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List of Abbreviations

Abbreviation	Full name
5-HT	serotonin
A	aspartate
ACh	acetylcholine
aCSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA-receptor
ANOVA	analysis of variance
AP	action potential
ATP	adenosine triphosphate
Bic	bicuculline
BoNT	botulinus neurotoxin
ceb	cellubrevin
DIV	days *in vitro*
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
E	glutamate
EDTA	ethylenediaminetetraacetic acid
E*n*	embryonic day *n*
EPSC	excitatory postsynaptic current
G	giga
GABA	γ-aminobutyric acid
Gln	glutamine
HBSS	Hank's balanced salt solution
het	heterozygous
IPSC	inhibitory postsynaptic current
KD	knock down
KO	knock out
M	mol/L
mGluR	metabotropic glutamate receptor
Munc-18	mammalian uncoordinated-18
NMDA	N-methyl-D-aspartate
NSF	N-ethylmaleimide-sensitive factor
NT	neurotransmitter
PBS	phosphate buffered saline
Phe	phenylalanine
P*n*	postnatal day *n*

Abbreviation	Full name
PPR	paired-pulse ratio
PSC	postsynaptic current
RNA	ribonucleic acid
rpm	rounds per minute
RRP	readily-releasable pool
S	serine
Sec1	secretory protein 1
SEM	standard error of the mean
shRNA	small hairpin RNA
siRNA	small interfering RNA
SNARE	soluble N-ethylmaleimide-sensitive-factor attachment receptor
stx	syntaxin
SV	synaptic vesicle
syb	synaptobrevin
syp	synaptophysin
syt	synaptotagmin
T	threonine
t-SNARE	target SNARE
TeNT	tetanus neurotoxin
TMD	transmembrane domain
V	volts
v-SNARE	vesicular SNARE
VAMP	vesicle associated membrane protein
VCHaT	vesicular acetylcholine transporter
VGLUT	vesicular glutamate transporter
VMAT	vesicular monoamine transporter
VTA	ventral tegmental area
WT	wild type

1. Introduction

1.1 The Chemical Synapse

The nervous system consists of millions of neurons that communicate with each other through synapses. The most abundant type of synapse is called “chemical synapse”. An electrical impulse (the action potential) in the axon of a presynaptic cell is transformed into chemical signals – the neurotransmitters (NTs). NTs are released into the synaptic cleft and bind to receptors on the postsynaptic cell. This leads to a conversion of the chemical signal back to an electrical signal or modulates the production of chemical messengers. The entire process needs to be extremely rapid, highly adaptable and very reliable. This work focuses primarily on processes in the presynapse, which will be described in the following paragraphs.

1.2 The Synaptic Vesicle Cycle – Overview

In the presynaptic terminal NTs are actively transported into synaptic vesicles (SVs), where they are stored until the SV fuses with the neuronal plasma membrane ((1) this number and the following in parenthesis refer to Figure 1). Several proteins are involved in this transport. The vesicular ATPase (V-ATPase), located in the vesicle membrane, hydrolyzes adenosine triphosphate (ATP) in order to pump protons into SVs, thus creating both a proton gradient and an electrical potential across the vesicular membrane. This electrochemical gradient is used by NT transporters to pump NTs into the vesicles. In order to be ready for fusion the SVs must get in close proximity to the active zone (2). With the help of numerous proteins the vesicle is tethered to the active zone where it “docks”, awaiting fusion. The cluster of SVs that has been primed for exocytosis is termed the readily-releasable-pool (RRP). Three proteins of the soluble N-ethylmaleimide-sensitive-factor attachment receptor family (SNARE) are thought to form the core of the fusion machinery: Synaptobrevin (also known as vesicle associated membrane protein, VAMP) is anchored with its transmembrane domain to the SV, whereas syntaxin and SNAP25 are tethered to the plasma membrane. If an action potential (AP) travels down the axon and reaches the synaptic terminal, voltage gated Ca^{2+}-channels open and Ca^{2+} flows into the cell (3). The binding of Ca^{2+} to the Ca^{2+}-sensing protein synatpotagmin (syt) leads to conformational changes in this protein, which in turn activates the fusion machinery. The two lipid bilayers of

