. Representative current traces of ten eIPSCs during the last 10 s are shown for Chal1 Ctrl (black, upper), Chal1 ALX (dark blue, lower), Chal10 Ctrl (gray, upper), and Chal10 ALX (light blue, lower). In Chal1 for Ctrl and ALX, the amplitude of every eIPSC reaches the detection threshold of 23 pA or 25, respectively (see Section 2.5.1). In Chal10, for each Ctrl and ALX, there is one amplitude that did not reach the detection threshold, this eIPSC amplitude failure was indicated by an arrow (Figure 3.24A1-A2). Overlay of fidelity time courses showed similar time courses under Ctrl (upper) and ALX (lower) conditions. For Chal9 and Chal10, the fidelity time course decreased continuously from 20 s to 80% by the end of the trace, whereas the other trials started with failures at ~40 s (Figure 3.24A3). Fidelity_{s50-60} decreased to 97-85% in all ten trials for Ctrl and to 99-80% for ALX, with Chal4 and Chal5 that showed no failure at the steady-state phase. No significant difference in Fidelity_{s50-60} was found between Ctrl and ALX across all ten trials (Figure 3.24B; Table 3.17).

Similar results were observed for the fidelity with BMS. In Chal1, each eIPSC amplitude (dark green) reached the detection threshold of 20 pA. In Chal10, two out of ten amplitudes did not reach the detection threshold (Figure 3.25A1-A2). Overlay of fidelity time courses showed a reduction of fidelity beginning in the early seconds of the challenge periods, decreasing to 87-77% across all ten trials (Figure 3.25A3; lower). Fidelity_{s50-60} decreased to ~80% by the end of the marathon experiments. However, no significant differences in Fidelity_{s50-60} were found between Ctrl and BMS across all ten trials (Figure 3.25B; Table 3.17).

For the combined application of ALX + BMS, in Chal1 all eIPSC amplitudes (brown) reached the detection threshold of 10 pA. In contrast, the Chal10 representative trace (orange) showed four failures, marked with arrows (Figure 3.26A1-A2). Overlay of time courses revealed a progressive reduction in fidelity across trials, decreasing from 89 to 56%, with failures beginning early in the challenge periods (Figure 3.26A3). Quantification of Fidelity_{s50-60} showed a reduction to 61% at the end of the marathon experiments. However, there is no significant difference compared to Ctrl (Figure 3.26B; Table 3.17).

Taken together, none of the conditions (ALX, BMS, and ALX + BMS) led to a significant reduction in fidelity compared to Ctrl. eIPSC amplitudes consistently reached the detection threshold, indicating that MNTB-LSO synapses can reliably transmit signals even under glycine transporter blockade. This suggests the presence of a large SV pool supporting sustained transmission.

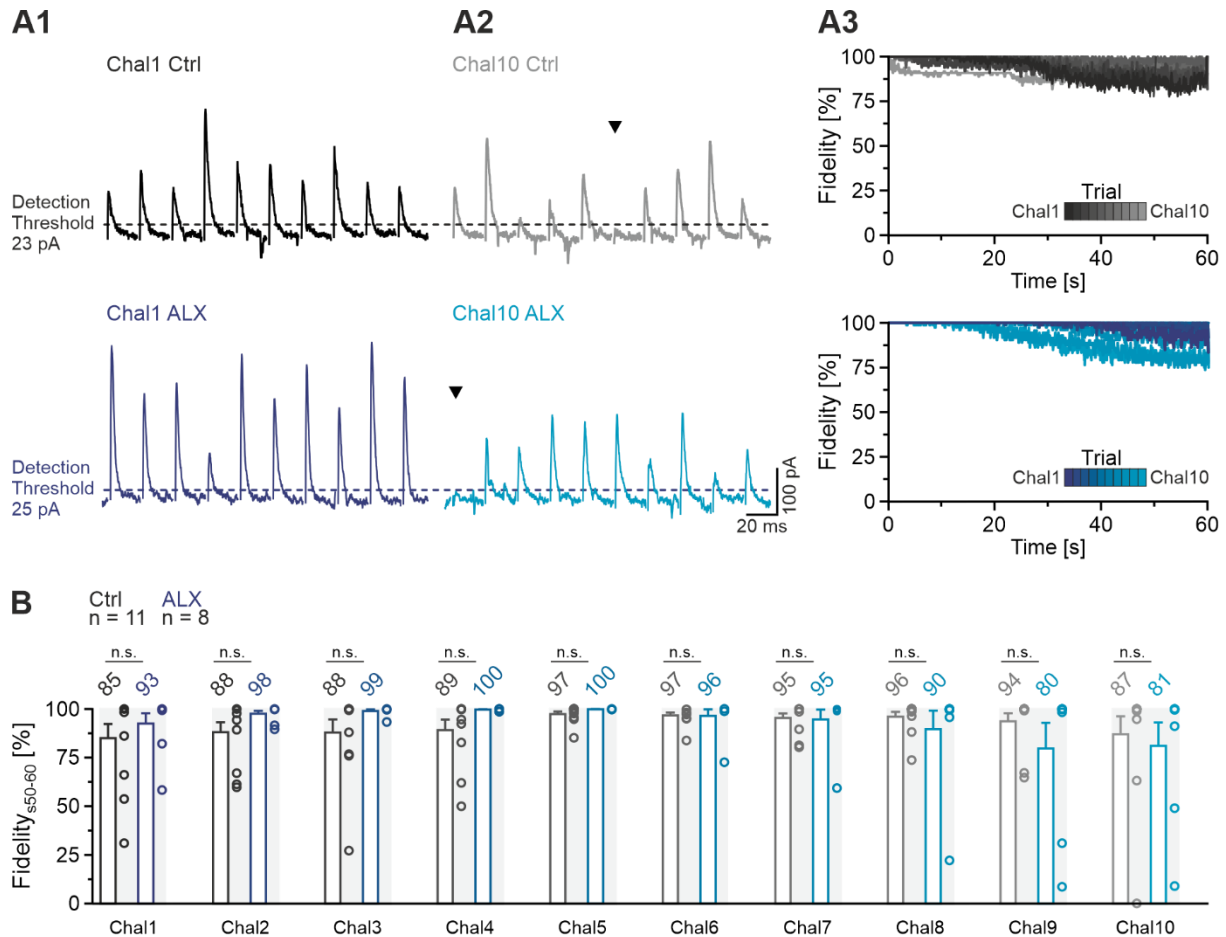


Figure 3.24 Unchanged fidelity during marathon experiments under GlyT2 inhibition in WT. (A1) Ten representative eIPSCs during the 50 Hz Chal1 period for Ctrl (upper, black) and ALX (lower, dark blue) of the last 10 s. Dashed lines show the detection threshold for each cell. Arrows mark a synaptic failure (see Material and Methods for details). Artifacts were blanked for clarity. (A2) same as A1 but for Chal10 for Ctrl (upper, gray) and ALX (lower, light blue). (A3) Time course of mean fidelity of all 10 trials for Ctrl (upper) and ALX (lower). Trials 1-10 are color-coded from dark to light. Time courses are simple moving averages. (B) Statistics for Fidelity_{s50-60} of all 10 trials. Mean values on top of the bars. Values are shown as mean $\pm$ SEM. Ctrl, n = 11; ALX, n = 8. See Table 3.17 for details, including statistics.

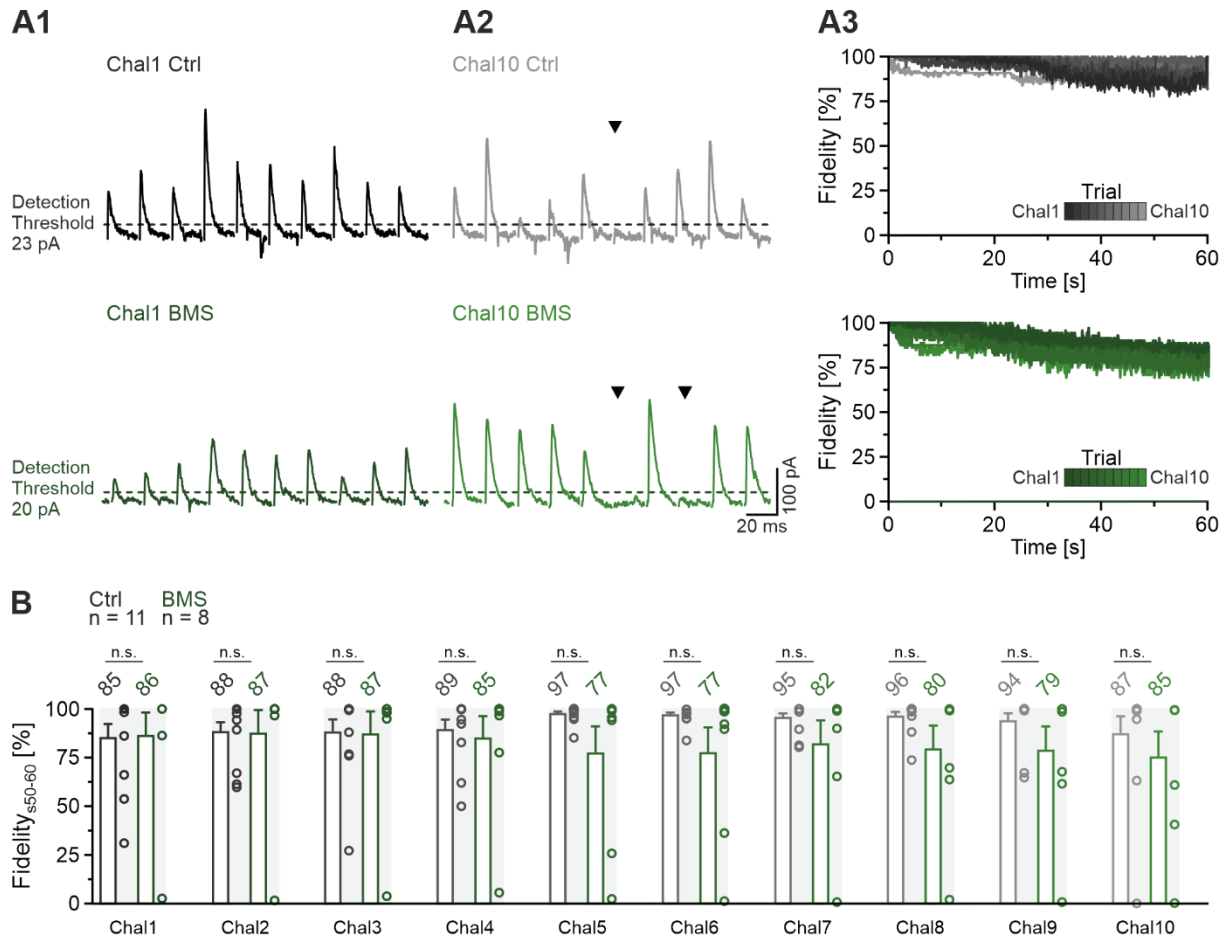


Figure 3. 25 Unchanged fidelity during marathon experiments under Asc-1 inhibition in WT. (A1) Ten representative eIPSCs during the 50 Hz Chal1 period for Ctrl (upper, black) and 50 μ M BMS (lower, dark green) of the last 10 s. Dashed lines show the detection threshold for each cell. Arrows mark a synaptic failure (see Material and Methods for details). Artifacts were blanked for clarity. (A2) same as A1 but for Chal10 for Ctrl (upper, gray) and BMS (lower, light green). (A3) Time course of mean fidelity of all 10 trials for Ctrl (upper) and BMS (lower). Trials 1-10 are color-coded from dark to light. Time courses are simple moving averages. (B) Statistics for Fidelity_{s50-60} of all 10 trials. Mean values on top of the bars. Values are shown as mean $\pm$ SEM. Ctrl, n = 11; BMS, n = 8. See Table 3.17 for details, including statistics.

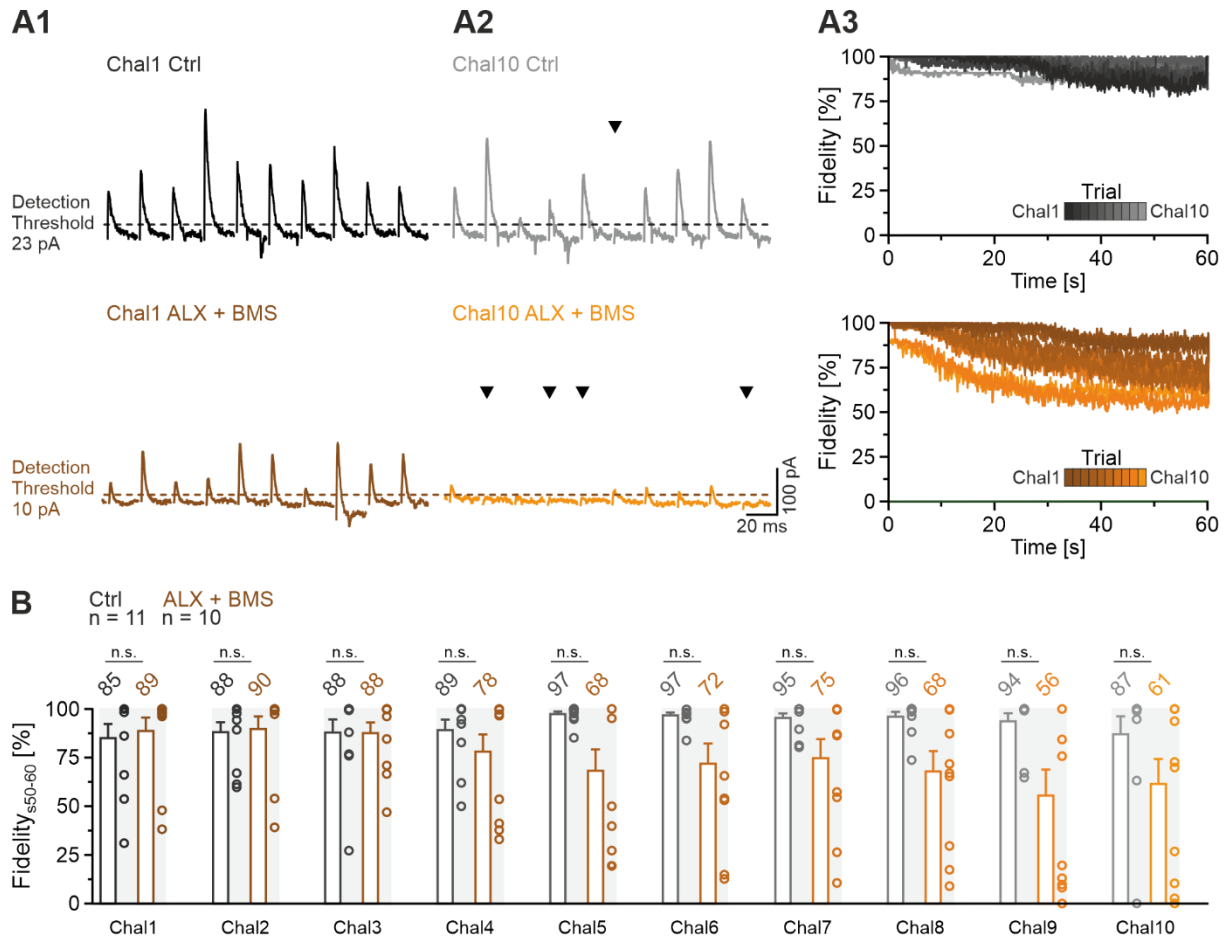


Figure 3.26 Unchanged fidelity during marathon experiments under combined GlyT2 and Asc-1 inhibition in WT. **(A1)** Ten representative eIPSCs during the 50 Hz Chal1 period for Ctrl (upper, black) and ALX + BMS (lower, brown) of the last 10 s. Dashed lines show the detection threshold for each cell. Arrows mark a synaptic failure (see Material and Methods for details). Artifacts were blanked for clarity. **(A2)** same as A1 but for Chal10 for Ctrl (upper, gray) and ALX + BMS (lower, orange). **(A3)** Time course of mean fidelity of all 10 trials for Ctrl (upper) and ALX + BMS (lower). Trials 1-10 are color-coded from dark to light. Time courses are simple moving averages. **(B)** Statistics for Fidelity_{s50-60} of all 10 trials. Mean values on top of the bars. Values are shown as mean $\pm$ SEM. Ctrl, n = 11; ALX + BMS, n = 10. See Table 3.17 for details, including statistics.

Table 3.17: eIPSC Fidelity_{s50-60} during marathon experiments for Ctrl, ALX, BMS, and ALX + BMS WT neurons.

		Ctrl n = 11			ALX n = 8			BMS n = 8			ALX + BMS n = 10			Ctrl vs. ALX	Ctrl vs. BMS	Ctrl vs. ALX + BMS
		Mean	SEM	Figure	Mean	SEM	Figure	Mean	SEM	Figure	Mean	SEM	Figure	p-value	p-value	p-value
Fidelity _{s50-60} [%]	Chal1	85	7	3.24B,	93	5	3.24B	86	12	3.25B	89	7	3.26B	>0.999	>0.999	>0.999
	Chal2	88	5	3.25B,	98	2		87	12		90	7		>0.999	>0.999	>0.999
	Chal3	88	7	3.26B	99	1		87	12		88	6		>0.999	>0.999	>0.999
	Chal4	89	5		100	1		85	12		78	9		>0.999	>0.999	>0.999
	Chal5	97	1		100	1		77	14		68	11		>0.999	>0.999	>0.999
	Chal6	97	1		96	3		77	13		72	10		>0.999	>0.999	>0.999
	Chal7	95	2		95	5		82	12		75	10		>0.999	>0.999	>0.999
	Chal8	96	2		90	10		79	12		68	10		>0.999	>0.999	0.419
	Chal9	94	4		80	13		79	12		56	13		>0.999	>0.999	0.099
	Chal10	87	9		81	12		75	14		61	13		>0.999	>0.999	0.601

Data are presented as mean $\pm$ SEM. Kruskal-Wallis test with Dunn's correction. P-values are adjusted values after correction.

3.3.2 Pharmacological blockade of GlyT2 or Asc-1 alone did not affect spontaneous release, but combined inhibition reduced q

Reduced eIPSC amplitudes suggest presynaptic glycine depletion and possibly fewer or empty SVs, which should be reflected by a decrease in q or sIPSC frequency. These parameters were analyzed during BL and all recovery periods (Recov1-Recov10). Representative sIPSCs current traces are shown for BL Ctrl (black, upper) and BL ALX (dark blue, lower), with events marked by arrows (Figure 3.27A1). A histogram of sIPSC peak amplitudes was plotted, and q was determined using a Gaussian fit for each condition (Figure 3.27A2). The same analysis was performed for Recov10 Ctrl (gray) and Recov10 ALX (light blue; Figure 3.27A3-A4). Across all ten trials, q remained stable (Ctrl: ~19 pA, ALX: ~19 pA), with no significant differences between ALX and Ctrl (Figure 3.27B; Table 3.18). sIPSC frequency also showed no change throughout all ten trials (Ctrl: ~8 Hz, ALX: ~12 Hz) and did not differ significantly between conditions (Figure 3.27C; Table 3.18).

Similar results were observed for BMS. Representative sIPSC traces and the corresponding histograms for BL BMS in dark green and Recov10 in light green showed similar distribution and amplitudes of sIPSCs (Figure 3.28A1-A4). q values remained stable across all ten trials at ~18 pA and did not differ significantly from Ctrl (Figure 3.28B; Table 3.18). sIPSC values were also stable at ~6 Hz throughout all ten trials and showed no significant difference compared to Ctrl (Figure 3.28C; Table 3.18).

Differences were found in the ALX + BMS group. Representative sIPSC traces and the corresponding histogram during BL ALX + BMS appeared the same as Ctrl (Figure 3.29A1-A2). In Recov10, event amplitudes decreased, and the corresponding histogram showed a peak at 9 pA (Figure 3.29A3-A4). During the first recovery periods (Recov1-Recov3), q values were ~15 pA. From Recov4 onward, q decreased to ~13 pA and was significantly different from Ctrl (Figure 3.29B; Table 3.18). As seen with ALX or BMS alone, sIPSC frequency remained stable at ~7 Hz and did not differ significantly from Ctrl (Figure 3.29C; Table 3.18). Compared to all conditions together, q values at trial 10 are 19 pA (Ctrl), 18 pA (ALX), 18 pA (BMS), 14 pA (ALX + BMS), corresponding to 100, 95, 95, and 74%.

Taken together, q remained unchanged with ALX or BMS but decreased with ALX + BMS. This suggests that glycine loading of SVs in MNTB terminals is maintained when either GlyT2 or Asc-1 is active but becomes impaired when both are inhibited in WT mice. sIPSC frequency stayed constant across all conditions, indicating that SVs are still released at the same rate, even if not fully filled.

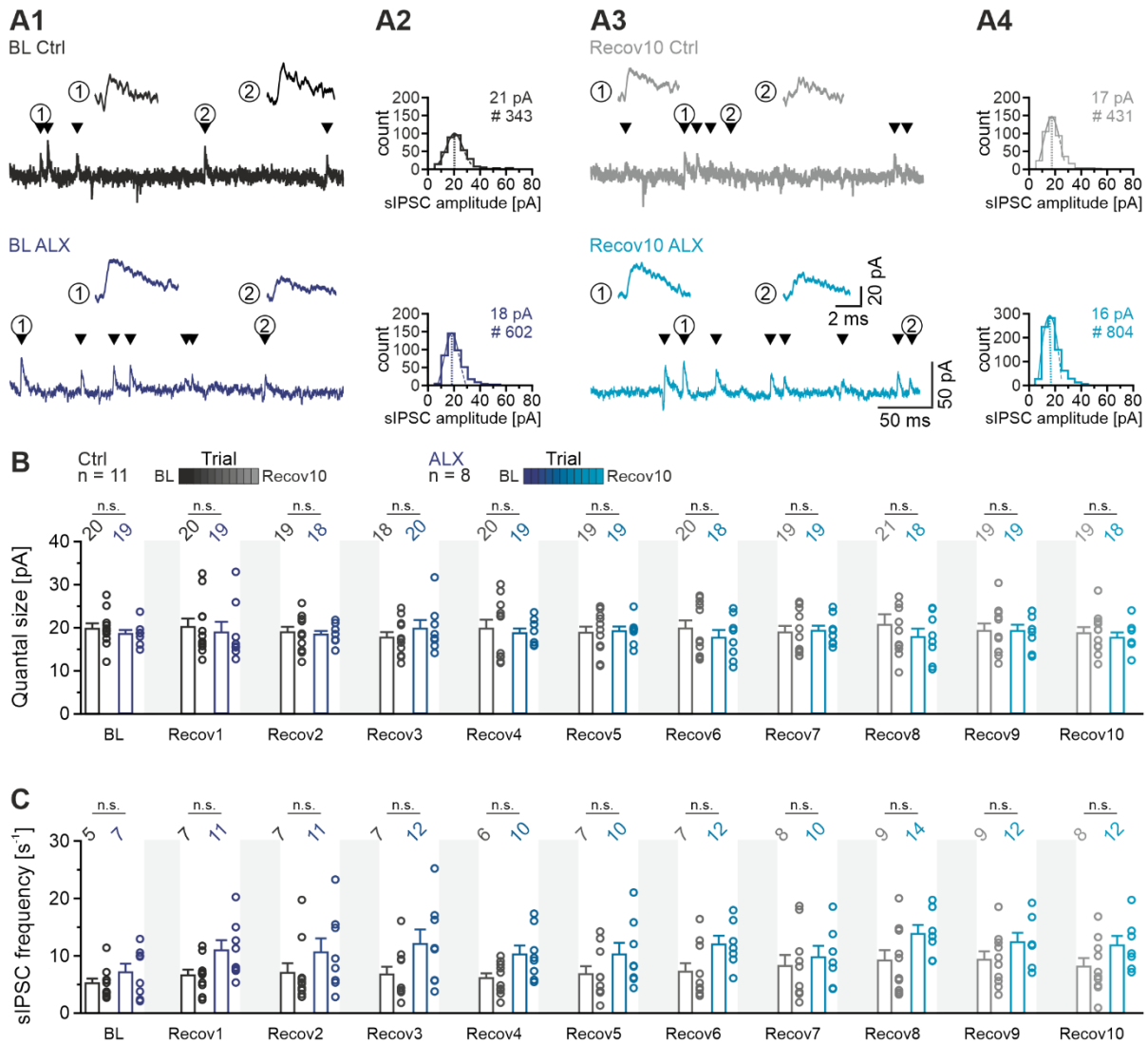


Figure 3.27 Unaffected spontaneous release under GlyT2 inhibition in WT. (A) Analysis to determine q . **(A1)** Original current traces of sIPSCs from a representative neuron for Ctrl (upper, black) and ALX (lower, dark blue) during BL. sIPSC amplitudes are highlighted with an arrow. Numbers over arrows mark two example sIPSCs, which are shown zoomed over traces. **(A2)** Estimation of q . Corresponding distribution histograms of sIPSC amplitudes to current traces in A1 for Ctrl (upper, black) and ALX (lower, dark blue) during BL. A Gaussian curve was fitted from 0 pA to the bin after the first local maximum (dotted curves; Krächan et al., 2017). Numbers in panels represent q and the number of sIPSCs (for details, see Material and Methods). **(A3, A4)** same as A1, A2, but during Recov10 for Ctrl (upper, gray) and ALX (lower, light blue). **(B)** Statistics for q during BL-Recov10. Mean values above bars. Gray areas display challenge periods. **(C)** Same as B, but for sIPSC frequency. Values are shown as mean $\pm$ SEM. Ctrl, $n = 11$; ALX, $n = 8$. See Table 3.18 for details, including statistics.

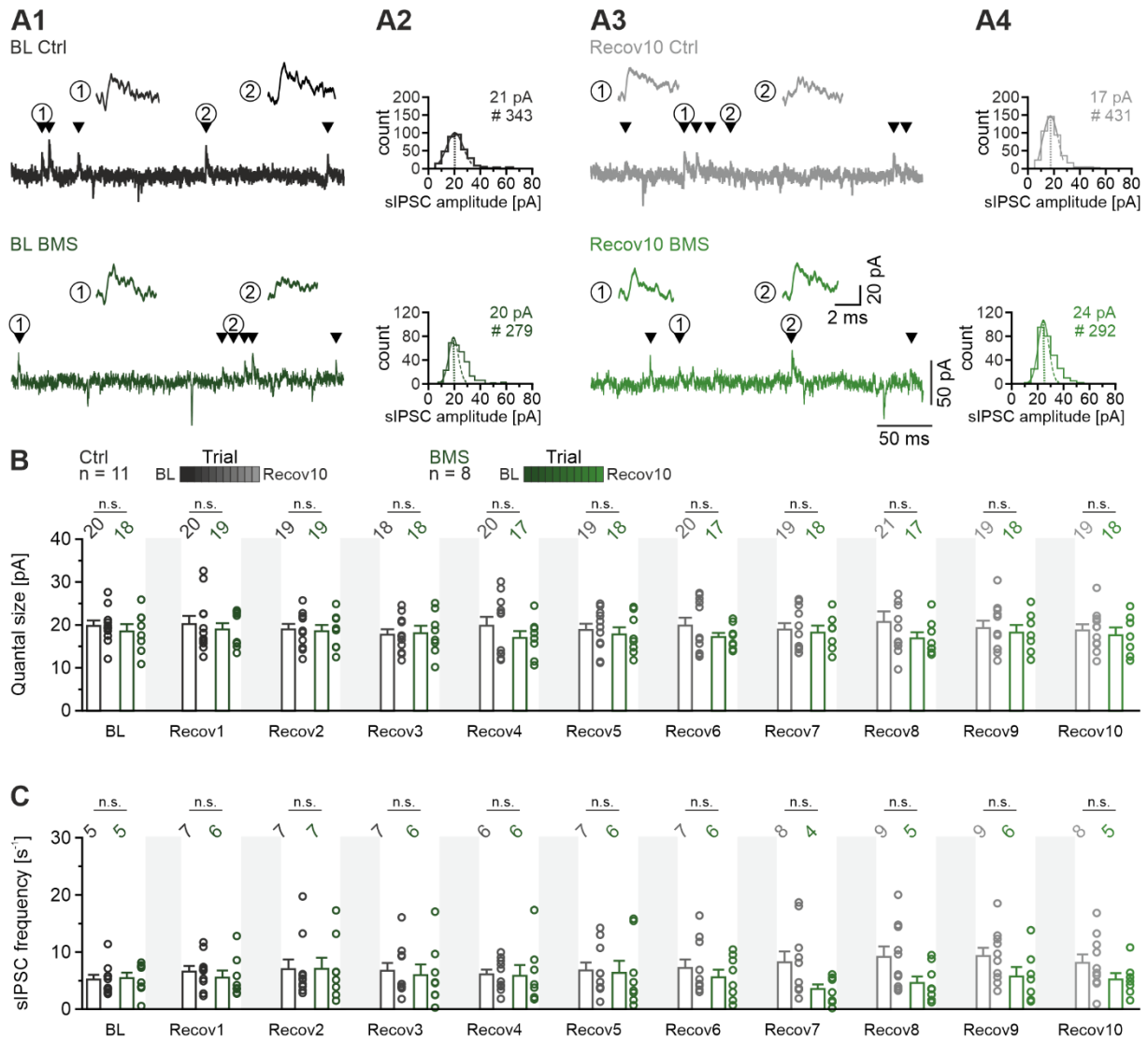


Figure 3.28 Unaffected spontaneous release Asc-1 inhibition in WT. (A) Analysis to determine q . **(A1)** Original current traces of sIPSCs from a representative neuron for Ctrl (upper, black) and BMS (lower, dark green) during BL. sIPSC amplitudes are highlighted with an arrow. Numbers over arrows mark two example sIPSCs, which are shown zoomed over traces. **(A2)** Estimation of q . Corresponding distribution histograms of sIPSC amplitudes to current traces in A1 for Ctrl (upper, black) and BMS (lower, dark green) during BL. A Gaussian curve was fitted from 0 pA to the bin after the first local maximum (dotted curves; Krächan et al., 2017). Numbers in panels represent q and the number of sIPSCs (for details, see Material and Methods). **(A3, A4)** same as A1, A2, but during Recov10 for Ctrl (upper, gray) and BMS (lower, light green). **(B)** Statistics for q during BL-Recov10. Mean values above bars. Gray areas display challenge periods. **(C)** Same as B, but for sIPSC frequency. Values are shown as mean $\pm$ SEM. Ctrl, $n = 11$; BMS, $n = 8$. See Table 3.18 for details, including statistics.

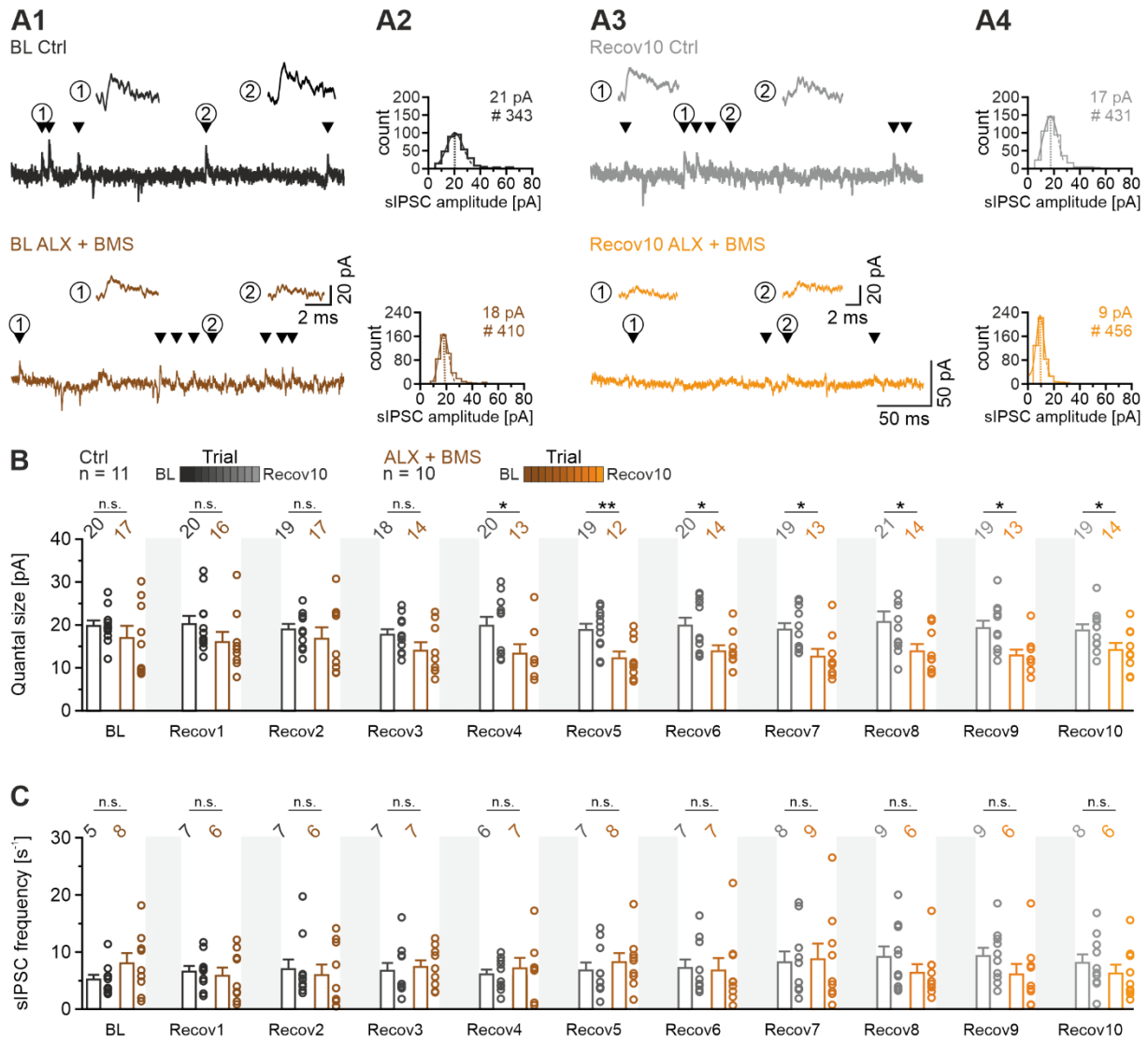


Figure 3.29 Reduced q under combined GlyT2 and Asc-1 inhibition in WT. (A) Analysis to determine q . **(A1)** Original current traces of sIPSCs from a representative neuron for Ctrl (upper, black) and ALX + BMS (lower, brown) during BL. sIPSC amplitudes are highlighted with an arrow. Numbers over arrows mark two example sIPSCs, which are shown zoomed over traces. **(A2)** Estimation of q . Corresponding distribution histograms of sIPSC amplitudes to current traces in A1 for Ctrl (upper, black) and ALX + BMS (lower, brown) during BL. A Gaussian curve was fitted from 0 pA to the bin after the first local maximum (dotted curves; Krächan et al., 2017). Numbers in panels represent q and the number of sIPSCs (for details, see Material and Methods). **(A3, A4)** same as A1, A2, but during Recov10 for Ctrl (upper, gray) and ALX + BMS (lower, orange). **(B)** Statistics for q during BL-Recov10. Mean values above bars. Gray areas display challenge periods. **(C)** Same as B, but for sIPSC frequency. Values are shown as mean $\pm$ SEM. Ctrl, $n = 11$; ALX + BMS, $n = 10$. See Table 3.18 for details, including statistics.

Table 3.18: sIPSC properties during marathon experiments for Ctrl, ALX, BMS, and ALX + BMS WT neurons.

		Ctrl n = 11			ALX n = 8			BMS n = 8			ALX + BMS n = 10			Ctrl vs. ALX	Ctrl vs. BMS	Ctrl vs. ALX + BMS
		Mean	SEM	Figure	Mean	SEM	Figure	Mean	SEM	Figure	Mean	SEM	Figure	p-value	p-value	p-value
q [pA]	BL	20	1	3.27B,	19	1	3.27B	18	2	3.28B	17	3	3.29B	>0.999	>0.999	>0.999
	Recov1	20	2	3.28B,	19	2		19	2		16	2		>0.999	>0.999	0.110
	Recov2	19	1	3.29B	18	1		19	1		17	3		>0.999	>0.999	0.565
	Recov3	18	1		20	2		18	2		14	2		>0.999	>0.999	0.322
	Recov4	20	2		19	1		17	2		13	2		>0.999	>0.999	0.048
	Recov5	19	1		19	1		18	2		12	2		>0.999	>0.999	0.006
	Recov6	20	2		18	2		17	1		14	1		0.989	0.706	0.027
	Recov7	19	2		19	1		18	2		13	2		>0.999	>0.999	0.015
	Recov8	21	2		18	2		17	1		14	2		0.923	0.548	0.049
	Recov9	19	2		19	1		18	2		13	1		>0.999	>0.999	0.016
	Recov10	19	1		18	1		18	2		14	1		>0.999	>0.999	0.048
sIPSC frequency [Hz]	BL	5	1	3.27C,	7	1	3.27C	5	1	3.28C	8	2	3.29C	0.916	>0.999	0.357
	Recov1	7	1	3.28C,	11	2		6	1		6	1		0.083	>0.999	>0.999
	Recov2	7	1	3.29C	11	2		7	2		6	2		0.349	>0.999	>0.999
	Recov3	7	1		12	3		6	2		7	1		0.156	>0.999	>0.999
	Recov4	6	1		10	2		6	2		7	2		0.173	>0.999	>0.999
	Recov5	7	1		10	2		6	2		8	2		0.510	>0.999	>0.999
	Recov6	7	1		12	2		6	1		7	2		0.224	>0.999	>0.999
	Recov7	8	2		10	2		4	1		9	3		>0.999	0.319	>0.999
	Recov8	9	2		14	2		5	1		6	2		0.800	0.060	0.242
	Recov9	9	1		12	2		6	2		6	2		0.976	0.239	0.261
	Recov10	8	2		12	2		5	1		6	1		0.610	0.278	0.662

Data are presented as mean $\pm$ SEM. q: Kruskal-Wallis test with Dunn's correction. sIPSC frequency: One-way ANOVA with Bonferroni correction. P-values are adjusted values after correction. Significant P-values are marked in green.

3.3.3 Altered synaptic release properties by combined inhibition of GlyT2 and Asc-1, but not by GlyT2 or Asc-1 inhibition alone

To assess the effects of GlyT2 and/or Asc-1 blockade on the synaptic strength of MNTB-LSO synapses, I_{RRP} , m_{s50-60} , and P_v were analyzed (Figure 3.30-3.32; Table 3.19). In the Ctrl group, all parameters remained stable across ten trials: I_{RRP} (~12 nA), m_{s50-60} (~12 SVs), and P_v (~11%). GlyT2 inhibition with ALX did not significantly affect the synaptic strength. The I_{RRP} , estimated by the method of [Elmqvist & Quastel \(1965\)](#); Figure 3.30-3.32A1-A3), remained stable at ~14 nA (Figure 3.30B). Similarly, m_{s50-60} was ~12 SVs, and P_v was ~9% over all ten trials (Figure 3.30C-D; Table 3.19).

Similar results were observed with Asc-1 inhibition by BMS. I_{RRP} (~10 nA), m_{s50-60} (~7 SVs), and P_v (~8%), were stable throughout all ten trials. During challenge periods, m_{s50-60} showed a trend toward reduction compared to Ctrl, but none of the analyzed parameters were significantly different from Ctrl (Figure 3.31B-D; Table 3.19).

The combination of GlyT2 and Asc-1 inhibition by ALX + BMS had effects on the analyzed parameters. I_{RRP} continued to decline with each trial, starting at 10 nA in Chal1 and reaching 3 nA in Chal10. However, there was only a significant difference from Ctrl at the end of the stimulation, beginning with Chal 8 onward (Figure 3.32B; Table 3.19). Similar results were observed for m_{s50-60} . From Chal1-Chal7, ~5 SVs were released per event. From Chal8 to Chal10, release dropped to ~4 SVs and was significantly different from Ctrl (Figure 3.32C; Table 3.19). Additionally, P_v increased by ~15% across all 10 trials. However, only three out of 10 comparisons (Chal2, Chal9, and Chal10) were significantly different from Ctrl (Figure 3.32D; Table 3.19).

Taking a closer look at trial 10, it was found that I_{RRP} decreased to 90% in ALX and BMS and to 30% in ALX + BMS, compared to Ctrl. Similar results were observed for m_{s50-60} for trial 10 with Ctrl, ALX, BMS, and ALX + BMS at 100, 75, 67, and 33%, respectively. For P_v in trial 10, the values were 10% (Ctrl), 10% (ALX), 7% (BMS), and 19% (ALX + BMS). For ALX + BMS, the value increased to nearly double that of the other conditions.

Taken together, only a combination of GlyT2 and Asc-1 inhibition significantly affected synaptic strength, and only at the end of the stimulation. This suggests that MNTB-LSO synapses can sustain glycinergic synaptic transmission even under (re)uptake blockade, likely due to sufficient initial glycine availability and vesicle reserves.

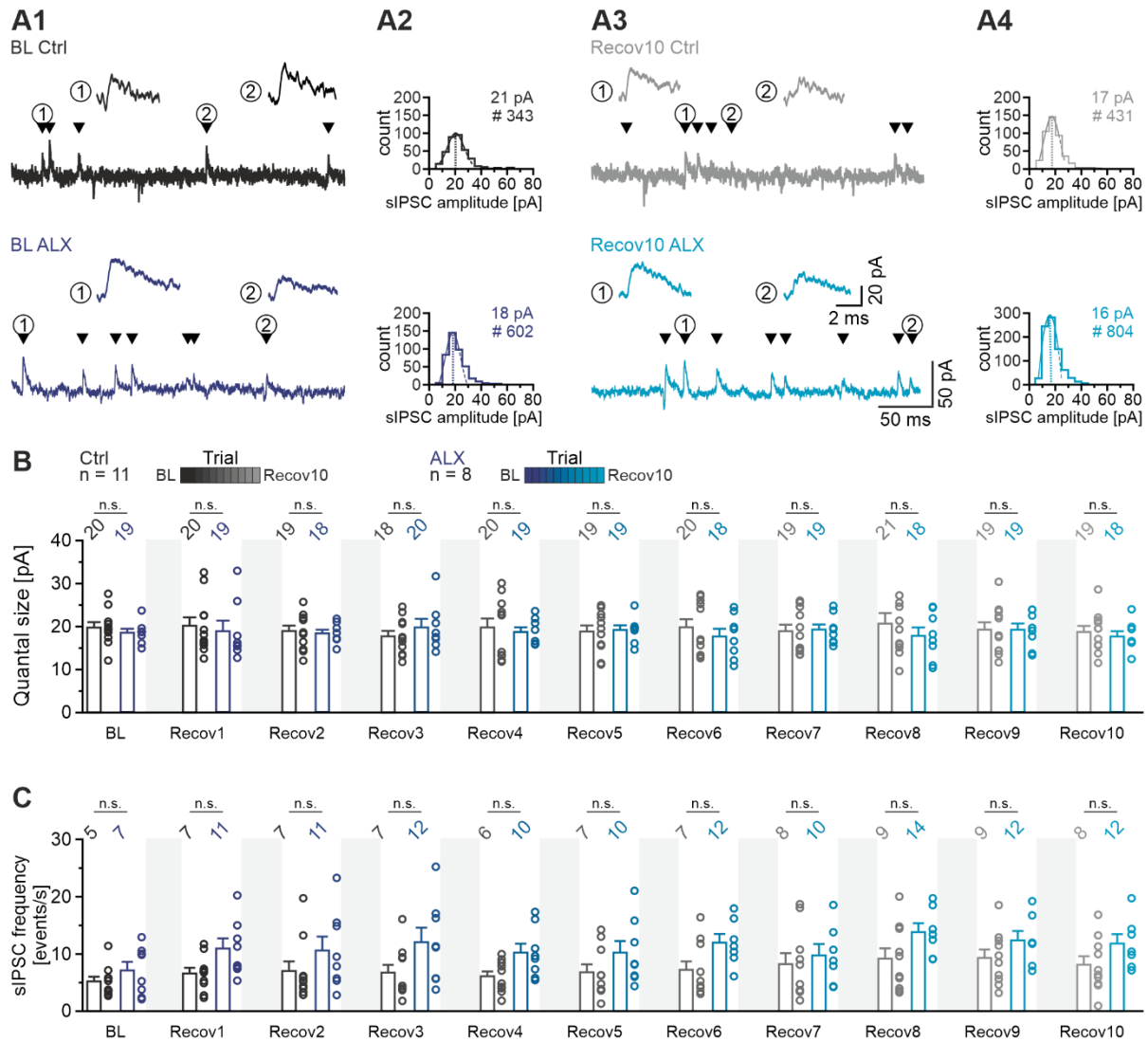


Figure 3. 30 Unchanged synaptic parameters under GlyT2 inhibition in WT. (A1) Close-up of first 20 eIPSC amplitudes during 50 Hz Chal1 period for Ctrl (black) and ALX (dark blue). **(A2)** same as A1 but for Chal10 for Ctrl (gray) and ALX (light blue). Artifacts were blanked for clarity. **(A3)** Corresponding Elmqvist & Quastel plots used to determine I_{RRP} . Linear regression was applied to the first five eIPSCs (darker shade in A). See Materials and Methods for details. **(B)** Statistics for I_{RRP} **(C)**, m_{s50-60} **(D)**, and P_v of all 10 trials. Mean values on top of the bars. Values are shown as mean $\pm$ SEM. Ctrl, $n = 11$; ALX, $n = 8$. See Table 3.19 for details, including statistics.