the vesicle and the plasma membrane fuse and the NTs are being released into the synaptic cleft, where they can bind to postsynaptic receptors or activate autoreceptors (located in the presynaptic membrane) (4). The membrane patch of the emptied vesicle with its proteins specialized for fusion has to be retrieved in order to regulate the amounts of plasma membrane and to replenish the pool of synaptic vesicles at the active zone. This process of endocytosis is not part of my work and will therefore not be discussed in great detail in this thesis. Briefly, recent works suggest that vesicles are retrieved in an actin- and dynamin-dependend process called ultrafast endocytosis to form endosome-like structures (Watanabe *et al.*, 2013) (5). These bigger endosome-like structures bud of newly formed synaptic vesicles in a clathrin-dependent manner (Watanabe *et al.*, 2014) (6). After shedding off the clathrin coat the vesicles are ready to undergo a new cycle (7) (for detailed reviews on the SV cycle, see e.g. Südhof, 2004, Rizo and Rosenmund, 2008, Jahn and Fasshauer, 2012).

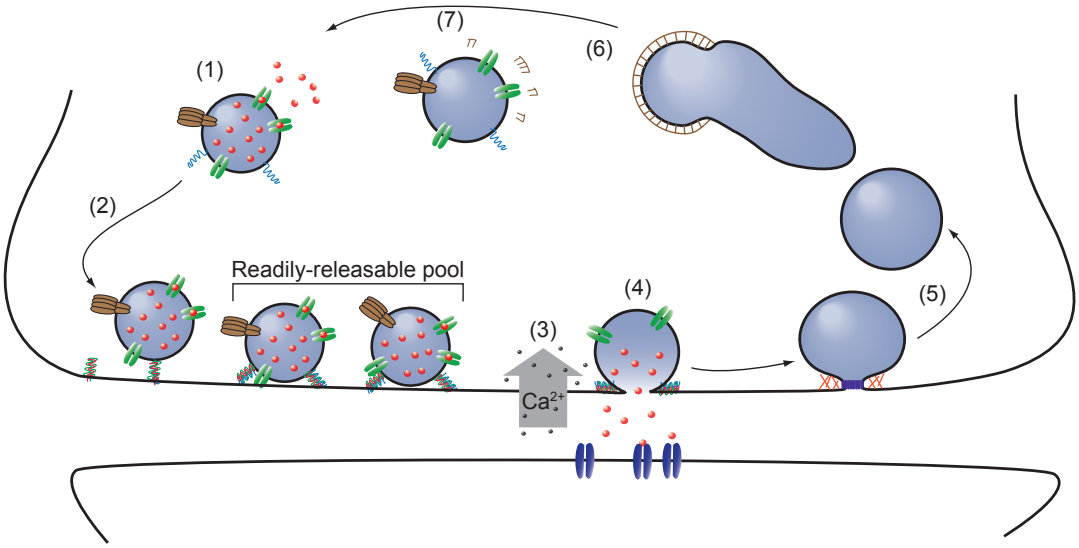

Figure 1: The synaptic vesicle cycle.
Vesicles are filled with NTs (red globes) by a concerted action of the V-ATPase (brown) and NT transporters (green) (1, see also text). The SVs dock at the presynaptic plasma membrane (2) and the SNARE proteins (blue, red and green spirals) form the SNARE complex. Upon Ca^{2+} influx (3) they zipper up from N- to C-terminus, thus fusing the two membranes. NTs are released into the synaptic cleft, where they bind to postsynaptic receptors (dark blue) to relay the signal (4). After fusion the membrane is being retrieved by dynamin- (blue) and actin- (red) dependent endocytosis (5). With the help of clathrin coats newly formed SVs bud of endosomal structures (6) and the cycle can restart (7). Many more proteins are involved in this process which have been omitted for visual clarity.

This work focusses on two different proteins in the vesicular membrane and their role in release: the glutamate transporters VGLUTs and the vesicular SNAREs. I will highlight their function in the following paragraphs.