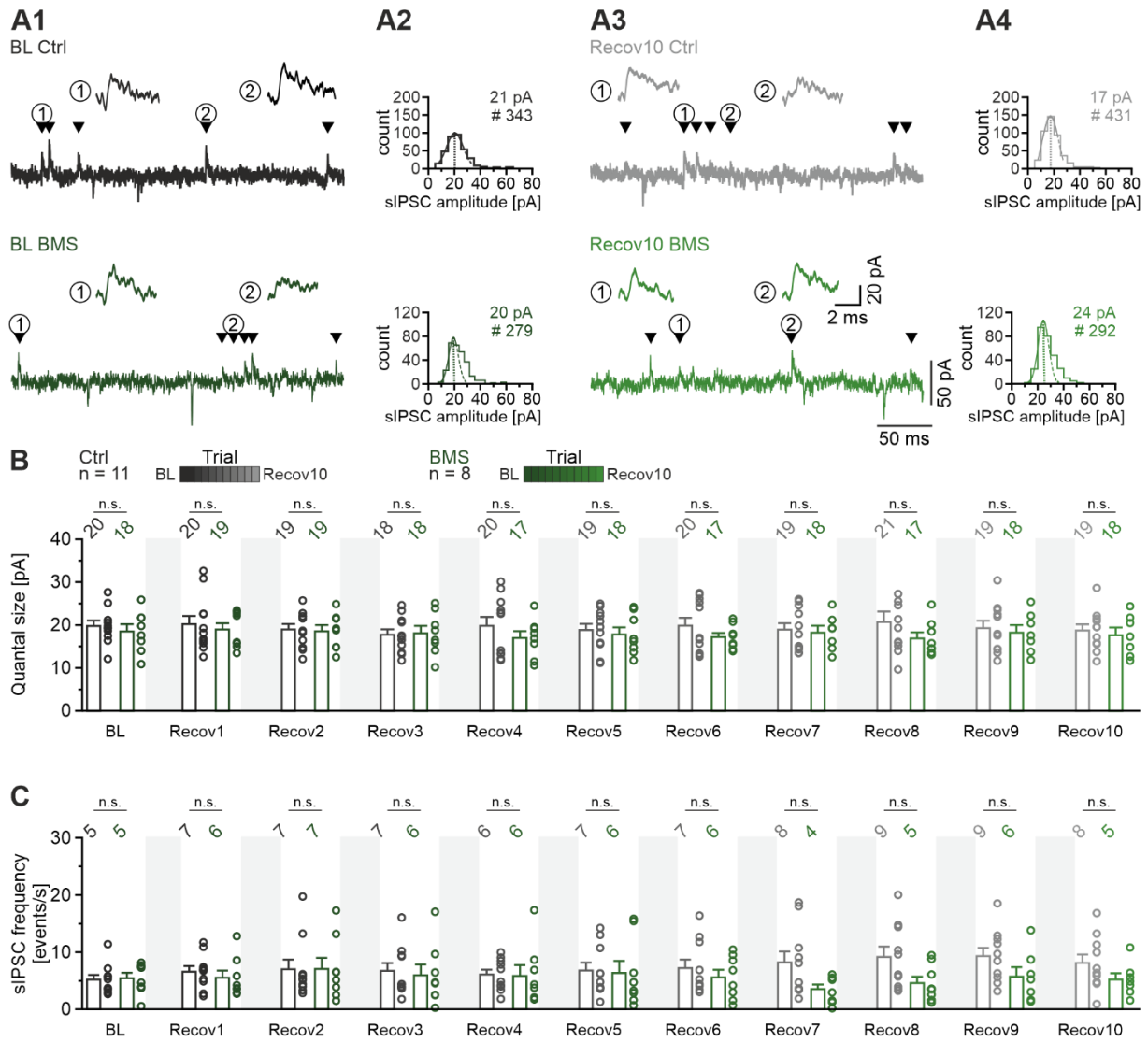


Figure 3. 31 Unchanged synaptic parameters under Asc-1 inhibition in WT. (A1) Close-up of first 20 eIPSC amplitudes during 50 Hz Chal1 period for Ctrl (black) and BMS (dark green). **(A2)** same as A1 but for Chal10 for Ctrl (gray) and BMS (light green). Artifacts were blanked for clarity. **(A3)** Corresponding Elmqvist & Quastel plots used to determine I_{RRP} . Linear regression was applied to the first five eIPSCs (darker shade in A). See Materials and Methods for details. **(B)** Statistics for I_{RRP} **(C)**, m_{s50-60} **(D)**, and P_v of all 10 trials. Mean values on top of the bars. Values are shown as mean $\pm$ SEM. Ctrl, n = 11; ALX, n = 8. See Table 3.19 for details, including statistics.

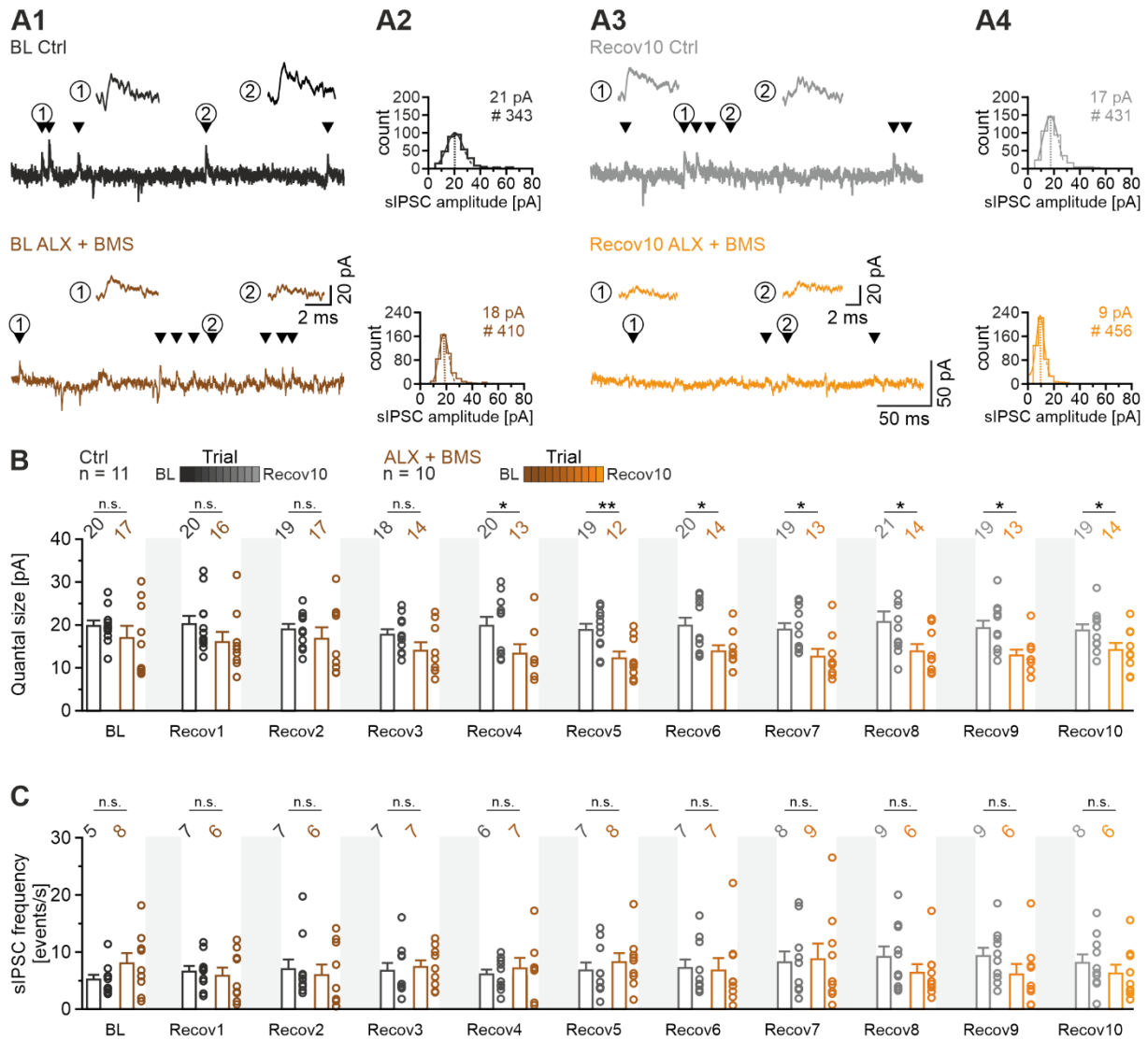


Figure 3.32 Altered synaptic parameters under combined GlyT2 and Asc-1 inhibition in WT. (A1) Close-up of first 20 eIPSC amplitudes during 50 Hz Chal1 period for Ctrl (black) and ALX + BMS (brown). (A2) same as A1 but for Chal10 for Ctrl (gray) and ALX + BMS (orange). Artifacts were blanked for clarity. (A3) Corresponding Elmqvist & Quastel plots used to determine I_{RRP} . Linear regression was applied to the first five eIPSCs (darker shade in A). See Materials and Methods for details. (B) Statistics for I_{RRP} (C), m_{50-60} (D), and P_v of all 10 trials. Mean values on top of the bars. Values are shown as mean $\pm$ SEM. Ctrl, $n = 11$; ALX + BMS, $n = 8$. See Table 3.19 for details, including statistics.

Table 3.19: I_{RRP} , m_{s50-60} , and P_v during marathon experiments for Ctrl, ALX, BMS, and ALX + BMS WT neurons.

		Ctrl n = 11		ALX n = 8			BMS n = 8			ALX + BMS n = 10			Ctrl vs. ALX	Ctrl vs. BMS	Ctrl vs. ALX + BMS
		Mean	SEM	Mean	SEM	Figure	Mean	SEM	Figure	Mean	SEM	Figure	p-value	p-value	p-value
I_{RRP} [nA]	Chal1	11	3	17	4	3.30B, 3.31B, 3.32B	11	3	3.31B	10	4	3.32B	0.804	>0.999	>0.999
	Chal2	13	3	14	4		11	3		6	2		>0.999	>0.999	0.128
	Chal3	11	3	19	5		16	5		6	2		0.481	>0.999	>0.999
	Chal4	14	5	14	3		11	4		5	2		>0.999	>0.999	0.195
	Chal5	11	2	16	4		11	4		4	1		0.683	>0.999	0.342
	Chal6	14	4	14	3		6	2		5	2		>0.999	0.162	0.059
	Chal7	10	2	13	3		8	2		5	1		>0.999	>0.999	0.186
	Chal8	12	2	13	4		9	3		3	1		>0.999	>0.999	0.011
	Chal9	13	3	10	2		8	1		4	1		>0.999	>0.999	0.018
	Chal10	10	2	9	2		9	2		3	1		>0.999	>0.999	0.012
m_{s50-60} [SV]	Chal1	11	3	12	4	3.30C, 3.31C, 3.32C	10	3	3.31C	6	1	3.32C	>0.999	>0.999	0.591
	Chal2	13	4	15	4		9	2		5	1		>0.999	0.939	0.246
	Chal3	14	4	14	4		8	2		6	2		>0.999	0.568	0.177
	Chal4	12	2	15	4		7	2		6	2		>0.999	0.684	0.426
	Chal5	11	3	14	3		6	2		5	2		>0.999	0.382	0.204
	Chal6	12	3	14	4		6	2		5	2		>0.999	0.298	0.052
	Chal7	13	3	10	2		6	2		5	2		>0.999	0.393	0.057
	Chal8	12	2	10	2		6	1		4	1		>0.999	0.088	0.016
	Chal9	13	3	8	2		6	2		4	2		0.394	0.215	0.028
	Chal10	12	3	9	3		8	2		4	1		0.963	0.722	0.039
P_v [%]	Chal1	9	2	7	1	3.30D, 3.31D, 3.32D	8	0.3	3.31D	13	3	3.32D	>0.999	>0.999	>0.999
	Chal2	9	1	9	1		10	1		16	3		>0.999	>0.999	0.031
	Chal3	14	2	8	1		7	1		15	3		0.163	0.084	>0.999
	Chal4	10	2	8	1		10	2		15	2		>0.999	>0.999	0.183
	Chal5	10	1	9	2		8	1		13	1		>0.999	>0.999	0.371
	Chal6	11	2	9	1		9	2		14	3		>0.999	>0.999	0.669
	Chal7	14	2	9	1		8	1		14	3		0.267	0.066	>0.999
	Chal8	12	2	9	2		8	1		16	3		>0.999	0.510	0.364
	Chal9	10	1	11	2		8	1		18	3		>0.999	>0.999	0.017
	Chal10	10	1	10	1		7	1		19	4		>0.999	0.973	0.015

Data are presented as mean $\pm$ SEM. Kruskal-Wallis test with Dunn's correction. P-values are adjusted values after correction. Significant P-values are marked in green.

3.3.4 Reduced vesicle replenishment by combined inhibition of GlyT2 and Asc-1, but not by GlyT2 or Asc-1 inhibition alone

q remained unchanged during GlyT2 or Asc-1 inhibition but decreased with combined GlyT2 and Asc-1 inhibition within marathon experiments. Suggesting that glycine loading of SVs in MNTB axon terminals is not abolished if either GlyT2 or Asc-1 is still functional. To investigate the impact of GlyT2 and/or Asc-1 mediated glycine (re)uptake on glycinergic transmission at MNTB-LSO synapses, the cumulative SVs and RR were analyzed during marathon experiments (Figure 3.33-3.35).

The Ctrl condition showed consistent slopes throughout all ten trials. Cumulative vesicle release at s_{60} did not differ significantly when tested against Chal1, with each trial releasing ~45,106 SV (Figure 3.33A1; Table 3.20). For ALX, the slopes remained similar, but the last three seemed reduced. Cumulative vesicle release at s_{60} with each trial was ~51,007 SV for Chal1-Chal7, followed by 41,740 SV (Chal8), 34,968 SV (Chal9), and 36,720 SV (Chal10). Despite this trend, cumulative vesicle release at s_{60} did not differ significantly across trials. For BMS, the slopes were consistent across all ten trials but were overall lower compared to Ctrl (Figure 3.34A2; Table 3.20). For ALX + BMS, slopes were further reduced compared to Ctrl (Figure 3.35A2; Table 3.20). In total, Ctrl synapses released 451,062 SVs, whereas ALX, BMS, and ALX + BMS values were 470,479, 300,561, and 267,734 SVs, respectively (corresponding to 100, 104, 67, 59%).

Next, the RR_{s50-60} during challenge periods was analyzed to quantify transmission at the last 10 s. Values remained stable within the ten trials for each condition ~728 SVs for Ctrl, ~752 SVs for ALX, ~484 SVs for BMS, and ~303 SVs for ALX + BMS. Neither the ALX nor the BMS group showed any differences compared to Ctrl (Figure 3.33B; Figure 3.34B). For the ALX + BMS group, there were no significant differences compared to the Ctrl during Chal1-Chal6 (~728 vs. ~348 SV). However, from Chal7 onward, there was a significant difference (806 vs. 327 (Chal7), 726 vs. 201 (Chal8), 819 vs. 183 (Chal9), and 686 vs. 228 SV (Chal10); Figure 3.35B). At the very end of the marathon experiments (Trial 10), the RR_{s50-60} values for Ctrl, ALX, BMS, and ALX + BMS are 686, 588, 468, and 228 SV, respectively (corresponding to 100, 86, 68, and 33%).

In summary, vesicle replenishment is reduced in the late phases of marathon experiments when both GlyT2 and Asc-1 are inhibited, but not when either transporter is blocked alone. This suggests that these transporters act in parallel to maintain glycine supply for sustained synaptic transmission.

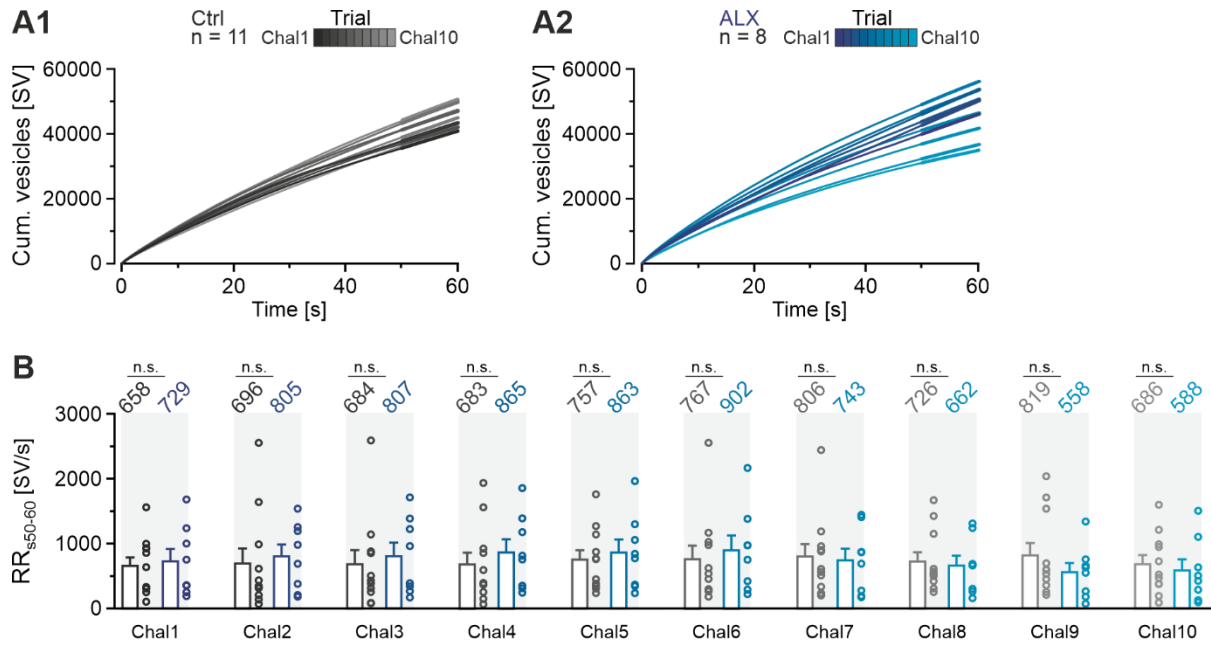


Figure 3.33 Unchanged released SVs under GlyT2 inhibition in WT. (A1) Time course of mean cumulative released synaptic vesicles for WT Ctrl (black), all ten trials. Thicker lines at s₅₀₋₆₀ marks period in which RR was quantified. Trials are color-coded from dark to light. **(A2)** Same as A1 but for WT ALX (blue). **(B)** Statistics for RR at s₅₀₋₆₀ of challenge periods (Chal1-Chal10). Mean values above bars. Values are shown as mean ± SEM. WT Ctrl, n = 11 and WT ALX, n = 8. See Table 3.20 for details, including statistics.

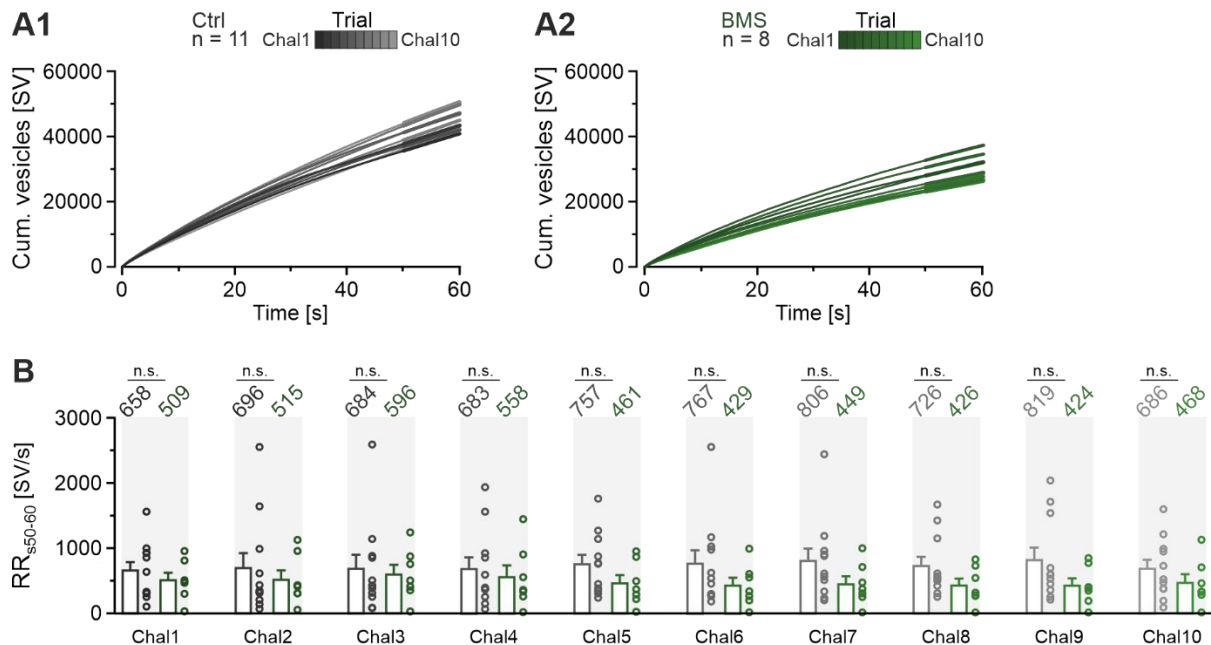


Figure 3.34 Unchanged released SVs under Asc-1 inhibition in WT. (A1) Time course of mean cumulative released synaptic vesicles for WT Ctrl (black), all ten trials. Thicker lines at s₅₀₋₆₀ marks period in which RR was quantified. Trials are color-coded from dark to light. **(A2)** Same as A1 but for WT BMS (green). **(B)** Statistics for RR at s₅₀₋₆₀ of challenge periods (Chal1-Chal10). Mean values above bars. Values are shown as mean ± SEM. WT Ctrl, n = 11 and WT BMS, n = 8. See Table 3.20 for details, including statistics.

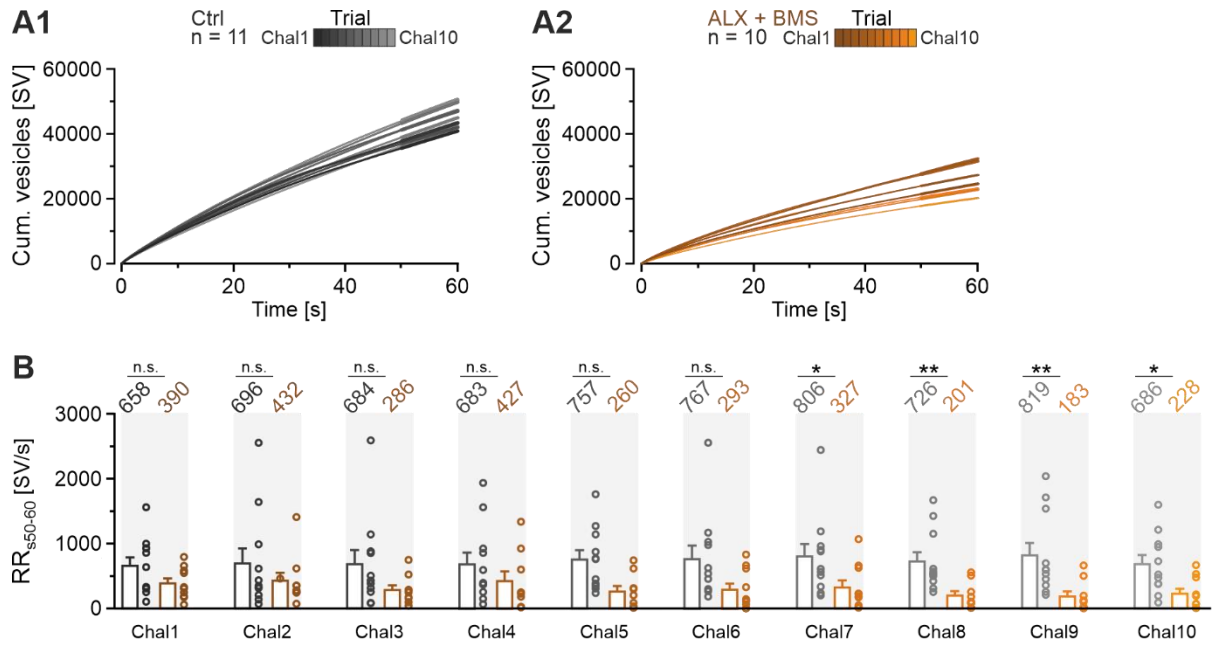


Figure 3.35 Reduced released SVs under combined GlyT2 and Asc-1 inhibition in WT. **(A1)** Time course of mean cumulative released synaptic vesicles for WT Ctrl (black), all ten trials. Thicker lines at s₅₀₋₆₀ marks period in which RR was quantified. Trials are color-coded from dark to light. **(A2)** Same as A1 but for WT ALX + BMS (brown). **(B)** Statistics for RR at s₅₀₋₆₀ of challenge periods (Chal1-Chal10). Mean values above bars. Values are shown as mean ± SEM. WT Ctrl, n = 11 and WT ALX + BMS, n = 10. See Table 3.20 for details, including statistics.

Table 3.20: Cumulative vesicles_{s60} and RR_{s50-60} during marathon experiments for Ctrl, ALX, BMS, and ALX + BMS WT neurons.

		Ctrl n = 11			ALX n = 8			BMS n = 8			ALX + BMS n = 10					
		Mean	SEM	p-value	Mean	SEM	p-value	Mean	SEM	p-value	Mean	SEM	p-value			
Cum. vesicles _{s60} [SV]	Chal1	40850	7891		46090	12062		31966	6998		24771	4749				
	Chal2	43451	14014	>0.999	50244	11231	>0.999	32239	8994	>0.999	27381	7323	>0.999			
	Chal3	43277	13286	>0.999	50737	12833	>0.999	37288	9183	>0.999	31926	10354	>0.999			
	Chal4	41971	10534	>0.999	53757	12565	0.359	34571	10950	>0.999	33922	9317	>0.999			
	Chal5	46927	8777	>0.999	53558	12315	0.431	28973	7900	>0.999	31466	12044	>0.999			
	Chal6	47268	12551	>0.999	56198	14432	>0.999	26703	7430	>0.999	24513	8267	>0.999			
	Chal7	49725	11462	>0.999	46467	11333	>0.999	27665	7406	>0.999	27411	10138	>0.999			
	Chal8	44972	8630	>0.999	41740	9771	>0.999	26398	6563	>0.999	22805	8323	>0.999			
	Chal9	50423	11689	>0.999	34968	8670	>0.999	26184	6833	>0.999	23256	9510	0.584			
	Chal10	42198	8440	>0.999	36720	10549	>0.999	28574	8069	>0.999	20283	7974	0.071			
		Ctrl n = 11			ALX n = 8			BMS n = 8			ALX + BMS n = 10			Ctrl vs. ALX	Ctrl vs. BMS	Ctrl vs. ALX + BMS
		Mean	SEM	Figure	Mean	SEM	Figure	Mean	SEM	Figure	Mean	SEM	Figure	p-value	p-value	p-value
RR _{s50-60} [SV/s]	Chal1	658	132	3.27B	729	191	3.27B	509	116	3.27B	390	76	3.39B	>0.999	>0.999	>0.999
	Chal2	696	231		805	182		515	145		432	118		>0.999	>0.999	>0.999
	Chal3	684	216		807	207		596	149		286	74		>0.999	>0.999	0.324
	Chal4	683	180		865	203		558	180		427	149		>0.999	>0.999	0.445
	Chal5	757	145		863	205		461	129		260	89		>0.999	>0.999	0.050
	Chal6	767	206		902	229		429	122		293	97		>0.999	>0.999	0.072
	Chal7	806	190		743	180		449	122		327	110		>0.999	>0.999	0.045
	Chal8	726	142		662	152		426	109		201	68		>0.999	>0.999	0.008
	Chal9	819	192		558	142		424	113		183	80		>0.999	>0.999	0.007
	Chal10	686	139		588	174		468	135		228	78		>0.999	>0.999	0.040

Data are presented as mean ± SEM. Cum. vesicles_{s60}: Ctrl: Friedman test with Dunn's correction, ALX, BMS, and ALX + BMS: One-way ANOVA with Bonferroni correction. RR_{s50-60}: Kruskal-Wallis test with Dunn's correction. P-values are adjusted values after correction. Significant P-values are marked in green.

3.3.5 Residual synaptic transmission is glycinergic

Combined GlyT2 and Asc-1 inhibition affected multiple parameters, including eIPSC amplitudes during challenge and recovery periods, q , I_{RRP} , m_{s50-60} , P_v , and RR_{s50-60} . However, these effects appeared no earlier than the fourth trial. At this point, MNTB fibers had been stimulated for nine minutes, including 60-s BL and four times at 50-Hz | 60-s challenge, followed by 1-Hz | 60-s recovery, in total 12,300 stimuli before any differences emerged. Despite the observed reductions, synaptic transmission continued, with eIPSC amplitudes reaching 56% during Recov10. To confirm whether this residual transmission was glycinergic and not because of other inhibitory neurotransmitters e.g., GABA. Additional experiments were performed using a wash-in of 100 nM strychnine (Stry), a GlyR antagonist. MNTB fibers were stimulated using Protocol 4 (Figure 2.4C), which consisted of Protocol 3 (50-Hz | 60-s challenge, followed by 1 Hz | 60-s recovery repeated ten times) followed by 1-Hz | 15-min stimulation under 100 nM Stry wash-in (Figure 3.36A1-A2).

In this set of experiments, ALX + BMS treatment showed similar results to those observed with Protocol 3 (Figure 3.36A1 vs. Figure 3.23A). eIPSC amplitudes started at 745 pA during BL. During the ten challenge periods, the eIPSC amplitudes decreased from 27% to 7% in the steady-state phase, but each of the 10 trials recovered. At Recov10, eIPSC amplitudes recovered to 64% (477 pA) of the initial BL. Following Recov10, wash-in of Stry led to a continuous reduction in eIPSC amplitudes over 15 minutes. eIPSC amplitudes were 78% during the first 10 s of Stry, 35% at $s_{440-450}$, and 27% at $s_{890-900}$, corresponding to 581, 261, and 201 pA, respectively (Figure 3.36A2). Quantification showed no significant difference between Recov10 and s_{0-10} or between Recov10 and $s_{440-450}$, but a significant reduction was observed between Recov10 and $s_{890-900}$ (Figure 3.36B; Table 3.21). These results confirm a prominent glycine component in the residual synaptic transmission.

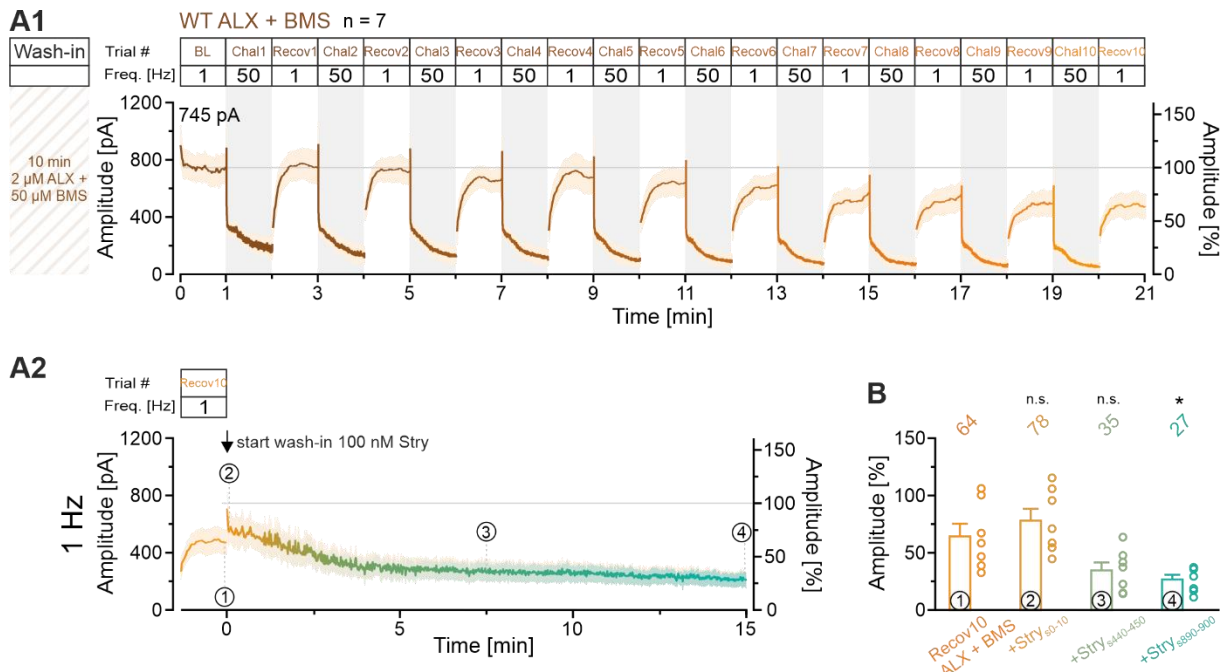


Figure 3.36 Further reduced synaptic transmission under GlyT2 and Asc-1 inhibition with 100 nM strychnine application in WT. (A1) Time course of absolute and normalized mean eIPSC peak amplitudes under ALX + BMS condition obtained with Protocol 4 (see Figure 2.4C). Trials are color-coded from brown to orange. The diagonally striped region indicates perfusion periods of ALX + BMS for 10 mins before recording. Horizontal lines show normalization (WT ALX + BMS BL = 100 %). Gray areas highlight the challenge periods. **(A2)** Same as A1, but for ALX + BMS + Stry condition (turquoise) during 1-Hz | 15-min recording period. For clarity, the preceding Recov10 is shown at the beginning. The arrow marks the start of strychnine (Stry) wash-in. Numbers in circles indicate time points corresponding to statistical data in B. **(B)** Statistics for normalized amplitudes analysis at different time points. Mean normalized values above bars. Values are shown as mean ± SEM. Time courses are simple moving averages (SEM lightly shaded). WT ALX + BMS and WT ALX + BMS + Stry, n = 7 same neurons. See Table 3.21 for details, including statistics.

Table 3.21: Normalized eIPSC amplitudes for marathon experiments + Stry application in WT.

Trial	ALX + BMS + Stry			Figure
	Mean [%] n = 7	SEM	p-value	
1 (Recov10 _{s50-60})	64	11		3.36B
2 (+ Stry _{s0-10})	78	10	0.057	
3 (+ Stry _{s440-450})	35	7	0.062	
4 (+ Stry _{s890-900})	27	4	0.043	

Data are presented as mean ± SEM. One-way ANOVA with Bonferroni correction. P-values are adjusted values after correction. Significant P-values are marked in green.

3.4 Contribution of V-ATPase and possible role of NHE on glycinergic transmission in MNTB-LSO synapses

To assess the contribution of V-ATPase to inhibitory synaptic transmission at MNTB-LSO synapses, synaptic responses in LSO neurons were recorded following MNTB fiber stimulation in P11±1 WT mice. The high-affinity V-ATPase inhibitor, Bafilomycin A1 (Bafi) was applied to brainstem slices. Bafi blocks proton (H⁺) transport through V-ATPase into newly endocytosed SVs, thereby preventing vesicle reacidification (Bowman *et al.*, 1988; Zhou *et al.*, 2000; Forgac, 2007). MNTB fibers were stimulated using Protocol 5 (Figure 2.4C), consisting of a 1-Hz | 60-s BL, followed by a 60-s challenge, and a 60-s recovery. Four stimulation frequencies were used: 10, 50, 100, and 200 Hz. The protocol lasted 9 minutes and included 21,900 stimuli. The first round of stimulation was recorded under ACSF (Ctrl). After the 200-Hz recovery period, Bafi was bath-applied for 10 min under 0.5-Hz stimulation. Then the protocol was repeated with continuous application of Bafi (Bafi10). After that new cell was patched, and after ≥30 min Bafi application, the protocol was applied again (Bafi30; Figure 3.37).

3.4.1 Sustained transmission is affected after long V-ATPase inhibition

eIPSC amplitudes of a representative neuron under Ctrl (black), Bafi10 (magenta), and Bafi30 (purple) are shown in Figure 3.37A1-A3. Under Ctrl conditions, BL eIPSC amplitudes were stable with a mean of 951 pA. During the 10-Hz challenge, peak amplitudes showed synaptic depression, decreasing to 629 pA, and recovered to 99% (942 pA) of the BL level. At 50 Hz, depression was stronger, with amplitudes reduced to 178 pA. Recovery exceeded BL, reaching 126% (1,199 pA). During the 100-Hz challenge, eIPSC amplitudes dropped to 63 pA, followed by a robust recovery to 108% of BL (1,023 pA). At 200 Hz, depression was most pronounced, with eIPSC amplitudes decreased to 13 pA, showing frequency-dependent depression is stronger with higher frequencies. But recovery after 200 Hz remained strong at 114% of BL (1,080 pA, Figure 3. (114% of BL; Figure 3.37A1).

During Bafi10, another BL was established with mean eIPSC amplitudes of 865 pA. Steady-state eIPSC amplitudes (s_{50-60}) during 10, 50, 100, and 200-Hz challenge periods were reduced to 550, 229, 49, and 12 pA, respectively. Despite this, recovery remained intact: 10 Hz returned to 100% (867 pA), 50 Hz to 113% (977 pA), 100 Hz to 104% (899 pA), and 200 Hz to 96% (827 pA; Figure 3.37A2). Another representative neuron showed a stronger effect on eIPSC amplitudes under Bafi30. After an additional 11 mins of Bafi wash-in, the third BL decreased to 300 pA, corresponding to 32% of the Ctrl BL. eIPSC amplitudes s_{50-60} during the challenge periods were 175 pA (10 Hz), 37 pA (50 Hz), 13 pA (100 Hz), and 3 pA (200 Hz). Despite the

collapse of synaptic transmission at 100 and 200 Hz, recovery remained partially intact under Bafi30. eIPSC amplitudes recovered to 30% (281 pA) at 10 Hz, 25% (236 pA) at 50 Hz, 22% (206 pA) at 100 Hz, and 23% (221 pA) at 200 Hz (Figure 3.37A3). Representative eIPSC amplitudes for the three conditions during Chal 3 and Recov 3 (100 Hz), showing eIPSC₁ and the overlay of individual eIPSCs and graphical means for eIPSC_{s50-60} and eIPSC_{s110-120} (Figure 3.37A4).

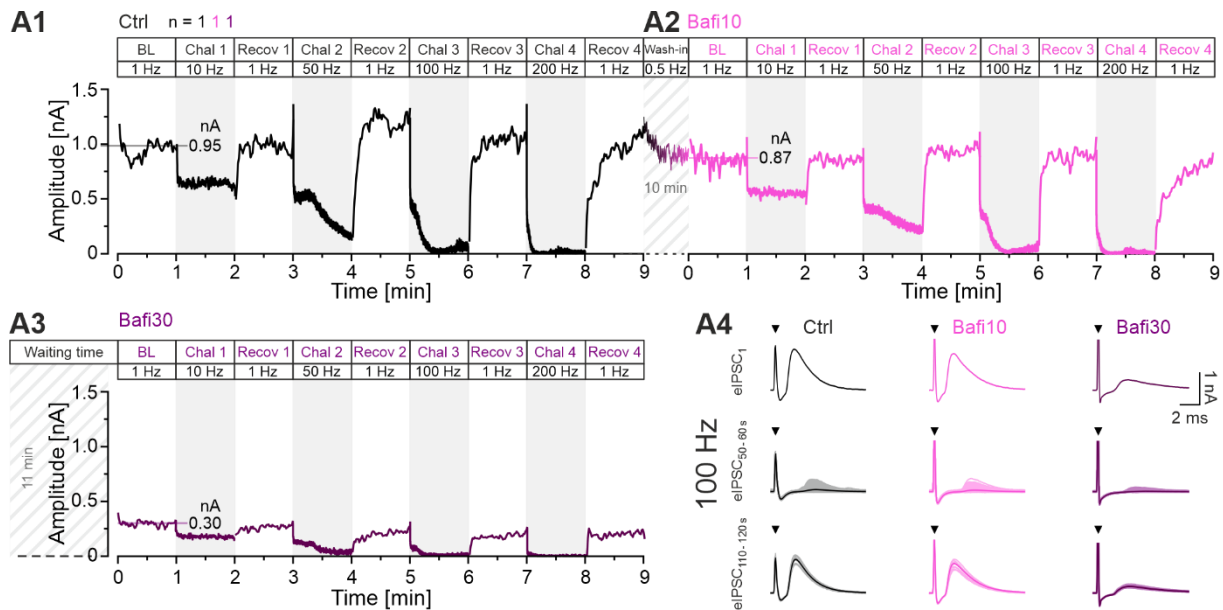


Figure 3.37 Reduced transmission under V-ATPase inhibition with 2 μ M Bafi in WT. (A1) Time course of absolute peak eIPSC amplitudes obtained with Protocol 5 (see Figure 2.4C) from a P11 WT LSO neuron Ctrl (black, ACSF). BL (0-1 min); dotted horizontal line indicates mean peak amplitude. **(A2)** Same as A1, but for Bafi10 (2 μ M, 10-min wash-in, magenta) **(A3)** Same as A1, but for Bafi30 (2 μ M, $\geq$ 30-min treatment, purple). A 6-s pause was introduced after the BL and each 60-s recovery period. Diagonally striped regions indicate perfusion periods of 2 μ M Bafi (time indicated in the figure; after perfusion, the drug was maintained in the bath throughout the entire recording period). Time courses are simple moving averages. Data in A1 and A2 are from the same neuron, whereas A3 is from a different neuron. **(A4)** Representative eIPSCs for the three cohorts during Chal 3 and Recov 3, showing eIPSC₁ and the overlay of individual eIPSCs and graphical means for eIPSC_{s50-60} and eIPSC_{s110-120} (lightly and darkly shaded). Arrows indicate stimulation artifacts. WT Bafi, n = 1.

The results from the two representative neurons were reflected in the population data. Under the Ctrl condition, eIPSC amplitudes showed frequency-dependent depression but recovered fully at all stimulation frequencies. Bafi10 showed similar time courses to Ctrl, with no major differences. In contrast, Bafi30 led to a clear reduction in eIPSC amplitudes across all frequencies (Figure 3.38A1-A3).

Time courses and quantification of BL confirm these results, with mean eIPSC amplitudes of 1023 pA for Ctrl, 914 pA for Bafi10, and 354 pA for Bafi30, corresponding to 100%, 89%, and 35%, respectively. A significant reduction was observed for Ctrl vs. Bafi30, while no significant difference was found between Ctrl and Bafi10 (Figure 3.38A4-A5). Similar results were observed across all challenge periods. Steady-state amplitudes were not significantly different between Ctrl and Bafi10. However, for 10 Hz, 50 Hz, and 100 Hz, a significant reduction was found for Ctrl vs. Bafi30. No significant difference was observed at 200 Hz, where eIPSC amplitudes_{s50-60} were reduced to 3% (29 pA), 2% (18 pA), and 1% (10 pA), indicating near-complete depletion of transmitter release, which may account for the lack of statistical significance. (Figure 3.38B1-B2, D1-D2, F1-F2, H1-H2; Table 3.22). During all recovery periods, eIPSC amplitudes recovered rapidly and reliably in all three conditions. No significant differences were observed between Ctrl and Bafi10 at any stimulation frequency. In contrast, all comparisons between Ctrl and Bafi30 were significantly different. Overall, only Bafi30 showed a strong effect (Figure 3.38C1-C2, E1-E2, G1-G2, I1-I2; Table 3.22). Taken together, MNTB-LSO synapses are functionally reliable during sustained high-frequency stimulation and recover robustly and precisely from synaptic depression, even under V-ATPase inhibition.