1.3 Vesicular Glutamate Transporters

Glutamate is the major NT at excitatory synapses in the mammalian brain. Three different isoforms of glutamate transporters have been identified, which pump NTs from the cytosol into SVs. Vesicular glutamate transporters (VGLUTs) 1 and 2 have been discovered first and account for the glutamate transport in the majority of excitatory neurons throughout the brain (Figure 2). VGLUT1 is predominantly expressed in the cerebral cortex, hippocampus and cerebellar cortex (Ni *et al.*, 1995 and Bellocchio *et al.*, 1998). VGLUT2 on the other hand transports glutamate into SVs in neurons of the thalamus, hypothalamus, colliculi and the brainstem (Hisano *et al.*, 2000, Fremeau *et al.*, 2001, Herzog *et al.*, 2001 and Varoqui *et al.*, 2002). The population of neurons expressing VGLUT3 is smaller and very diverse (Fremeau *et al.*, 2002, Gras *et al.*, 2002, Schafer *et al.*, 2002, Takamori *et al.*, 2002). It is present in subgroups of primarily glutamatergic neurons of the habenula, hypothalamus, raphe, olfactory tubercles and in inner hair cells of the cochlea (Ruel *et al.*, 2008, Seal *et al.*, 2008, Commons, 2009, Jackson *et al.*, 2009). Interestingly, most of the VGLUT3-expressing neurons were not considered to be primarily glutamatergic. Serotonergic (5-hydroxytryptamine (5-HT)) neurons in raphe nuclei, dopaminergic neurons of the ventral tegmental area (VTA), cholinergic striatal neurons as well as a subpopulation of hippocampal and cerebral GABAergic basket cells express VGLUT3 (Herzog *et al.*, 2004, Somogyi *et al.*, 2004, Gras *et al.*, 2005). It was soon speculated that the expression of a glutamate transporter in these neurons might result in glutamate co-release in addition to the primary NT.

The "classical" action of a VGLUT is to use the energy created by an electrochemical potential ($\Delta\mu$) across the vesicle membrane to pump glutamate molecules from the cytoplasm into the SV (Figure 3). $\Delta\mu$ is produced by the vacuolar-type H^+-ATPase (Maycox *et al.*, 1988). It hydrolyzes ATP and harvests the energy to pump protons into SVs. Whereas the cytoplasm has a pH of 7.4 the pH inside of SVs is around 5.7 (Miesenböck *et al.*, 1998). The concentration gradient (ΔpH) causes also an electrical potential ($\Delta\psi$) across the vesicular membrane. Glutamate is anionic at neutral pH, so its transport by VGLUTs depends largely on $\Delta\psi$ and less on

ΔpH (Edwards, 2007). If a glutamate molecule is exchanged for nH^+ (the exact stoichiometry of coupling is not known) it creates an efflux of $n + 1$ charge. This would produce an imbalance between charge and protons and implicates additional mechanisms to balance the two components of Δμ.

Interestingly, the transport of the various NTs depends to different amounts on Δψ and ΔpH (Edwards, 2007). Monoamines (e.g. Dopamine, 5-HT) and acetylcholine (ACh) are positively charged in the cytosol. They are transported into the SV lumen in exchange for 2 protons by the vesicular monoamine transporter (VMAT) and the vesicular ACh transporter (VAChT), respectively (Johnson *et al.*, 1981, Knoth *et al.*, 1981, Nguyen *et al.*, 1998). The exchange