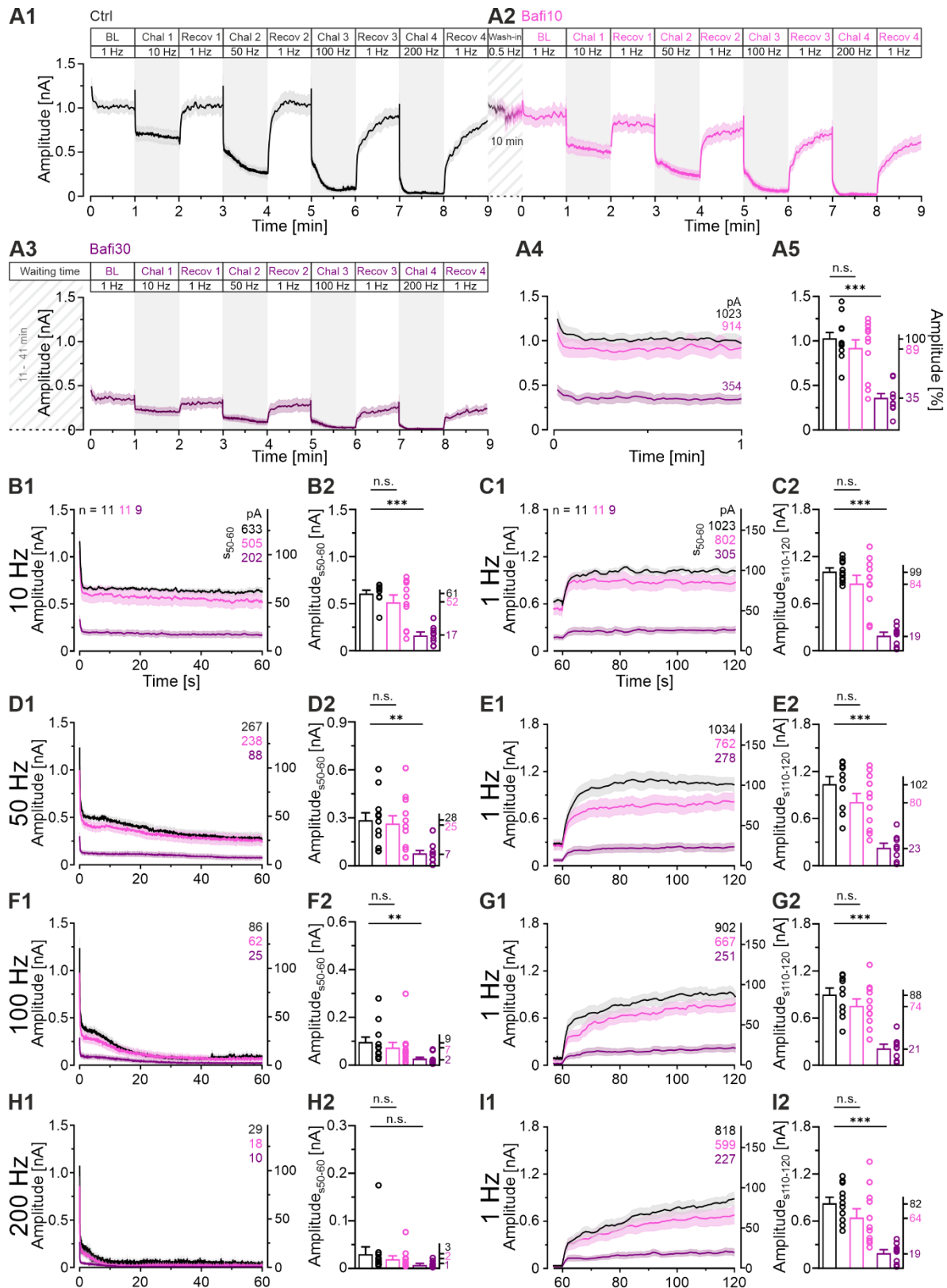


Figure 3.38 Reduced transmission under ≥ 30 min V-ATPase inhibition but not under 10 min V-ATPase inhibition. (A1, A2) Time course of mean eIPSC amplitudes obtained from paired recordings with Protocol 5 (see Figure 2.4C) for Ctrl (black) and Bafi10 (magenta). **(A3)** Time course for Bafi30 (purple). Data in A3 are from a different neuron than in A1 and A2. The BL mean of the Ctrl was set to 100%. Diagonally striped regions indicate perfusion periods of 2 μ M Bafi. A 6-s pause was introduced after the BL and each 60-s recovery period. **(A4)** Close-

up of the BL traces. Mean values are shown on the right. **(A5)** Statistics for BL analysis. **(B1)** Time course of absolute and normalized eIPSC peak amplitudes during 10-Hz | 60-s challenge. The upper right numbers indicate the mean amplitudes during s₅₀₋₆₀. **(B2)** Statistics for challenge analysis at s₅₀₋₆₀. Mean normalized values are shown on the right y-axis. **(C1)** Same as B1, but during 1-Hz | 60-s recovery periods. **(C2)** Same as B2, but for recovery analysis at s₁₁₀₋₁₂₀. Note the different amplitude scaling in B1-C2. **(D1-E2)** Same as B1-C2 but for 50 Hz **(F1-G2)**, 100 Hz **(H1-I2)**, and 200 Hz. Time courses are simple moving averages over three (1 Hz) or nine (10-200 Hz) data points, with SEM lightly shaded. Ctrl and Bafi10, n = 11; Bafi30, n = 9. See Table 3.22 for details, including statistics. The recordings in this figure are from Erika Pizzi (former postdoc, Animal Physiology, RPTU Kaiserslautern-Landau) and myself.

3.4.2 Reduction of I_{RRP}, q, and m and increased P_v under V-ATPase inhibition

Reduced eIPSC amplitudes during Bafi30 may result in fewer released SVs or a smaller q. V-ATPase inhibition is expected to impair SV (re)filling. To test this different parameter, like I_{RRP}, q, m, and P_v were analyzed. q was analyzed as from BL-Recov4. Representative current traces for BL and Recov4 under all three conditions are shown in Figure 3.39A1-A6. Peak sIPSC amplitudes were used to calculate q with a Gaussian fit (Figure 3.39B1-B6). No significant difference in q was found between Ctrl and Bafi10 (26 pA vs. 27 pA). However, a significant reduction was observed between Ctrl and Bafi30 (26 pA vs. 20 pA; Figure 3.39C3). The other synaptic parameters were calculated at 100 Hz. For I_{RRP}, mean values were 11.3 nA (Ctrl), 8.5 nA (Bafi10), and 2.4 nA (Bafi30; Figure 3.39C1-C2). Corresponding N_{RRP} values were 434, 389, and 125 SVs for Ctrl, Bafi10, and Bafi30, respectively (Figure 3.39C2, left y-axis). The number of SVs released per stimulus (m) was calculated as $m1 = eIPSC1/q$, resulting in 45 SVs (Ctrl), 35 SVs (Bafi10), and 21 SVs (Bafi30; Figure 3.39C4). No significant differences were found between Ctrl and Bafi10. However, I_{RRP}, q, and m were significantly reduced in Bafi30 compared to Ctrl. P_v was calculated as $P_v = m1/I_{RRP}$, yielding values of 11% (Ctrl), 12% (Bafi10), and 16% (Bafi30). A significant increase in P_v was observed between Ctrl and Bafi30 (Figures 3.39C5; Table 3.22). In summary, the Bafi30 treatment reduced synaptic parameters but did not abolish the (re-)filling process.

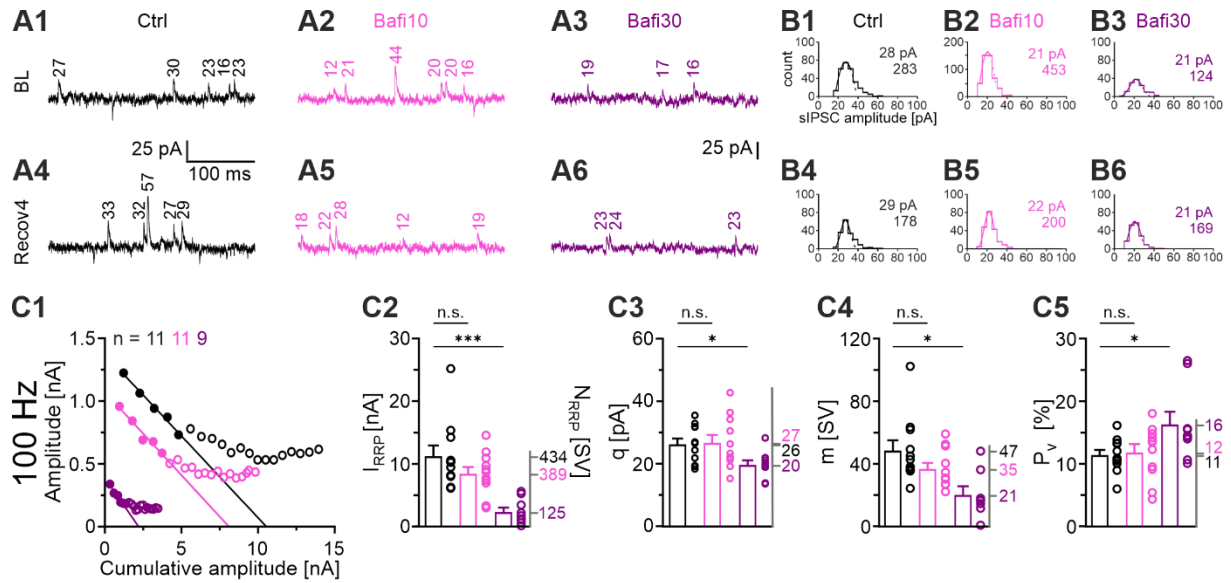


Figure 3.39 Reduced q , I_{RRP} , and m under V-ATPase inhibition, whereas P_v is increased. (A) Original sIPSC traces for Ctrl (black), Bafi10 (magenta), and Bafi30 (purple). (A1) sIPSC amplitudes during BL (A1) and Recov4 (A4) for Ctrl. Same as A1 and A4, but for Bafi10 (A2, A5), and Bafi30 (A3, A6). Numbers indicate the sIPSC amplitudes. (B) Estimation of q . (B1, B4) Distribution histograms of sIPSC amplitudes from current traces in A during BL (B1) and Recov4 (B4) for Ctrl. (B2-B6) Same as B1 and B4, but for Bafi10 (B2, B5) and Bafi30, respectively (B3, B6). A Gaussian curve was fitted from 0 pA to the bin after the first local maximum (dashed curves; Krächan et al., 2017). Numbers in panels represent q values and number of sIPSCs for BL (B1, B2, B3) and Recov4 (B4, B5, B6). Data for Ctrl and Bafi10 are from one neuron, while data for Bafi30 are from another neuron. (C) Analysis of synaptic parameters. (C1) Elmqvist & Quastel plots are used to determine I_{RRP} . The stimulation frequency was 100 Hz, and a linear fit was applied to eIPSCs₁₋₅ of the cohort mean values. (C2) Statistics for I_{RRP} . Means of N_{RRP} on the right y-axis. (C3) Statistics for q (obtained from a 300 s period over BL and Recov1-Recov4). Means on the right y-axis. Statistics for m (C4), and P_v (C5). Mean values on the right y-axis. Note that $N_{RRP} = I_{RRP}/q$. Variables on the left y-axis in B2-B6 are the same as in B1. Ctrl and Bafi10, n = 11; Bafi30, n = 9. See Table 3.22 for details, including statistics. The recordings in this figure are from Erika Pizzi (former postdoc, Animal Physiology, RPTU Kaiserslautern-Landau) and myself.

3 Results

Table 3.22: Synaptic transmission and parameters with 2 μ M Bafi in WT.

2 μ M Bafi		Ctrl n = 11		Bafi10 n = 11		Bafi30 n = 9		Ctrl vs. Bafi10	Ctrl vs. Bafi30	
Stimulation freq. [Hz]		mean	SEM	mean	SEM	mean	SEM	p-value		Figure
1	BL [pA]	1023	72	914	99	354	56	0.358	<0.001	3.38A5
10	Chal1 [pA]	633	62	505	70	202	43	0.196	<0.001	3.38B2
50	Chal2 [pA]	267	48	238	46	88	29	0.647	0.003	3.38D2
100	Chal3 [pA]	86	21	62	19	25	9	0.042	0.002	3.38F2
200	Chal4 [pA]	29	14	18	5	10	3	0.465	<u>0.025</u>	3.38H2
10	Recov1 [pA]	1023	95	802	85	305	54	0.154	<0.001	3.3CB2
50	Recov2 [pA]	1034	110	762	87	278	70	0.311	<0.001	3.38E2
100	Recov3 [pA]	902	91	685	65	251	58	0.197	<0.001	3.38G2
200	Recov4 [pA]	819	71	599	83	227	50	0.089	<0.001	3.38I2
100	I _{RRP} [nA]	11.3	2	8.5	1	2.4	1	0.102	<0.001	3.39C2
1	q [pA]	26	2	27	3	20	1	0.715	0.014	3.39C3
100	m [SV]	47	7	35	4	21	5	0.126	0.014	3.39C4
100	P _v [%]	11	0.1	12	0.1	16	0.2	0.794	0.024	3.39C5

Data are presented as mean $\pm$ SEM. Ctrl vs. Bafi10: Paired 2-tailed t-test; Ctrl vs. Bafi30: Unpaired 2-tailed t-test. If bold: Wilcoxon test. If underlined: Mann-Whitney test. Bonferroni post hoc correction (k = 2). Significant P-values are marked in green.

3.4.3 NHE6 localization in presynaptic axon terminals of MNTB-LSO synapses in WT

Due to the residual transmission observed despite V-ATPase inhibition (Bafi30), the involvement of an alternative proton pump for SV acidification was considered. A potential candidate is the Na^+/H^+ exchanger NHE6, which may support the proton gradient required for VIAAT-mediated glycine loading into SVs (see Section 1.1). I found strong immunofluorescence for NHE6 in all major SOC nuclei (MNTB, SPN, and LSO; Figure 3.40A1). Co-distribution of NHE6 and GlyT2 was observed in the MNTB (Figure 3.40A2). Both NHE6 and GlyT2 signals were prominent in the cytoplasm (Figure 3.40A3). Immunosignals for NHE6 and GlyT2 were also detected in the LSO (Figure 3.40A4). In contrast to the MNTB, the signals surrounded the LSO neurons (Figure 3.40A5). The characteristic punctate perisomatic pattern of GlyT2 colabeling helped to identify LSO neurons (Friauf *et al.*, 2019). A close-up of the white frame shown in Figure 3.40A5 highlights the co-distribution of NHE6 and GlyT2 as white puncta (Figure 3.40A6). The intensity profiles of NHE6 and GlyT2 were analyzed to assess their spatial distribution and co-distribution. Both profiles overlapped, indicating colocalization of the two proteins at MNTB terminals (Figure 3.40B1). Further quantification using Pearson's coefficient (0.68 ± 0.00 , $n = 16$; Figure 3.40B2) and the P_{Costes} coefficient (0.68 ± 0.00 , $n = 16$; Figure 3.40B3) confirmed co-distribution. Taken together, these results suggest that NHE6 is present in glycinergic MNTB axon terminals.

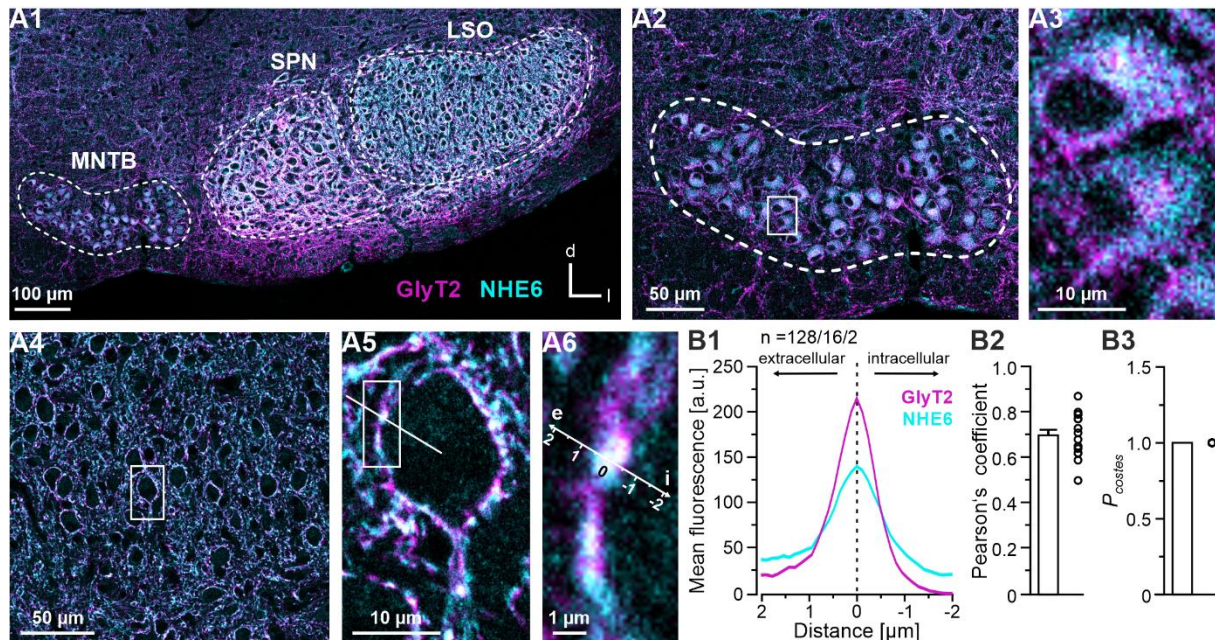


Figure 3.40 NHE6 is abundant in the MNTB and LSO and co-distributed with GlyT2 in the LSO in WT. (A) Immunohistochemical analysis of NHE6 and GlyT2 in a coronal brainstem at P11. **(A1)** Double-labeling for NHE6 (cyan) and GlyT2 (magenta) in the SOC. MNTB, SPN, and LSO are outlined by dotted lines. **(A2)** NHE6 and GlyT2 immunosignals in the MNTB. **(A3)** Closer view of two representative MNTB neurons from the framed area in A2. **(A4)** Double-labeling in the LSO. **(A5)** Close-up of a representative LSO neuron in the framed area

in A4. A representative line scan is shown. **(A6)** Close-up of the framed area in A5. The spatial axis is also used in B1. **(B)** Quantification of NHE6 and GlyT2 co-distribution. **(B1)** Averaged intensity profiles for NHE6 (magenta) and GlyT2 (cyan). Before averaging, each profile was aligned to the position of the maximum GlyT2 signal. See Materials and Methods for details. Negative and positive μm values indicate intracellular and extracellular signals, respectively. n, number of profiles/cells/animals. **(B2)** Pearson's coefficients and **(B3)** corresponding P_{Costes} values. The Pearson's coefficient was 0.70 ± 0.02 (16 cells), and the P_{Costes} value was 1.00 ± 0.00 (16 cells; NB: co-distribution is indicated by $P_{\text{Costes}} > 0.95$). Optical thickness: $1 \mu\text{m}$. Abbreviations: d, dorsal; e, extracellular; i, intracellular; l, lateral; LSO, lateral superior olive; MNTB, medial nucleus of the trapezoid body; SPN, superior paraolivary nucleus.

3.4.4 NHE inhibition reduces glycinergic transmission and synaptic parameters

To test whether NHE contributes to SV replenishment by supporting SV acidification and glycine (re)filling, LSO neurons were recorded while stimulating MNTB fibers with Protocol 6 (see Figure 2.4C). Protocol 6 consisted of an initial 1-Hz | 60-s BL, followed by a 60-s challenge and a 60-s recovery period, applied at 50 and 100 Hz. The total duration of the stimulation protocol was 5 min and included 9,180 stimuli. EIPA was then washed in for 10 min before the protocol was applied again (Figure 3.41A1-A2). No selective antagonist for NHE is currently available; the Na^+/H^+ exchanger inhibitor EIPA ($100 \mu\text{M}$) was used to assess synaptic transmission at MNTB-LSO synapses.

In the Ctrl condition, synaptic depression and recovery followed the expected pattern (Figure 3.41A1). During EIPA wash-in, eIPSC amplitudes decreased progressively, and the same trend continued under EIPA treatment (Figure 3.41A2). Time courses and quantification of BL amplitudes showed a significant reduction with EIPA compared to Ctrl (1450 vs. 799 pA), corresponding to 100% and 55%, respectively (Figure 3.41A3-A4). During challenge periods, eIPSC amplitudes were significantly reduced by EIPA. At 50 Hz, eIPSC amplitudes decreased from 30% (439 pA) in Ctrl to 8% (109 pA) in EIPA. At 100 Hz, eIPSC amplitudes dropped from 9% (131 pA) to 2% (34 pA; Figure 3.41B1-B2, D1-D2; Table 3.23). Despite the strong reduction in synaptic transmission, eIPSC amplitudes partially recovered, reaching 41% of Ctrl BL (598 pA) at 50 Hz and 35% (507 pA) at 100 Hz (Figure 3.41 C1-C2, E1-E2; Table 3.23).

Synaptic parameters were analyzed at Chal2 (100 Hz). I_{RRP} was significantly reduced ~ 4 -fold from 30.9 nA (Ctrl) to 7.3 nA (EIPA10). Corresponding N_{RRP} values were 930 SVs (Ctrl) and 254 SVs (EIPA10; Figure 3.41F1-F2). q remained unchanged, with 27 pA for Ctrl and 32 pA for EIPA10 (Figure 3.41F3; Table 3.23). m was significantly decreased ~ 3 -fold in EIPA10 (24 SVs) compared to Ctrl (67 SVs; Figure 3.41F4; Table 3.23). P_v did not differ significantly between conditions, with 11% for Ctrl and 12% for EIPA10 (Figure 3.41F5; Table 3.23). Surprisingly, the EIPA10 caused a ≥ 2 -fold reduction in eIPSC amplitudes and a ≥ 3 -fold

decrease in released SVs compared to Ctrl, showing a stronger effect than for V-ATPase inhibition.

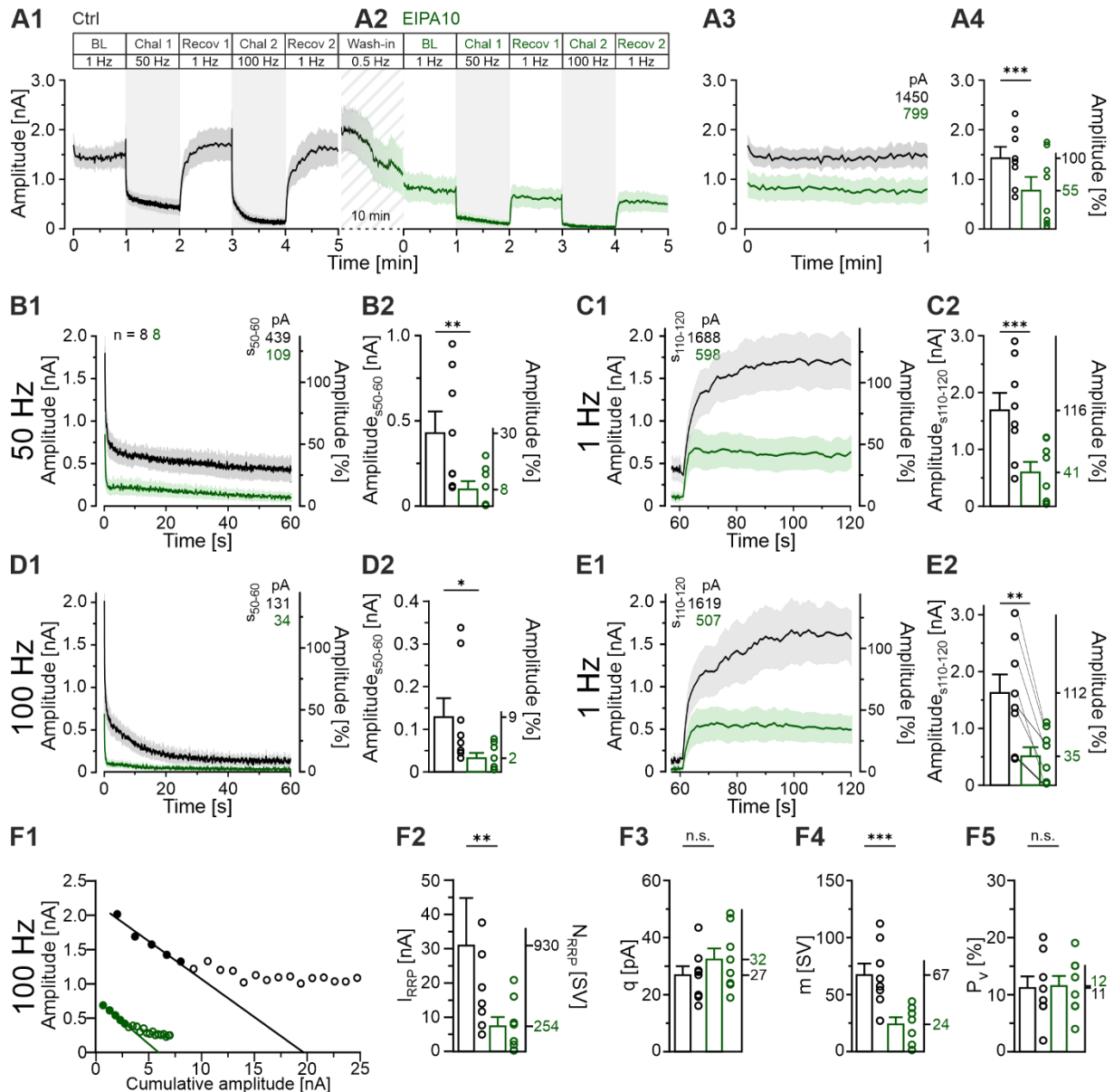


Figure 3.41 Reduced transmission and synaptic parameters under NHE inhibition with 100 μ M EIPA. (A1, A2) Time course of eIPSC amplitudes (mean $\pm$ SEM) for Ctrl (black) and EIPA10 (100 μ M, green), obtained with Protocol 6 (see Figure 2.4C). The diagonally striped region represents the wash-in period for EIPA. **(A3)** Close-up of the BL traces. The numbers in the upper right indicate the absolute mean amplitudes. **(A4)** Statistical analysis for the BL periods (mean Ctrl = 100%, EIPA10: 55%). **(B1)** Time course of absolute and normalized eIPSC peak amplitudes during 50-Hz | 60-s challenge. The upper right numbers indicate the mean amplitudes during s_{50-60} . **(B2)** Statistics for challenge analysis at s_{50-60} . Mean normalized values are shown on the right y-axis. **(C1)** Same as B1, but during 1-Hz | 60-s recovery periods. **(C2)** Same as B2, but for recovery analysis at $s_{110-120}$. Note the different amplitude scaling in B1-C2. **(D1-E2)** Same as B1-C2, but for 100 Hz. **(F)** Analysis of synaptic parameters. **(F1)** Elmqvist and Quastel plots were used to determine I_{RRP} ; forward extrapolation was applied

to the linear fit through eIPSCs₁₋₅ evoked at 100 Hz. Cohort values. **(F2)** Statistical analysis for I_{RRP} . Mean values of N_{RRP} on the right y-axis. **(F3-F5)** Statistics for q (obtained from a 300 s period over BL and Recov1-Recov4). **(F3)**, m **(F4)**, and P_v **(F5)**. Means on the right y-axis. Variables on the left y-axis in B2-D2 are the same as in B1. Time courses are simple moving averages (SEM lightly shaded). Ctrl, $n = 8$; EIPA10, $n = 8$. See Table 3.23 for details, including statistics. The recordings in this figure are only from Erika Pizzi (former postdoc, Animal Physiology, RPTU Kaiserslautern-Landau).

Table 3.23: Synaptic transmission and parameters with 100 μ M EIPA in WT.

100 μ M EIPA		Ctrl $n = 8$		EIPA10 $n = 8$		Ctrl vs. EIPA10	
Stimulation freq. [Hz]		mean	SEM	mean	SEM	p-value	Figure
1	BL [pA]	1450	205	799	251	<0.001	3.41A4
50	Chal1 [pA]	439	120	109	41	0.009	3.41B2
100	Chal2 [pA]	131	43	34	11	0.022	3.41D2
50	Recov1 [pA]	1688	307	598	183	<0.001	3.41C2
100	Recov2 [pA]	1619	328	507	160	<0.001	3.41E2
100	I_{RRP} [nA]	31	14	7	3	0.008	3.39F2
1	q [pA]	27	3	32	4	0.077	3.39F3
100	m [SV]	67	10	24	6	<0.001	3.39F4
100	P_v [%]	11	2	12	2	0.881	3.39F5

Data are presented as mean $\pm$ SEM. Paired 2-tailed t-test. If bold: Wilcoxon test. Bonferroni post hoc correction ($k = 2$). Significant P-values are marked in green.

3.4.5 NHE inhibition by EIPA showed effects on action potential generation/conduction

During EIPA experiments, a difference in the quality check of neurons (Figure 2.3) after EIPA wash-in was observed. In the Ctrl condition, principal LSO neurons fired a typical onset action potential (AP) in response to the current injection (Sternborg *et al.*, 2010; Friauf *et al.*, 2019). Under EIPA, this onset AP was absent, indicating impaired excitability (Figure 3.42A1-A2). To test whether EIPA affects AP generation or conduction from the MNTB neuron to the LSO neuron, antidromic recordings were performed. MNTB neurons were recorded while stimulating MNTB fibers near the LSO using Protocol 6 (Figure 2.4A2, C; Figure 3.42C1-C2). AP failures were defined as peak amplitudes <1 mV. In the Ctrl condition, mild AP failures were observed during 50 and 100 Hz stimulation, with AP amplitudes remaining stable. In contrast, under EIPA and EIPA wash-in, a high AP failure rate and a strong reduction of AP peak amplitude were observed at both 50 and 100 Hz (Figure 3.42B1-B2, D). Already at BL, the AP fidelity was only 56% compared to Ctrl BL (Figure 3.42C1-C2). At 50 Hz, AP fidelity was 86% in Ctrl and significantly decreased to 24% with EIPA. Similar results were observed at 100 Hz, with 74% in Ctrl and 11% under EIPA (Figure 3.42E; Table 3.24). Thus, the strong reduction in eIPSC amplitudes and released SVs under EIPA is likely due to impaired AP generation and conduction rather than a failure of synaptic transmission. Due to the strong impairment of AP generation and conduction under NHE inhibition with EIPA, antidromic experiments were

repeated under V-ATPase inhibition with Bafi using Protocol 5 (Figure 2.4B, C; Figure 3.42F1-F3) to determine whether the effect of Bafi results from the drug itself and not from a failure of APs reaching MNTB terminals. During the challenge periods, AP Fidelity_{s50-60} in the Ctrl condition was 100% at 10 Hz, 84% at 50 Hz, 70% at 100 Hz, and 33% at 200 Hz. Under Bafi10, AP Fidelity_{s50-60} was 89%, 82%, 61%, and 35%, respectively. For Bafi30, values were 88%, 66%, 44%, and 19%, respectively. None of these values differed significantly from Ctrl (Figure 3.42G1-G4; Table 3.24). Taken together, Bafi showed no effect on AP generation and conduction, in contrast to EIPA.

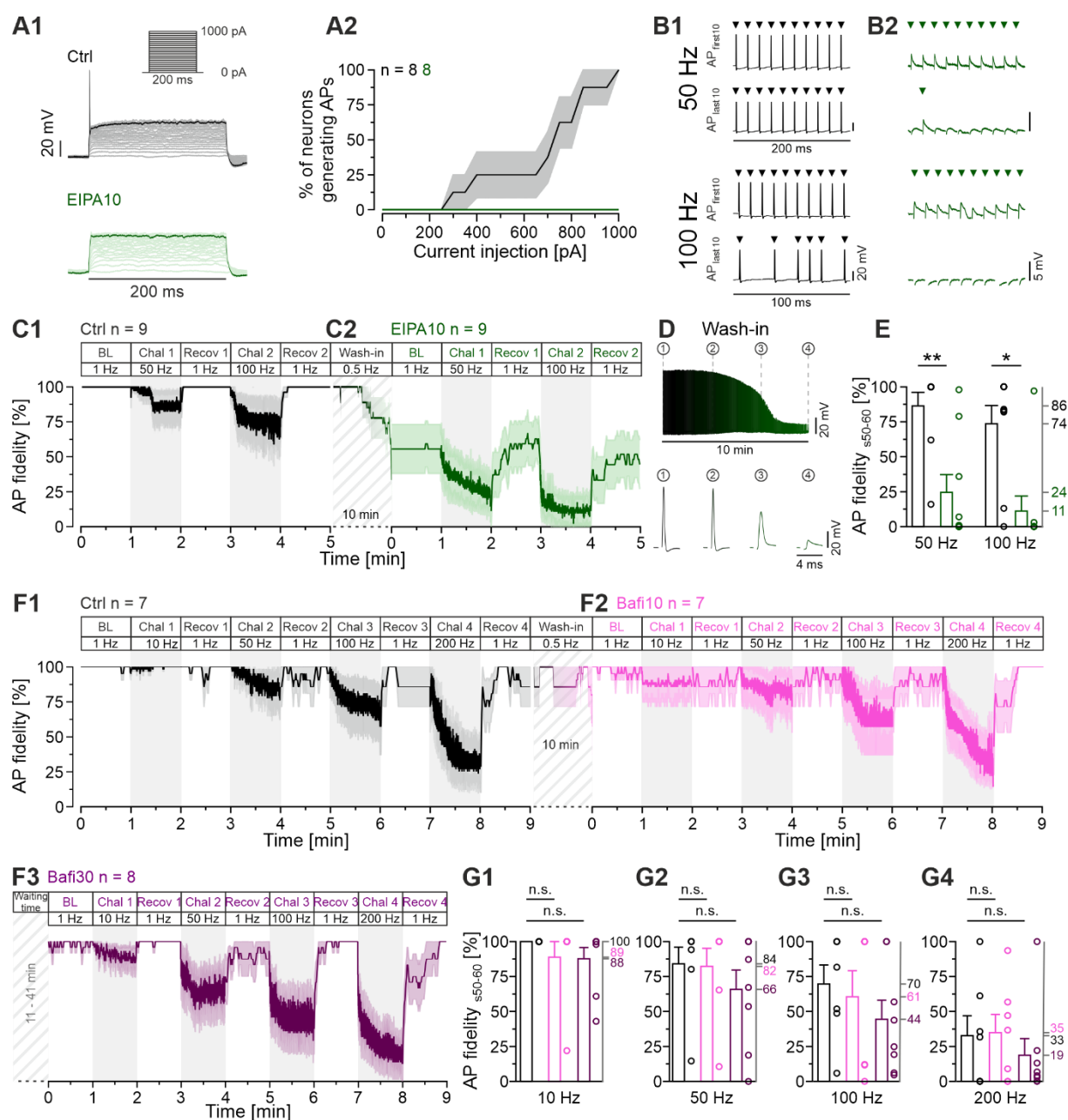


Figure 3.42 Application of EIPA reduces the reliability of AP firing, whereas Bafi does not. (A1) Representative voltage traces from a Ctrl MNTB neuron (black, top) and after application of EIPA10 (100 μ M; green, bottom) at rheobase 850 pA. The traces show voltage responses to rectangular current pulses (inset: 0 to 1,000 pA; 50 pA increments; 200 ms duration). **(A2)** Percentage of neurons generating APs (mean $\pm$ SEM) as a function of the current amplitude. Note the absence of APs upon EIPA treatment. Same neurons as shown in Figure 9. **(B)** Representative antidromic APs during Chal 1 and Chal 2 periods, showing the first and last 10 APs for Ctrl **(B1)** and EIPA10 **(B2)**. Each arrow marks an AP. Note the different amplitude scaling in B1 and B2. **(C1-C2)** Time course of AP fidelity (mean $\pm$ SEM) for Ctrl **(C1)** and EIPA10 **(C2)** obtained with Protocol 6 (see Figure 2.1C, antidromic stimulation). Diagonally striped regions represent the wash-in period of EIPA. A 6-s long pause was introduced after the BL and each 60-s recovery period. Ctrl, n = 9; EIPA10, n = 9. **(D)** AP amplitudes during the 10-min wash-in period (top) and magnification of four representative APs at different time points (bottom). **(E)** Statistical analysis for AP fidelity during s_{50-60} . **(F)** Time course of AP fidelity (mean $\pm$ SEM) for Ctrl (black) **(F1)** and Bafi10 (magenta) **(F2)** obtained from paired recordings with Protocol 5 (see Figure 2.4C, antidromic stimulation). **(F3)** Time course for Bafi30 (purple). Data in F3 are from a different neuron than in F1 and F2. Diagonally striped regions indicate perfusion periods of 2 μ M Bafi. A 6-s pause was introduced after the BL and each 60-s recovery period. **(G)** Statistics for AP fidelity during s_{50-60} for 10 Hz **(G1)**, 50 Hz **(G2)**, 100 Hz **(G3)**, and 200 Hz **(G4)**. Variables on the left y-axis in G2-G4 are the same as in G1. Mean percentages on the right y-axis. Ctrl, n = 7; Bafi10, n = 7; Bafi30, n = 8. See Table 3.24 for details, including statistics.

Table 3.24: Effect of 2 μ M Bafi and 100 μ M EIPA on the AP fidelity of MNTB neurons

2 μ M Bafi or 100 μ M EIPA		Ctrl n = 9		EIPA10 n = 9				Ctrl vs. EIPA10		
Stimulation freq. [Hz]	AP Fidelity [%]	mean	SEM	mean	SEM			p-value		Figure
50	S ₅₀₋₆₀	86	10	24	13			0.004		3.42E
100		74	13	11	11			0.023		
		Ctrl n = 7		Bafi10 n = 7		Bafi30 n = 8		Ctrl vs. Bafi10	Ctrl vs. Bafi30	
Stimulation freq. [Hz]	AP Fidelity [%]	mean	SEM	mean	SEM	mean	SEM	p-value		
10	S ₅₀₋₆₀	100	0	89	11	88	8	>0.999	0.20	3.42G
50		84	12	82	13	66	14	>0.999	0.368	
100		70	13	61	19	44	14	0.875	0.213	
200		33	14	35	13	19	11	0.863	0.885	

Data are presented as mean $\pm$ SEM. Paired 2-tailed t-test; unpaired 2-tailed t-test in *italic*. If bold: Wilcoxon test. If underlined: Mann-Whitney test. Bonferroni post hoc correction (Fig. 3.42E: k = 1, Fig. 10G: k = 2). Significant P-values are marked in green.

A mathematical analysis was performed to determine the influence of AP failures on synaptic transmission. This analysis was used to distinguish between effects on AP generation and conduction and those caused by synaptic fatigue due to the drug. For that, mean eIPSC amplitudes during the challenge periods were corrected by assuming 100% AP fidelity. This allows to attribute changes in eIPSC amplitudes to changes in presynaptic release mechanisms using *Synaptic transmission corrected = eIPSC amplitude/AP fidelity rate* (Figure 3.43). Confirming previous results (Figure 3.42F1-G4), corrected Bafi time courses and eIPSC amplitudes_{s50-60} were similar to uncorrected values (Figure 3.43A1-A2, C1-C2). This confirms that the reduction in eIPSC amplitudes, I_{RRP} , q , and m , and the increase in P_v under Bafi30 were due to drug effects and not caused by AP generation or conduction failures.

In contrast, eIPSC amplitudes during EIPA returned to the same level as Ctrl after correction, indicating that the reduction in eIPSC amplitudes and released SV was primarily due to AP failures rather than a defect in the presynaptic release machinery (Figure 3.43B1-B2, D1-D2).

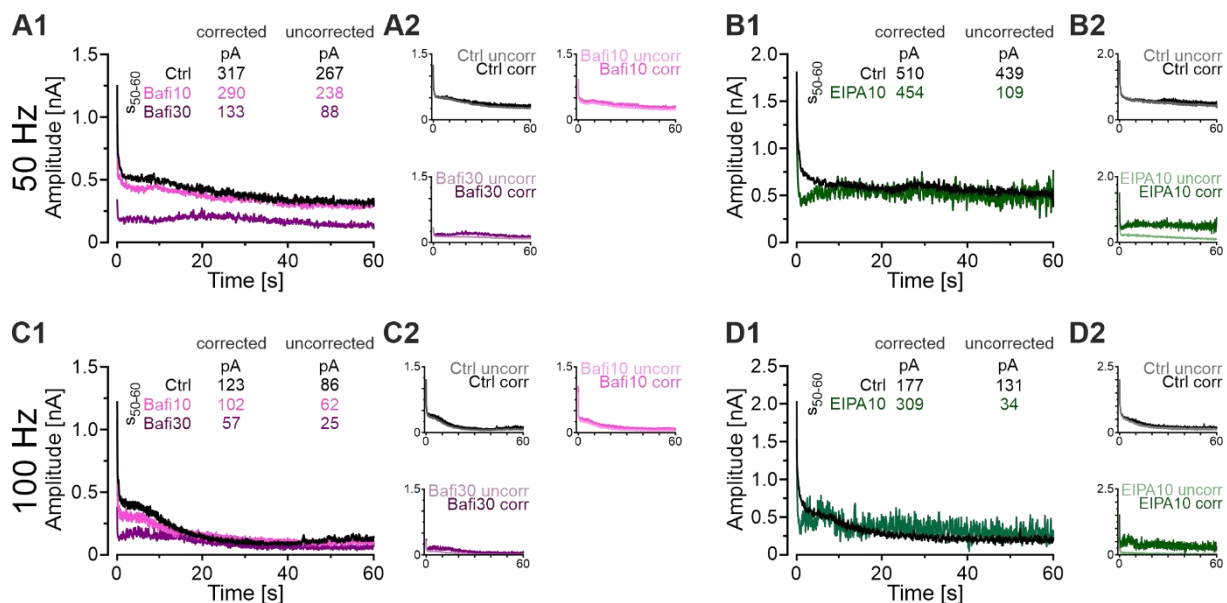


Figure 3.43 When AP failures are corrected, EIPA no longer affects synaptic transmission. (A1) Time course of eIPSC mean peak amplitudes upon AP correction for Ctrl (black), Bafi10 (magenta), and Bafi30 (purple) during 50-Hz | 60-s challenge. The upper right numbers indicate the mean corrected and uncorrected amplitudes during s_{50-60} . (A2) Time course of corrected amplitudes (dark color) and uncorrected amplitudes (light color) for Ctrl (upper left), Bafi10 (upper right), and Bafi30 (lower left). (B1) Same as A1, but for EIPA10 (green). (B2) Same as A2, but for Ctrl (top) and EIPA (bottom). (C1-D2) Same as A1-B2, but for 100 Hz. Variables on the left y-axis in A2, B2, C2, and D2 are the same as in A1. Note the different amplitude scaling in B1, B2, D1, and D2.

4 Discussion

Part 1: Localization of Asc-1 and contribution to glycine (re)uptake in WT and KO

In part 1 (see Section 3.1-3.3), I investigated the localization of Asc-1 at MNTB-LSO synapses to address whether Asc-1 contributes to synaptic transmission by supplying glycine for SV (re)filling at presynaptic terminals. Further, I tested how Asc-1 and GlyT2 together shape synaptic strength during sustained activity by pharmacologically inhibiting both transporters. For the first time, Asc-1 localization was analyzed at an auditory brainstem synapse, along with functional contribution under evoked stimulation conditions.

Major results part 1:

1. Asc-1 is highly expressed in the MNTB and LSO, surrounding the LSO somata indicating the presence of Asc-1 in MNTB terminals in WT and GlyT2 KO.
2. Asc-1 contributes in an activity-dependent manner to glycinergic transmission at MNTB-LSO synapses (Figure 4.1).
3. Asc-1 cause residual glycinergic transmission in GlyT2 KO but q remains unaffected upon Asc-1 inhibition, suggesting SV loading is maintained. However, the SV pool size is mildly reduced in WT and strongly reduced in GlyT2 KO (Figure 4.1).
4. GlyT2 or Asc-1 inhibition had minor effects on synaptic performance. However, combined inhibition of GlyT2 and Asc-1 resulted in a pronounced impairment of synaptic performance (Figure 4.2).

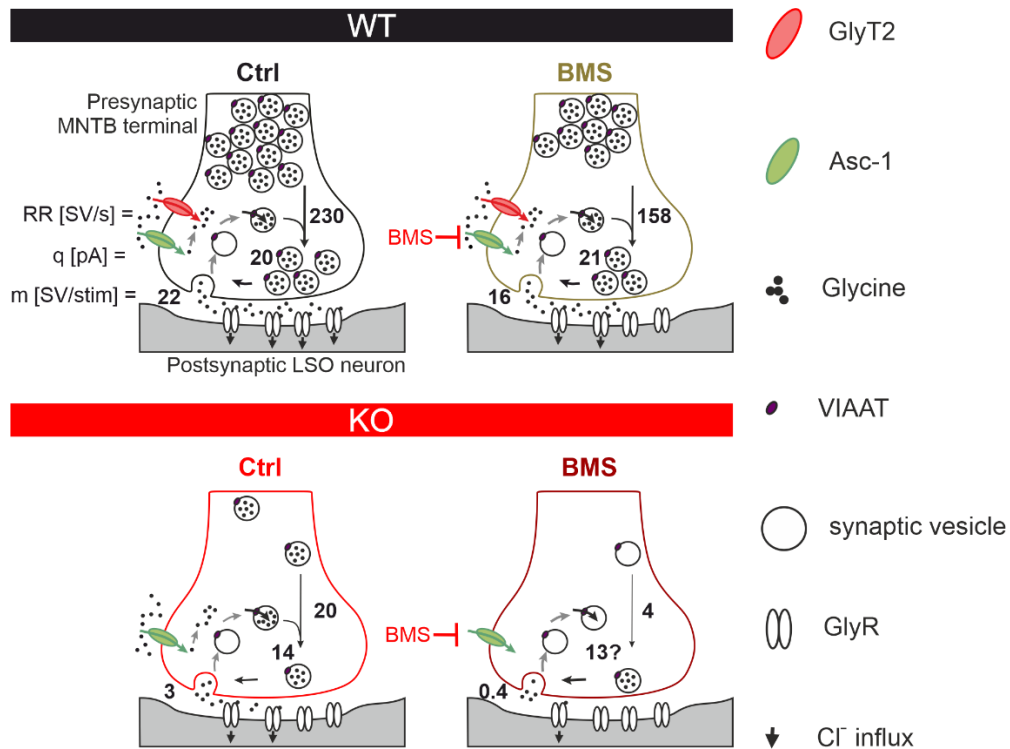


Figure 4.1 Graphical summary of Asc-1 localization and function. Schematic overview of the MNTB-LSO synapse. Both Asc-1 (green) and GlyT2 (red) are localized in MNTB terminals in both WT and GlyT2 KO. During sustained activity, WT Ctrl (black) synapses maintain synaptic transmission, with an RR of 230 SV/s, q of 20 pA, and m of 22 SV. Under Asc-1 inhibition (WT BMS, olive), the RR (158 SV/s) and m (16 SV) are moderately reduced, but q (21 pA) remains unaffected. In GlyT2 KO Ctrl (red), the RR (20 SV/s), q (14 pA), and m (3 SV) are already severely compromised compared to WT, but residual glycinergic transmission persists. When Asc-1 is inhibited in GlyT2 KO (GlyT2 KO BMS, dark red), synaptic transmission is completely abolished. [Section 4.4](#) explains the technical limitations indicated by question marks.