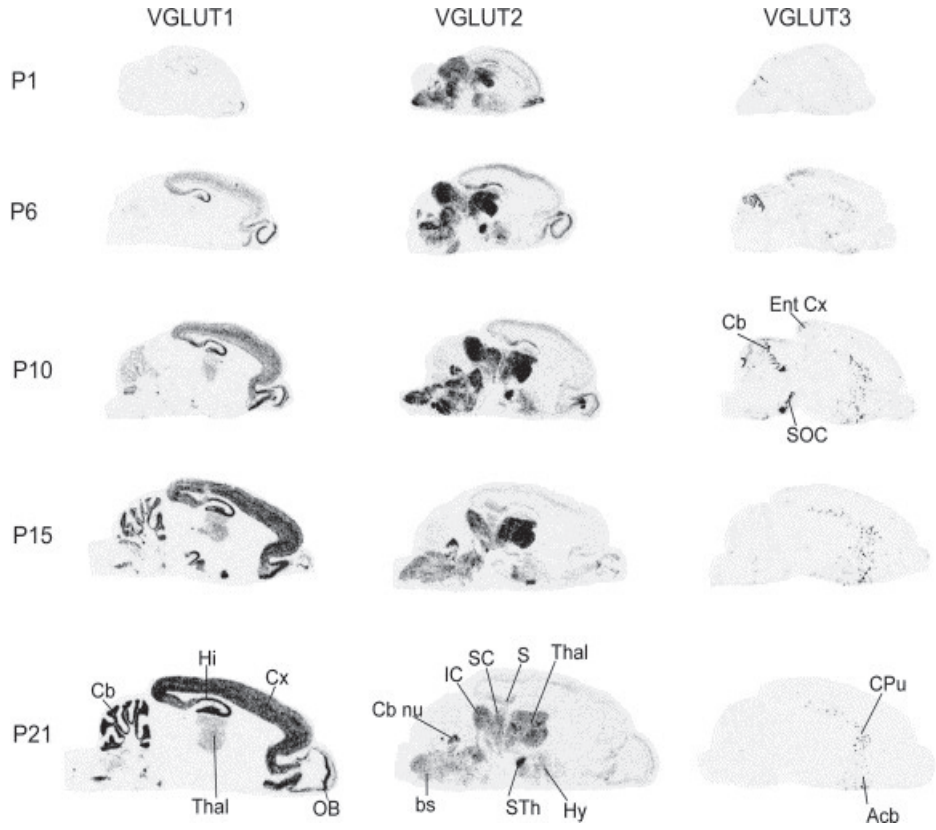

Figure 2: mRNA expression pattern of VGLUT1, 2 and 3 in a developing rat brain.
Distribution of VGLUT1–3 mRNA determined by radioactive in situ hybridization of sagittal sections from rat brain at different ages after birth (P1–P21), with [S^{35}]-labeled antisense oligonucleotides specific for VGLUT1–3 nucleic sequences. Acb, nucleus accumbens; bs, brainstem; Cb, cerebellum; Cb nu, deep cerebellar nuclei; CPu, caudate putamen; Cx, cerebral cortex; Ent Cx, entorhinal cortex; Hi, hippocampus; Hy, hypothalamus; IC, inferior colliculus; OB, olfactory bulb; S, subiculum; SC, superior colliculus; STh, subthalamic nucleus; Thal, thalamus nuclei; and SOC, superior olivary complex. Gras *et al.*, 2005

leads to a net outflow of only +1 charge but two H^+ and thus depends more on ΔpH than on Δψ. The zwitterions γ-aminobutyric acid (GABA) and glycine are electrically neutral and their transport by the vesicular GABA transporter (VGAT) depends equally on ΔpH and Δψ (Kish *et al.*, 1989, Hell *et al.*, 1990).

One way of dissipating the increasing Δψ across the SV membrane in order to maintain ATPase function is by transporting chloride anions into the vesicle (Maycox *et al.*, 1988, Tabb *et al.*, 1992). This can be achieved by chloride channels, which might potentially work as Cl-/H+ exchangers (Stobrawa *et al.*, 2001, Picollo and Pusch, 2005). Interestingly, it was found that VGLUTs themselves exhibit a chloride conductance (Juge *et al.*, 2006, Schenck *et al.*, 2009) and latest results suggest that the presence of the V-ATPase and VGLUT molecules is sufficient for efficient glutamate uptake into SVs (Preobraschenski *et al.*, 2014)

1.4 Vesicular Synergy

As mentioned above several types of neurons have been found to co-express VGLUTs with other NT transporters, e.g. VMAT and VAchT. Glutamate transport into SVs dissipates Δψ and therefore creates an increased ΔpH. This could potentially promote uptake of cationic NTs like dopamine, 5-HT and ACh, which rely largely on ΔpH. Indeed, several studies show this

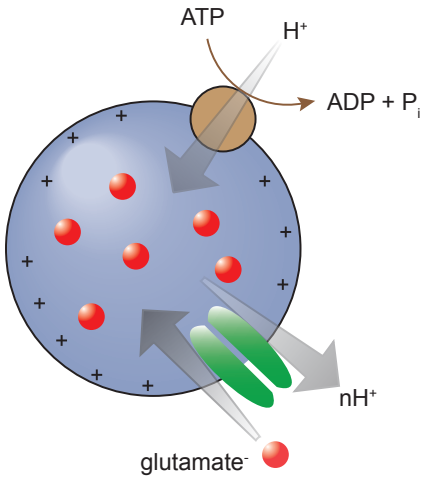

Figure 3: VGLUTs use an electrochemical gradient to pump glutamate into synaptic vesicles
The vesicular ATPase (brown cycle) hydrolyzes ATP to transport protons into SVs and acidifies the lumen. This creates an electrochemical gradient which is used by VGLUTs (green) to pump glutamate (red globes) into the vesicles. The VGLUTs act as antiporters: For each glutamate molecule an unknown number of protons leaves the SV (modified from Chaudhry *et al.*, 2008).