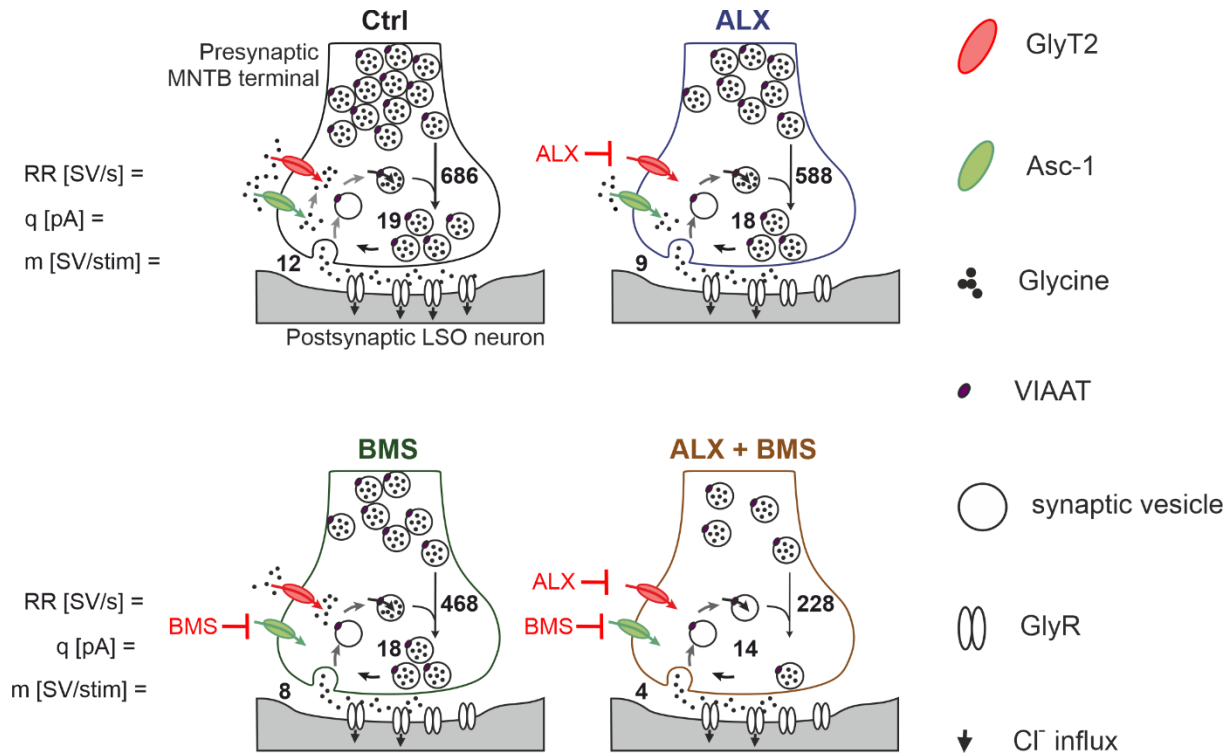


Figure 4.2 Graphical summary of GlyT2 and/or Asc-1 inhibition effects. Schematic overview of the MNTB-LSO synapse during the end of marathon experiments. In Ctrl (black), synaptic transmission is maintained. Inhibition of GlyT2 (ALX; blue) or Asc-1 (BMS; green) alone leads to a reduction of 14% (GlyT2) and 32% (Asc-1) in RR (686, 588, and 468 SV/s, respectively) and 25% (GlyT2) and 34% (Asc-1) in m (12, 9, and 8 SV, respectively), while q (19, 18, and 18 pA, respectively) remains unaffected. The combined inhibition of GlyT2 and Asc-1 (ALX + BMS; brown) causes a strong impairment of 67 % in RR and m and 26% in q, (RR = 228 SV/s; q = 14 pA, and m = 4 SV).

Part 2: Contribution of V-ATPase to glycinergic transmission and role of NHE in SV (re)filling

In part 2 (see Section 3.4), I examined the role of the vesicular H^+ pump V-ATPase in glycinergic transmission at MNTB-LSO synapses. In addition, I investigated the localization and potential function of NHE at these synapses to test whether it could support VIAAT function as an alternative or complementary proton source.

Major results part 2 (Figure 4.3):

1. V-ATPase inhibition strongly reduces synaptic performance but does not fully abolish synaptic transmission. This led to the idea of an alternative H^+ pump, NHE, which is abundant in MNTB terminals.
2. NHE inhibition causes side effects on AP conduction. AP failure correction analysis reveals no effect of EIPA on synaptic transmission.

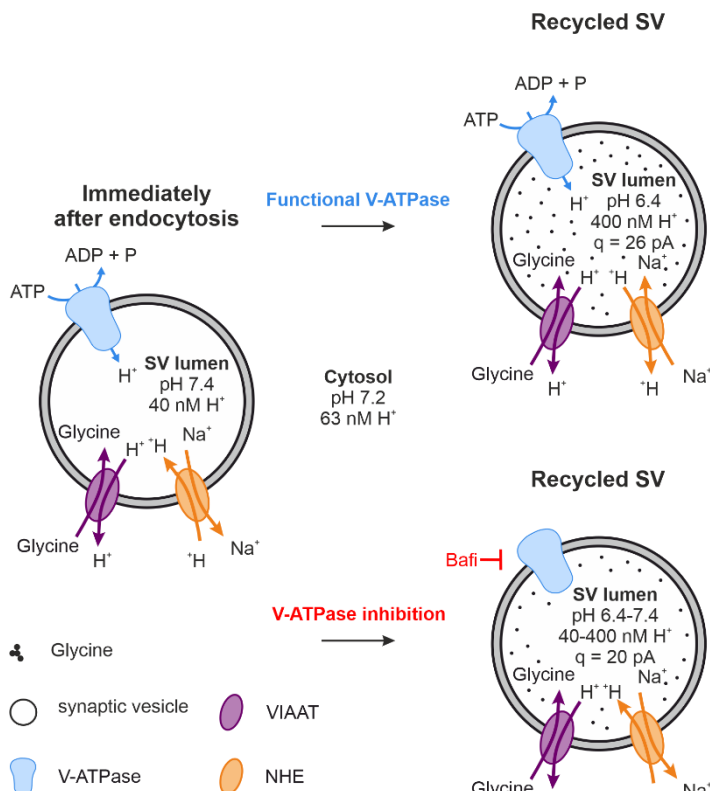


Figure 4.3 Graphical summary of SV acidification.

Schematic overview of SV acidification and glycine loading after endocytosis. Left: SV immediately after endocytosis. Key components for (re)acidification and NT (re)filling are the V-ATPase (blue) and the VIAAT (purple). To regulate the electrochemical gradient, as hypothesized, an additional mechanism is proposed. The H^+/Na^+ exchangers (NHE, orange). Top right: Under physiological conditions, V-ATPase continuously pumps H^+ into SV, generating the proton gradient that drives glycine uptake via VIAAT (H^+ /glycine antiport). Bottom right: Under V-ATPase inhibition with Bafi, NHE is hypothesized to operate in reverse mode immediately after

endocytosis, exchanging luminal Na^+ for cytoplasmic H^+ . This may support VIAAT-mediated glycine uptake even when H^+ pumping is impaired. Under these conditions, the quantal size was reduced from 26 pA to 20 pA.

4.1 Role of Asc-1 in synaptic transmission

4.1.1 Asc-1 is localized in MNTB terminals

This section referred to major result 1 of part 1. Asc-1 is broadly expressed throughout the brain but is concentrated in regions with a high density of glycinergic synapses (Helboe *et al.*, 2003; Ehmsen *et al.*, 2016; Nakahara *et al.*, 2023). Nothing is known about the localization of Asc-1 in MNTB-LSO synapses. I found co-localization of VIAAT and Asc-1 in both genotypes, in both MNTB and LSO. The LSO neurons were surrounded by co-localized puncta, which were attributed to glycinergic MNTB terminals. These results have been confirmed by transcriptomic sequencing data from Asc-1 in P11 MNTB in WT (Brill *et al.*, 2021). Additionally, the VIAAT staining pattern in the LSO was unchanged in GlyT2 KO compared to WT, suggesting that SV (re)filling with glycine is not abolished in GlyT2 KO animals (Latal *et al.*, 2010; Brill *et al.*, 2021).

However, its cellular localization remains debated, with studies reporting expression in either neurons or astrocytes. Ehmsen *et al.*, 2016 used a LacZ knock-in reporter under the control of the endogenous *Slc7a10* promoter with co-immunostaining from P16-21 old mice. This approach showed co-localization of Asc-1 and the astrocyte-specific marker GFAP, but not with the oligodendrocyte marker OLIG2 or the neuronal marker NeuN. Further evidence that Asc-1 is expressed in astrocytes was shown in single-cell RNA-sequencing data from lateral globus pallidus P60-90 mice, which showed that Olig2-astrocytes, a subpopulation of astrocytes, express a higher level of Asc-1 than to GFAP-astrocytes. These results were confirmed with immunohistochemistry of Asc-1 and Olig2-astrocyte markers (Tatsumi *et al.*, 2021). While Asc-1 has been reported in astrocytes, there is also evidence of neuronal expression of Asc-1. Immunohistochemistry using a C-terminal Asc-1 antibody showed co-localization with the presynaptic marker synaptophysin, but not with the astrocytic marker GFAP in adult mice throughout the brain (Helboe *et al.*, 2003). Using two independent polyclonal antibodies targeting N- and C-termini, Asc-1 expression was detected in the dendrites and somata of pyramidal neurons in the cerebral cortex and CA1 region of the hippocampus, as well as in Purkinje neurons of the cerebellum in both adult rats and mice. In astrocytes no expression was observed (Matsuo *et al.*, 2004). Functional studies showed that Asc-1 synaptosomes from mouse forebrain or cerebellum had no D-serine transfer (Rutter *et al.*, 2007). Asc-1 mediates D-serine release in a neuronal culture, but no D-serine efflux was detected in astrocytes under the same conditions in rat primary astrocyte cultures (Rosenberg *et al.*, 2010, 2013). Furthermore, quantitative PCR, which uses fluorescence probes to detect specific targets, was used to sort neurons and astrocytes and confirmed that Asc-1 is expressed in neurons (Sakimura *et al.*, 2016).

However, the results of immunohistochemistry depend heavily on antibodies. The specificity of antibodies can vary, and some may produce nonspecific staining. During my staining process, I discovered that heat-induced epitope retrieval was necessary for the specific detection of Asc-1. This approach also enables the detection of VIAAT expression in MNTB neurons that transport the protein to axon terminals. Discrepancies in the literature may be explained by technical factors. Depending on the cell type, Asc-1 may have different roles, such as providing glycine from astrocytes or contributing directly to synaptic transmission. Further, Asc-1 expression may change during development. Taken together, my data provide new insight into Asc-1 localization at MNTB-LSO synapses, specifically within MNTB terminals, and contribute to understanding synaptic organization in both WT and GlyT2 KO.

4.1.2 Glycine uptake via Asc-1

This section referred to major result 2 of part 1. Asc-1 inhibition with mild stimulation (Figure 3.2-3.5) did not affect synaptic transmission at WT and GlyT2 KO MNTB-LSO synapses. BMS blocks Asc-1-mediated glycine uptake with high potency ($IC_{50} \sim 10\text{-}400\text{ nm}$, depending on the *in vitro* system: HEK cells, primary cortical cultures, and rat brain synaptosomes), acting as a non-transported, full transporter inhibitor (Brown *et al.*, 2014). It blocks the transmembrane helices (TM) TM6, TM10, and TM11, which are essential for the conformational changes required for transport function (Torrecillas *et al.*, 2019; Mikou *et al.*, 2020). Based on previous studies using BMS in murine preadipocyte cultures to inhibit Asc-1 (Suwandhi *et al.*, 2021) and using another non-transportable Asc-1 inhibitor (LuAE00527) in brain slices (Sason *et al.*, 2017; Mesuret *et al.*, 2018; Bodner *et al.*, 2020), a concentration of 10 μM BMS was used first. Because 10 μM BMS showed no effect on synaptic transmission, the concentration was increased to 50 μM BMS to ensure effective Asc-1 inhibition under tissue conditions. Upon 50 μM BMS, there was still no effect on synaptic transmission, suggesting no contribution of Asc-1 in glycinergic transmission at MNTB-LSO synapses. However, it was shown that Asc-1 exchanges small neutral AA, including glycine and D-serine (Fukasawa *et al.*, 2000; Helboe *et al.*, 2003; Safory *et al.*, 2015). Various studies showed that pharmacological inhibition of Asc-1 reduces D-serine uptake in neurons (Rosenberg *et al.*, 2010; Rosenberg *et al.*, 2013; Sakimura *et al.*, 2016; Sason *et al.*, 2017; Billard & Freret, 2018; Mikou *et al.*, 2020). As well as increased extracellular glycine (Thomsen *et al.*, 2003). Other Asc-1 substrates like alanine are not effective counter-transport partners for D-serine. Microdialysis injection of alanine into the medial prefrontal cortex did not trigger significant D-serine release, whereas the transportable Asc-1 inhibitor SMLC induced D-serine efflux (Ishiwata *et al.*, 2013). Furthermore, another study showed substrate selectivity of Asc-1, glycine is the preferred substrate of Asc-1 (Li *et al.*, 2024). These data show that Asc-1 is

substrate-dependent (Fukasawa *et al.*, 2000; Mikou *et al.*, 2020; Li *et al.*, 2024) and supports the idea that glycine gets transported into the terminal by exchange of D-serine to extracellular space (Neame *et al.*, 2019).

These findings indicate that D-serine is required for glycine uptake through Asc-1. But where does the D-serine come from? In the hippocampus, L-serine is synthesized in astrocytes via an enzyme called PHGDH and exported by ASCT1/ASCT2. It can be taken up by neurons through yet unidentified transporter (Neame *et al.*, 2019). Inside neurons, L-serine can be converted by serine-racemase to D-serine (Wolosker *et al.*, 2008), providing the substrate for Asc-1 antiport. Supporting this, a study in mouse forebrain has shown that D-serine is primarily synthesized and released by neurons (Balu *et al.*, 2014). Taken together, even when mild stimulation has no effect on synaptic transmission upon Asc-1 inhibition, there is evidence that Asc-1 takes up glycine by releasing D-serine in neurons.

4.2 Activity-dependent role of Asc-1

This section referred to major result 2 of part 1. My immunohistochemical data, together with previous studies, show that Asc-1 is expressed in neurons, including presynaptic terminals. Asc-1 KO studies showed that Asc-1 is essential for regulating neuronal excitability (Xie *et al.*, 2005). Electrophysiology data either from Asc-1 KO mice or pharmacological inhibition of Asc-1 showed that Asc-1 is essential for glycinergic transmission (Safory *et al.*, 2015; Ehmsen *et al.*, 2016; Mesuret *et al.*, 2018).

The auditory brainstem synapses are known for reliable high-frequency transmission (Friauf *et al.*, 2015; Ngodup *et al.*, 2015; Luo & Südhof, 2017). This suggests the previously applied stimulation protocol (Figure 3.2-3.5) may not have been sufficient to fully unmask the consequences of Asc-1 inhibition. Therefore, activity was increased (Figure 3.6-3.10) and a significant reduction in eIPSC amplitudes was observed at the end of the stimulation protocol in WT. Synapses underwent 3,036 stimuli before Asc-1 inhibition had an effect. Compared to control experiments with 0.1% DMSO, the reduction in glycinergic transmission was drug-specific and not time-dependent. Control recordings showed a slight increase in recovery eIPSC amplitudes compared to BL, reaching values above 100%. It is known that DMSO concentrations below 1% can enhance synaptic transmission (Sawada & Sato, 1975).

Experiments under more challenging stimulation (Figure 3.22) show a stronger impairment of synaptic transmission during the end of stimulation when Asc-1 is inhibited. These results support my hypothesis that Asc-1 contributes to maintaining glycinergic transmission by providing glycine in presynaptic terminals. It should be mentioned that, as previously thought, Asc-1 only contributes during high-frequency activity, indicating an activity-dependent

mechanism (Eulenburg & Hülsmann, 2022a). My data convinces these thoughts, that synaptic performance is impaired after 9 mins of ongoing activity. Asc-1 is activity-dependent due to its antiporter function, which exchanges one AA for another based on concentration gradient (Fukasawa *et al.*, 2000; Mikou *et al.*, 2020; Li *et al.*, 2024). This led to the assumption, that with increased synaptic activity, more glycine is released, with increased extracellular glycine concentration in the presynaptic cleft. This may create a gradient that drives Asc-1-mediated glycine (re)uptake into presynaptic terminals. If Asc-1 is inhibited, glycine cannot be exchanged and remains in the extracellular space. Taken together, Asc-1 contributes to presynaptic glycine recycling during high-frequency activity. Suggests that Asc-1 works in an activity-dependent manner at MNTB-LSO synapses.

4.3 Equal contribution of GlyT2 and Asc-1

This section referred to major result 4 of part 1. My data show that during high synaptic activity (Figure 3.21-3.23), either GlyT2 or Asc-1 inhibition showed a similar effect. eIPSC amplitudes decreased significantly to ~60% at the end of the recovery periods without significantly impairing SV replenishment. The effect of GlyT2 inhibition with ALX-1393 is consistent with previous results at MNTB-LSO synapses (Brill *et al.*, 2021). Despite it was shown, that GlyT2 is the major transporter for glycine (re)uptake (Apostolides & Trussell, 2013), ALX-1393 does not impair amplitudes drastically (Xu *et al.*, 2005; Oyama *et al.*, 2017; Brill *et al.*, 2021). It has been proposed that this limited impact on synaptic transmission results from the presence of preformed SVs that can maintain sustained transmitter release independently from glycine recycling (Brill, 2018). A similar mechanism has been described at the ribbon synapse of inner hair cells, where replenishment from preformed SV from the cytoplasmic pool takes place (Griesinger *et al.*, 2005). The here uses concentration of 2 μ M ALX-1393 is sufficient for effective GlyT2 inhibition. The used concentration is similar to the concentration used in other studies (Jeong *et al.*, 2010; Oyama *et al.*, 2017). Leading to the conclusion, that MNTB-LSO synapses are independent of GlyT2 (re)uptake.

With either GlyT2 or Asc-1 inhibition alone, the remaining transporter is still functional and can partly compensate for the loss of the other. When both transporters GlyT2 and Asc-1 are simultaneously inhibited, synaptic impairment becomes stronger (Figure 3.23). eIPSC amplitudes, q , I_{RRP} , m , RR , and P_v are all affected. The number of released and replenished SVs is reduced, while P_v is increased, likely as a compensatory mechanism. Indicating that both transporters work cooperatively together. This dual transport system may ensure reliable and high-fidelity information transfer from LSO to higher auditory stations. My data suggests that after synaptic release of glycine, Asc-1 provides glycine into presynaptic MNTB terminals in the same

manner than GlyT2, especially under conditions of high synaptic activity. In contrast to my results, Apostolides & Trussell, 2013 performed recordings with the GlyT2 inhibitor ORG25543, which caused a nearly complete breakdown of glycinergic transmission in the cartwheel cells of the mouse dorsal nucleus. They propose that GlyT2 is the primary transporter for glycine (re)uptake for SV (re)filling and that there is no alternative glycine (re)uptake mechanism.

But why should Asc-1 contribute as much as GlyT2 to NT recycling? Asc-1 is classified as an AA transporter. It contributes to synaptic transmission with its substrate-driven exchange mechanism (Fukasawa *et al.*, 2000; Mikou *et al.*, 2020; Li *et al.*, 2024). Different from GlyT2, Asc-1 is a Na⁺-independent antiporter, that transports small AAs (Verrey *et al.*, 2004; Fotiadis *et al.*, 2013). GlyT2 maintains glycine levels in both basal and high-activity conditions by actively importing glycine into the presynaptic terminal (Gomez *et al.*, 2003b; Rousseau *et al.*, 2008; Apostolides & Trussell, 2013). In contrast, Asc-1 contributes when its substrate concentration is high, such as during high activity, when extracellular glycine concentrations increase and are available for exchange. Under these conditions, Asc-1 contributes to glycine (re)uptake and to SV (re)filling when GlyT2 capacity becomes limited. However, because Asc-1 is an antiporter, it cannot maintain high glycine levels in the presynaptic terminal alone. Therefore, it cannot compensate for the complete GlyT2 loss in KO but modulate synaptic strength in WT during high activity. It is shown that Asc-1 is involved in glycine supply, but GlyT2 function is still essential for surviving. Furthermore, Asc-1 contributes to glycine metabolism and homeostasis in the brain (Safory *et al.*, 2015). Asc-1 KO mice display a hyperekplexia phenotype (Xie *et al.*, 2005; Safory *et al.*, 2015), which was recently found to induce this disease (Drehmann *et al.*, 2023). This shows the role of Asc-1 in glycine regulation. Asc-1 is expressed in neurons and astrocytes (Tatsumi *et al.*, 2021). The early postnatal death of Asc-1 KO mice further supports the importance of Asc-1 in maintaining glycine homeostasis. Although Asc-1 is expressed throughout the whole brain (Helboe *et al.*, 2003) and is not limited to glycinergic synapses (Sason *et al.*, 2017). Its role in synaptic transmission is context-dependent. In the external globus pallidus, Asc-1 modulates NMDA receptor activity by regulating either D-serine (Nakahara *et al.*, 2023). This demonstrates that Asc-1 contributes to overall brain function.

4.4 Residual transmission in GlyT2 KO and the role of Asc-1

This section referred to major result 3 of part 1. GlyT2 KO mice and chronic GlyT2 inhibition show reduced sIPSCs amplitudes and frequency (Gomez *et al.*, 2003a; Rousseau *et al.*, 2008) and eIPSCs (Brill *et al.*, 2021). Despite this, glycine loading into SVs is not completely abolished (Gomez *et al.*, 2003a; Latal *et al.*, 2010; Brill *et al.*, 2021). This residual transmission suggests the presence of an alternative glycine (re)uptake mechanism. A reduction of eIPSC amplitudes and synaptic fidelity within the first 10 s was observed (Figure 3.14). This suggests that Asc-1 inhibition may impair presynaptic glycine recycling early during stimulation, while still maintaining transmission. This is contrary to the results discussed in section 4.2. However, stimulation Protocol 1 may not have been sufficient to fully unmask the functional consequences of Asc-1 inhibition in GlyT2 KO. In my data, synaptic transmission is stable in the GlyT2 KO ctrl situation and recovers back to 100% of the BL level under functional Asc-1. After the application of the Asc-1 inhibitor, a gradual reduction of eIPSC amplitudes is shown, leading to a nearly complete loss of synaptic transmission at the end of the stimulation (Figure 3.16).

The complete rundown of eIPSC amplitudes (during seven times 10 Hz stimulation followed by 0.1 Hz recovery) supports the role of Asc-1 in glycine (re)uptake required for SV (re)filling. Surprisingly, despite the reduction in transmission, *q* remains unchanged after Asc-1 inhibition. This indicates that SVs are still fully loaded with glycine and that SV (re)filling itself is not impaired. However, the estimated number of released SVs during the last 10 s of stimulation is decreased to <1 SV per stimulus, representing a near collapse of synaptic transmission. This suggests that although SVs are fully filled, significantly fewer SVs are available for release. Now one could say that SV availability from preformed SVs is disturbed, but previous data have shown that GlyT2 KO MNTB-LSO synapses can maintain transmission at 50 Hz for 60 s (Brill *et al.*, 2021), and these synapses fully recover at 0.1 Hz (Kurz, 2019). Moreover, control experiments in 0.1% DMSO showed no reduction of eIPSC amplitudes during sustained stimulation, showing drug-specific run-down. Furthermore, the *q* analysis used in this thesis is technically limited. Events smaller than ~10 pA may be lost in the recording noise, meaning less-filled SVs would not be detected. Additionally, my recordings are limited to postsynaptic current and cannot detect exocytosis of empty SVs. Therefore, an SV release without NT content would not be visible in my recordings. Indeed, empty SV releases take place (Granseth & Lagnado, 2008). Moreover, unchanged VIAAT immunohistochemical signal in GlyT2 KO mice suggests that glycine replenishment by VIAAT is not impaired (Latal *et al.*, 2010; Brill *et al.*, 2021). This means that SVs in GlyT2-KO mice could be filled with glycine produced by *de novo* synthesis. However, GlyT2 KO dies within the first two postnatal weeks, indicating that *de novo* synthesis cannot compensate for the loss of GlyT2.