"synergistic" effect of glutamate: Cholinergic interneurons in the striatum have reduced levels of vesicular ACh and show increased basal and cocaine-stimulated locomotor activity in absence of VGLUT3 (Gras *et al.*, 2008, Nelson *et al.*, 2014). A conditional knockout of VGLUT2 in dopaminergic neurons leads to a reduction in dopamine storage and release in VTA neurons (Hnasko *et al.*, 2010). Also, 5-HT neurons from the raphe nuclei have a significant decrease in NT release if VGLUT3 is knocked out (Amilhon *et al.*, 2010). Whether the synergistic effects are solely dependent on glutamate transport into the vesicle or if also the chloride and phosphate conductance of VGLUTs play a role is not yet understood.

Studies about synergy between GABA and glutamate are inconsistent. Zander *et al.*, 2010, report a 15 % decrease in GABA uptake after blocking VGLUT function in a SV preparation from whole brain. In contrast, a recent paper on the function of VGLUT3 in refinement of an inhibitory map negates synergy between GABA/glycine and glutamate (Case *et al.*, 2014). Since GABA (and glycine) are neutral zwitterions the acidification of SVs by VGLUTs seems to be less important than in the case of the positively-charged ACh, dopamine and 5-HT.

1.5 Glutamate co-release

Besides the possible role for glutamate in vesicular synergy there is the obvious consequence of co-release once these vesicles filled with two kinds of NT fuse with the membrane. Whether there is a physiological function for glutamate co-release has been subject to numerous studies: Using optogenetics it could be shown that dopaminergic neurons in the nucleus accumbens release glutamate *in vivo* (Tecuapetla *et al.,* 2010). The co-release was mediated by VGLUT2 as the glutamate signal disappeared in VGLUT2 KO animals (Stuber *et al.*, 2010). However, there is an ongoing debate whether the release from glutamate is from the same site as the dopamine release or whether it is a locally separated form of co-release (see e.g. Sulzer *et al.*, 1998 and Zhang *et al.*, 2015).

Co-release of glutamate and 5-HT was observed in autaptic culture (Johnson 1994) and *in vivo* using optogenetic stimulation of axonal fibres from mouse raphe nuclei (Varga *et al.*, 2009). The finding suggests that the signals mediated by glutamate and 5-HT, respectively, operate at different time scales with a fast, ionotropic component and a slower, metabotropic component (El Mestikawy *et al.* 2011).

Besides its role in promoting vesicular filling of ACh glutamate was also shown to activate ionotropic receptors on medium spiny neurons in striatal interneurons (Gras *et al.* 2008). The co-release disappeared in the absence of VGLUT3, which is normally expressed in these terminals (Higley *et al.*, 2011). Neurons of the medial habenula release glutamate and ACh in the interpeduncular nucleus of the midbrain (Ren *et al.*, 2011). Interestingly, transmission modes for the two NTs were different. Whereas brief photostimulation in cholinergic neurons expressing channelrhodopsin 2 led to excitatory postsynaptic currents mediated by ionotropic glutamate receptors, a tetanic stimulation protocol activated nicotinic receptors through ACh release. The two transmitters could thus have different roles in signaling.

Co-release of glutamate and GABA – two NTs that supposedly act oppositely – was particularly often observed in neuronal development. In the lateral superior olive glutamate release from GABA/glycinergic neurons is necessary for synaptic silencing and strengthening of GABA/glycinergic connections and is mediated by VGLUT3 (Noh *et al.*, 2010). Furthermore, single mossy fibre giant boutons in rat dentate gyrus – usually releasing glutamate – release GABA during development (Beltran *et al.*, 2012). At this age GABA acts excitatory and could potentially have a trophic effect to pyramidal cells (Ben-Ari *et al.*, 1994).

The previous paragraphs summarize only a few of the various functions that VGLUTs carry out in the nervous system (for reviews see also e.g. El Mestikawy *et al,* 2011 and Hnasko *et al.*, 2012). In the next part of this thesis I want to continue along the synaptic vesicle cycle towards fusion, drawing the attention to another type of SV protein, which was also part of my studies.