Furthermore, my results demonstrate that inhibition of Asc-1 leads to a gradual reduction in eIPSC amplitudes and impaired synaptic performance during repetitive stimulation. Asc-1 contributes to functionally maintaining residual synaptic transmission in GlyT2 KO mice at MNTB-LSO synapses. These results are consistent with previous studies that showed that Asc-1 is essential for glycine inhibitory transmission (Safory *et al.*, 2015; Ehmsen *et al.*, 2016; Mesuret *et al.*, 2018). Despite the functional role of Asc-1 in GlyT2 KO, it is still not enough to compensate for the loss of GlyT2. GlyT2 KO animals still die within the first two postnatal weeks.

4.5 Moderate impairment of V-ATPase

This section referred to major result 1 of part 2. I investigated the role of V-ATPase in the robustness of prolonged synaptic transmission at MNTB-LSO synapses. I continued this project, which is based on data originally collected by Erika Pizzi. The data she collected is marked accordingly. The V-ATPase creates an H⁺ gradient, which acidifies the SV lumen (Forgac, 2007). For example, VIAAT uses these electrochemical gradients for SV (re)filling with glycine. I hypothesized that inhibiting the V-ATPase would lead to a breakdown of these gradients, resulting in a collapse of SV (re)filling and impaired synaptic transmission. Surprisingly, V-ATPase inhibition significantly reduces synaptic performance, but only after the drug is applied for more than 30 min (Figure 3.38). The remaining synaptic transmission and SV (re)filling are unlikely due to insufficient V-ATPase inhibition by 2 μ M Bafi. The IC₅₀ of Bafi is in the nanomolar range (Bowman *et al.*, 1988; Roseth *et al.*, 1995; Wang *et al.*, 2021). Similar concentrations have been used in previous studies (Zhou *et al.*, 2000; Harrison & Jahr, 2003; Granseth & Lagnado, 2008; Satake *et al.*, 2012; Arnold & Peters, 2025). The same result of a significant eIPSC decrease occurring only after 30 min or more could also be detected with higher Bafi concentrations (5 μ M, data not shown). Higher concentrations were also used in previous studies (Muller *et al.*, 2013; Song *et al.*, 2013). These studies all showed a Bafi effect after 0.5-2.5 h. These results are consistent with our results. Only one study demonstrated a total loss of mPSC amplitudes after treatment with 4 μ M Bafi for >2.5 h in hippocampal neurons (Song *et al.*, 2013). The others found only moderately reduced q values, similar to ours. These results demonstrate SV (re)filling upon V-ATPase inhibition is present across different synapses. Despite strong stimulation, the effect of V-ATPase inhibition was only moderate. This suggests that SV (re)filling with V-ATPase is required, but not essential, for synaptic transmission at MNTB-LSO synapses. Residual synaptic transmission suggests the presence of another mechanism for acidification of the SV lumen that ensures proper SV (re)filling at high activity at MNTB-LSO synapses.

4.6 An additional H⁺ pump system besides V-ATPase

This section referred to major result 2 of part 2. After V-ATPase inhibition, synaptic transmission still occurs even under the prevention of glycine (re)filling. This suggests the presence of an alternative H⁺ pump that could acidify the SV for SV replenishment at MNTB-LSO synapses. One candidate is the NHE6 exchanger, which is highly abundant in the brain (Masereel *et al.*, 2003; Hori & Takamori, 2022). Under resting conditions with pH values of 6.4 in the SV lumen and 7.2 in the cytosol, NHE mediates H⁺ transport outside the SV lumen in counter exchange with Na⁺. This occurs against the H⁺ gradient mediated by V-ATPase (Ouyang *et al.*, 2013; Takamori, 2016). After endocytosis, the SV has a high Na⁺ concentration similar to that of the extracellular fluid (153 mM). This results in a higher Na⁺ concentration in the SV lumen than in the cytosol. Under these conditions, I hypothesize that NHE reverses its antiporter function, transporting H⁺ into the SV lumen in exchange for Na⁺ and thereby mediating an H⁺ gradient. This gradient is then used by VIAAT for SV (re)filling. Furthermore, after SV endocytosis, the pH is 7.4 in the SV lumen and 7.2 in the cytosol, corresponding to 40 vs. 63 nM. With this difference in pH, H⁺ is more likely to be transported inside the SV lumen. To test the hypothesis, an NHE antagonist EIPA, was used during high-frequency stimulation at MNTB-LSO synapses. The concentration used was 100 μ M, which is the same concentration used in Goh *et al.*, 2011; Huang & Trussell, 2014; and Li *et al.*, 2020. The synaptic performance was drastically reduced after 10 minutes of EIPA wash-in. EIPA is a non-specific blocker of several NHE channels (NHE1-7; (Masereel *et al.*, 2003). Antidromic experiments revealed an influence of EIPA on AP conduction. I could not block NHE6 specifically, so changes in synaptic performance are more likely due to AP failures than to a failure of the presynaptic release machinery. Supporting this, q remained unchanged. If the NHE function were truly blocked, a reduction in q could be expected. In addition, after correcting for AP failures, eIPSC amplitudes did not differ from Ctrl eIPSC amplitudes. These results suggest that NHE inhibition by EIPA does not impair SV (re)filling. Further studies are needed to investigate whether another H⁺ pump, besides V-ATPase, is involved in supplying H⁺ for proper VIAAT function.

4.7 Preformed SVs from the reserve pool are unlikely to replenish the RRP

The idea that the residual synaptic transmission is maintained by preformed SVs from the reserve pool is unlikely. In Section 3.3, I inhibited glycine (re)uptake into the presynaptic terminals. With that, glycine availability decreased inside the presynaptic terminal, and VIAAT was not able to (re)fill SVs. Stimulated with Protocol 3 (marathon experiments), 30,660 stimulus pulses were applied with a total number of ~451,000 released SVs in Ctrl conditions. With an I_{RRP} of ~12 nA and q of ~19 pA, this results in an N_{RRP} of ~600 SV. This means during the whole

stimulation, the RRP was released ~750 times. In Section 3.4, I inhibited V-ATPase, which means the stop of SV acidification and VIAAT activity to (re)fill SVs. This means endocytosed SVs are unable to contribute to the replenishment of the RRP (Sankaranarayanan & Ryan, 2001). The corresponding values for Protocol 5, where 21,600 stimulus pulses were delivered, are ~109,000 released SVs, 430 SVs in the N_{RRP} , and the RRP has to be released ~250 times. To ensure sustained synaptic transmission at high activity, very robust replenishment mechanisms are essential.

SVs are stored in three different pools. The RRP, the recycling pool, and the reserved pool. The RRP amounts to 0.15-5% of the total number of SVs (Rizzoli & Betz, 2005; Nouvian *et al.*, 2006; Gan & Watanabe, 2018). The recycling pool contains ~5-20% of all SVs, while the reserve pool is the largest pool with ~80-90% of all SVs (Rizzoli & Betz, 2005). The total number of SVs in MNTB-LSO synapses is unknown (Cant, 1984; Helfert & Schwartz, 1986; Helfert *et al.*, 1992; Brunso-Bechtold *et al.*, 1994; Gjoni *et al.*, 2018). But for the calyx of Held synapse, a synapse one station before the MNTB-LSO synapses, data are known. The total number of SVs in this synapse is ~190,000 SVs. The RRP contains ~3000 SVs, the recycling pool ~7,000 SVs, and the reserve pool ~180,000 SVs, corresponding to 1.5%, 3.5%, and 95%, respectively (Rizzoli & Betz, 2005). To assume the same ratio in MNTB-LSO synapses, the total SV pool will contain ~42,000 (Section 3.3) or ~29,000 SVs (Section 3.4). With that 451,000 SVs or 109,000 SVs, cannot be released by available resources without replenishment. This means SVs have to be (re)filled. A model at MNTB-LSO synapses revealed that the total pool of SVs is released during the first 40 s of marathon experiments (Brill *et al.*, 2021). This value is consistent with the literature, which states that replenishment from the recycling pool via the recruitment of refilled SVs begins after tens of seconds (De Lange *et al.*, 2003; Rizzoli, 2014; Watanabe *et al.*, 2014). With that SV replenishment, because preformed SVs in the reserved pool are unlikely. Still, there is residual synaptic transmission, possible sources of the released glycine could be GABA, another inhibitory NT, or *de novo* synthesis of glycine.

4.8 Other possible glycine sources besides Asc-1

I increased the concentration to 50 μ M to ensure a full transporter block under slice conditions. However, synaptic transmission persisted even at 50 μ M, suggesting alternative glycine sources, because BMS is a selective inhibitor of Asc-1 (Brown *et al.*, 2014; Torrecillas *et al.*, 2019). Other glycine sources, such as uptake via different transporters or *de novo* synthesis, were considered (Eulenburg & Hülsmann, 2022a).

Many plasma membrane transporters that use glycine as a substrate have been identified (Pizzagalli *et al.*, 2021). The alanine, serine, cysteine, and threonine transporter 1 (ASCT1, *Slc1a4*) is a small AA exchanger in astrocytes, mediating bidirectional transport of D-serine and L-serine. ASCT1 KO mice exhibit decreased levels of D-serine and L-serine in the brain (Kaplan *et al.*, 2018). Glycine is a low-affinity substrate for ASCT1 (Kaplan *et al.*, 2018). Similar to ASCT1, the alanine, serine, and cysteine transporter 2 (ASCT2, *Slc1a5*) is also expressed in astrocytes and transports L-serine, L-glutamine, and glycine (Sakimura *et al.*, 2016; Liu *et al.*, 2023). Glycine transporter 1 (GlyT1, *Slc6a9*) is primarily found in astrocytes of the spinal cord and brainstem, especially in areas with high concentrations of glycinergic synapses (Zafra, 1995; Eulenburg & Hülsmann, 2022). Some evidence suggests that GlyT1 may also be expressed in glycinergic neurons of the brainstem (Raiteri *et al.*, 2005; Eulenburg & Hülsmann, 2022). However, this could not be confirmed by *in vivo* studies (Eulenburg *et al.*, 2018). The sodium-dependent neurotransmitter transporter 4 (NTT4, *Slc6a17*) transports neutral AAs such as glutamine, proline, alanine, and glycine (Parra *et al.*, 2008). A recent study by Nicoli *et al.*, 2025 demonstrated that NTT4 is localized to the plasma membrane of glutamatergic presynaptic terminals, suggesting a potential role in regulating the availability of extracellular AAs. The sodium-imino transporter (IMINO, *Slc6a20*) is known for its role in proline transport, though it was recently shown to transport glycine as well (Bae *et al.*, 2021). IMINO is expressed in both neurons and astrocytes, particularly in the cortical and hippocampus (Bae *et al.*, 2021). The *Slc38* family (SNAT1-5) is primarily localized to the plasma membrane of brain endothelial cells at the blood-brain barrier (Alves *et al.*, 2019). A study using HEK293 cells expressing *Slc38a5* confirmed this, identifying *Slc38a5* as a major L-serine transporter at the blood-brain barrier. *Slc38a5* primarily transports L-serine, with no significant transport of glycine (Radzishewsky *et al.*, 2023). All the mentioned transporters are either localized at astrocytes or absent from glycinergic synapses. In summary, so far Asc-1 is besides GlyT2 a glycine source at the presynaptic terminal at MNTB-LSO synapses.

De novo synthesis is not a possible source of glycine for very prolonged synaptic transmission. Whole-body *de novo* glycine synthesis via serine hydroxymethyltransferase has been estimated at ~15 mg/kg/h in humans (Alves *et al.*, 2019). This corresponds to ~0.075 mg/h in a 5 g P11 mouse, which equals ~1 μ mol/h glycine. This is far below the rate required to sustain

SV (re)filling during stimulation at 50 Hz stimulation. While presynaptic glycine concentrations after transmission are in the nanomolar range (~20-40 nM), the total glycine demand to support continuous SV (re)filling exceeds this level. Additionally, ~15mg/kg/h reflects systemic synthesis and not only brain synthesis. Axonal transport of glycine from the soma to the terminal would take hours. Furthermore, transcriptomic data from laser-dissected MNTB tissue showed that the expression levels of transcripts required for enzymes involved in *de novo* glycine synthesis are low or moderate at MNTB-LSO synapses (Brill *et al.*, 2021). Western blot analysis also showed only moderate expression levels of serine hydroxymethyltransferase (Neame *et al.*, 2019). Taken together, the data presented in this section suggest that alternative glycine transporters and *de novo* synthesis can be excluded.

4.9 Residual synaptic transmission is unlikely because of GABA

After prolonged stimulation with combined GlyT2 and Asc-1 inhibition, residual transmission remained at 64% (Figure 3.36). This raises the question of whether the residual transmission is due to the release of GABA at the MNTB-LSO synapses. To investigate this, I performed experiments in which I washed in 100 nM Stry for 15 min with 1 Hz stimulation after marathon experiments, similar to previously performed experiments (Fischer *et al.*, 2019). However, in my experiments, a significant decrease of eIPSC amplitudes to 27%, corresponding to ~200 pA, was observed at the end of the 1 Hz stimulation. VIAAT (also known as VGAT) can fill SVs with both GABA and glycine. Co-release of these two NTs could be reported at different synapses during development. (Jonas *et al.*, 1998; Kotak *et al.*, 1998; Nabekura *et al.*, 2004; Muller *et al.*, 2006). It was shown that MNTB-LSO synapses are GABAergic before hearing onset (Kotak *et al.*, 1998; Nabekura *et al.*, 2004). During early development in MNTB-LSO synapses, Cl⁻ currents are depolarizing because of high intracellular Cl⁻ concentrations of the LSO neuron. This excitatory effect plays a key role in the refinement of inhibitory connectivity (Kandler & Friauf, 1995; Kandler, 2004). Furthermore, Asc-1 KO showed significantly increased mIPSC amplitude and frequency in spinal cord motoneurons (Ehmsen *et al.*, 2016). Further, a switch from glycinergic to GABAergic upon GlyT2 inhibition was observed in the spinal cord in P12 mice. mIPSCs increased upon GlyT2 inhibition with ORG25543 (Rousseau *et al.*, 2008). Co-release of glycine and GABA by LSO neurons in the inferior colliculus in P19-35 mice occurs, a fraction of mIPSCs in control conditions were mixed and persisted during adulthood (Moore & Trussell, 2017). These studies gave a hint that the residual transmission after GlyT2 and Asc-1 block in marathon experiments could be GABAergic. My results confirm a prominent glycine component in the residual synaptic transmission. This is supported by other studies, the MNTB-LSO synapses underwent a functional and structural reorganization during early development (Kandler *et al.*, 2009). Before hearing onset,

MNTB-LSO synapses switch from co-releasing of GABA and glycine to only releasing glycine (Kotak *et al.*, 1998; Kandler, 2004; Nabekura *et al.*, 2004; Weisz *et al.*, 2016). Furthermore, it was shown that GABA is not a classical NT in MNTB-LSO synapses. A dominant glycine transmission in P12 LSO neurons was confirmed with immunohistochemistry and electrophysiology recordings (Fischer *et al.*, 2019). The remaining eIPSC amplitudes from 200 pA can be explained by the used Stry concentration of 100 nM. This concentration is insufficient to block GlyR completely, but the nonspecific block of GABA_A receptors is avoided (Jonas *et al.*, 1998). In a study that used a similar stimulation protocol of 1 Hz >20 min with 100 nM Stry they could also find remaining transmission ~50 pA, these eIPSC amplitudes could not be decreased further with GABAzin, an antagonist of GlyR (Fischer *et al.*, 2019). Higher concentrations of Stry blocks completely remaining transmission (unshown data, Fischer *et al.*, 2019). Furthermore, in MNTB-LSO synapses of P11 GlyT2 KO mice, no effect was detected on sIPSC amplitude and frequency after treatment with GABAzin (Ziegler, 2021). Previous data and my data show that synaptic transmission is unlikely because of a takeover of GABA.

4.10 Concluding remarks

This study provides new insights into the mechanism of glycine recycling and SVs (re)filling at MNTB-LSO synapses. When I started my PhD, the localization and functional contribution of Asc-1 under evoked synaptic activity were unexplored. I could demonstrate that Asc-1 is localized at MNTB terminals. My data show that Asc-1, in addition to GlyT2, supplies glycine to the presynaptic terminal in an activity-dependent manner in WT mice. I show that GlyT2 and Asc-1 together shape synaptic strength during sustained synaptic activity. Simultaneous GlyT2 and Asc-1 inhibition resulted in a reduction of synaptic strength. This indicates that Asc-1 supports glycine recycling and additionally acts cooperatively with GlyT2 to maintain sufficient glycine levels for SV (re)filling during high activity. Moreover, in GlyT2 KO mice, Asc-1 becomes functionally essential for maintaining residual glycinergic transmission.

Furthermore, the contribution of V-ATPase to sustained high activity at MNTB-LSO synapses was unexplored. My results suggest that V-ATPase is necessary for maintaining synaptic transmission under these conditions. However, the presence of residual transmission after V-ATPase inhibition suggests an additional H⁺ pump. One possible candidate is the Na⁺/H⁺ exchanger NHE6. NHE6 is located in MNTB terminals, which supports its potential role in SV acidification and SV (re)filling. The used NHE inhibitor EIPA is not selective for NHE6 and interferes with AP conduction. Due to these unspecific effects, the functional contribution to synaptic performance could not be directly assessed.

Together, these findings demonstrate that, in MNTB-LSO synapses, reliable information transfer is ensured by multiple complementary mechanisms, even under sustained activity.

4.11 Outlook

In this thesis, I addressed the impact of glycine recycling by GlyT2 and Asc-1 and I investigated glycine (re)filling in SVs. Further research is required to verify my conclusions and continue researching this topic. Ideas for further experiments are listed below.

- The organization and size of the SV subpools at the LSO synapse are poorly understood. Electron microscopy would allow the precise quantification of the number of SVs and the distribution of subpools.
- Fluorescence microscopy could be used to assess presynaptic uptake and release. After endocytosis, newly formed SVs remain labeled, which allows to determine activity-dependent SV recycling based on GlyT2 or Asc-1.
- Alternative inhibitors for GlyT2 and Asc-1 should be used, such as ORG25543 for GlyT2 or LuAE00527 for Asc-1 to confirm target specificity.
- If Asc-1 acts differently across synapse types, its expression and function at excitatory synapses in the LSO should be examined.
- To determine more precisely the exocytosis of SV in GlyT2 KO, membrane capacitance measurements should be done to detect empty-released SVs.
- Immunohistochemistry images should be acquired with identical laser settings to compare Asc-1 expression between WT and GlyT2 KO mice. To assess the potential upregulation of Asc-1 in GlyT2 KO mice as a compensatory response to the loss of GlyT2.
- Evoked stimulation protocols should be repeated on Asc-1 KO mice to evaluate synaptic performance.
- Assess developmental changes in the expression of Asc-1 to determine whether its role in synaptic transmission is age-dependent.
- Use fluorescently labeled glycine to directly monitor presynaptic uptake and SV (re)filling with Asc-1, GlyT2, and V-ATPase inhibitor.

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7 Index of abbreviations

AA	Amino acid
ACSF	Artificial cerebrospinal fluid
AN	Auditory nerve
AP	Action potential
Asc-1	Alanine-serine-cysteine Transporter 1
Bafi	Bafilomycin
BL	Baseline level
BMS	BMS-466442 (Bristol-Myers Squibb, Compound 1)
BSA	Bovine serum albumin
CC	Current clamp
Chal	Challenge
Cl ⁻	Chloride
CN	Cochlear nucleus
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
Ctrl	Control
d	Dorsal
DABCO	1,4-diazabicyclo[2.2.2]octane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EIPA	Ethylisopropylamiloride
eIPSC	Evoked inhibitory postsynaptic current
eIPSC ₁	First eIPSC amplitude
EtOH	Ethanol
freq	Frequency
GABA	γ -aminobutyric acid
GlyT2	Glycine transporter 2
GlyR	Glycine receptor
H ⁺	Proton
ILD	Interaural level differences
KO	GlyT2 Knock-out

I	Lateral
LSO	Lateral superior olive
m	Quantal content
mIPSC	Miniature inhibitory postsynaptic current
MNTB	Medial nucleus of trapezoid body
mRNA	Messenger ribonucleic acid
n	Number of cells
Na ⁺	Sodium
NHE	Na ⁺ /H ⁺ exchanger
N _{RRP}	Number of SVs in the RRP
NT	Neurotransmitter
P	Postnatal
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
Recov	Recovery
RR	Replenishment rate
RRP	Readily releasable pool
RT	Room temperature
s ₁₋₅	First 5 s
s ₅₀₋₆₀	Last 10 s (steady-state)
sIPSC	Spontaneous inhibitory postsynaptic current
SOC	Superior olive complex
Stry	Strychnine
SV	Synaptic vesicle
TM	Transmembrane helices
v	Ventral
VC	Voltage clamp
VGAT	Vesicular GABA transporter
VIAAT	Vesicular inhibitory amino acid transporter
WT	Wildtype

8 Curriculum vitae

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Pizzi, E., Hofmann, L. N., Singh, A., & Friauf, E. (2025). Robust inhibitory glycinergic transmission and the effect of bafilomycin, folimycin and EIPA: lessons from the auditory brainstem. Submitted to Frontiers in Neuroscience (submission process).

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DARLEGUNG ALLER BENUTZTEN HILFSMITTEL UND HILFESTELLUNGEN

Lina Hofmann

Glycinergic transmission at auditory brainstem synapses: The impact of glycine recycling and vesicle refilling

Ich erkläre hiermit, dass ich diese Arbeit selbst verfasst habe und keine weiteren Hilfsmittel als angegeben benutzt habe. Die Analyse der Daten erfolgte mit Hilfe von IGOR Pro (Version 6.37 und 9.01; WaveMetrics), Excel 2021 (Version 2105, Microsoft) und GraphPad Prism 9 (GraphPad Software). Die statistischen Analysen wurden ausschließlich unter Verwendung von GraphPad Prism 9 (GraphPad Software) durchgeführt. Die Arbeit wurde von Prof. Dr. Eckhard Friauf (Seniorprofessor, AG Tierphysiologie, RPTU Kaiserslautern-Landau) Korrektur gelesen. Ich habe ChatGPT und DeepL zur Übersetzungshilfe, sprachlichen Verbesserung und Stilverbesserung des Textes der Dissertation benutzt. Für eventuelle Fehler in dieser Arbeit trage ich alleinige Verantwortung.

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