1.6 SNARE-mediated fusion

The three SNARE proteins synaptobrevin (syb), syntaxin (stx) and SNAP25 are considered to be the core machinery of SV fusion in the mammalian brain (Sollner *et al.*, 1993, Weber *et al.*, 1998). They share a common coiled-coil stretch, which is called the SNARE motif and which serves as the interaction site between the three proteins (Sutton *et al.*, 1998, Poirier *et al.*, 1998). The SNARE motifs of syb and stx are connected to the carboxy-terminal transmembrane region via a short linker (Trimble *et al.*, 1988, Elferink *et al.*, 1989, Bennett *et al.*, 1992). SNAP25 on the other hand has two SNARE motifs, which are connected by a

short linker sequence. This sequence is palmitoylated and tethers SNAP25 to the plasma membrane (Chapman *et al.*, 1994). Whereas syb is located to SVs with its transmembrane domain (= a v-SNARE), stx (like SNAP25) is anchored to the plasma membrane (t-SNAREs, where t stands for target).

Even though there is still an ongoing debate about the molecular steps proceeding SV fusion (see Meriney *et al.*, 2014, for a detailed comparison of different models for Ca^{2+}-dependent fusion at nerve terminals) I will describe the most widely accepted model in this paragraph: Vesicle fusion requires the three SNARE proteins to form a tight complex with their α-helical SNARE motifs. In the mammalian brain the major SNARE isoforms are syb2 (also called VAMP2), stx1 and SNAP25. To form a SNARE complex syb2 and stx1 provide one SNARE motif each, whereas SNAP25 provides two. Since syb2 is located to SVs and stx1 and SNAP25 are anchored to the plasma membrane it is thought that the formation of the SNARE complex from the proteins' N- to C-terminus brings the two membranes in close proximity. The zippering of SNAREs is arrested in this metastable *trans*-state by yet unknown mechanisms, which involves a number of additional proteins from the SM family (SM stands for Sec1/Munc18-like). The number of SNARE complexes per vesicle, which are needed for fusion is still under debate and ranks from 1 to 15 complexes (Montecucco *et al.*, 2005, van den Bogaart *et al.*, 2010). Vesicles that are closely tethered to the plasma membrane and ready for fusion are called "primed vesicles". When an AP reaches the terminal, voltage-gated Ca^{2+}-channels open and Ca^{2+} rushes into the neuron at the active zone. The Ca^{2+}-sensor synaptotagmin1 is associated to the SNARE complex. Binding of Ca^{2+} to the C2 domains of syt1 leads to conformational changes in the protein structure causing an "unclamping" of the SNARE complex. This in turn results in full zippering of the SNARE complex, an exergonic reaction producing enough energy to fuse the two opposing membranes. NTs stored in the SV are released in the synaptic cleft. The SNAREs are now in a *cis*-state and can be disassembled by the N-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment proteins (SNAP) in an ATP consuming process for reuse (Wilson *et al.*, 1989, Clary and Rothman 1990).

1.7 Studies of NT release in absence of SNAREs

The importance of SNAREs for SV fusion has been shown in various studies for all three SNARE proteins and various organisms. In the nematode *caenorhabditis elegans* the t-SNARE syntaxin is essential for neurotransmitter release (Saifee *et al.*, 1998). Syntaxin null mutants in the fruit fly exhibit no neurotransmission except for rare asynchronous fusion events (Schulze *et al.*, 1995, Saitoe *et al.*, 2001). In mammals there are two functionally redundant isoforms of syntaxin 1 (Bennett *et al.*, 1992, Bennett *et al.*, 1993). Knocking out either syntaxin 1A (Fujiwara *et al.*, 2006) or 1B (Mishima *et al.*, 2014) results in only minor defects in synaptic release because of mutual redundancy. Hippocampal neurons deficient in syntaxin 1B show unaltered evoked responses but have a decreased frequency in excitatory and inhibitory spontaneous events (Mishima *et al.*, 2014).

SNAP25 dimerizes with syntaxin before the complex binds to v-SNAREs (Rickman *et al.*, 2004). Lack of SNAP25 inhibits neurotransmitter release in *Caenorhabditis elegans* (Miller *et al.*, 1996). In *drosophila melanogaster* an ablation of SNAP-25 impairs synaptic transmission but compensatory effects of SNAP homologs have been observed (Niemeyer and Schwarz, 2000, Vilinsky *et al.*, 2002). Homozygous knockout of SNAP25 in mice is embryonically lethal (Washbourne *et al.*, 2002). Evoked release at neuromuscular junctions is abolished, however spontaneous release can still be observed. These results were confirmed in cultured neurons as well as in cortical slices from embryonic SNAP25 knockout mice: The lack of SNAP25 has a greater impact on evoked than on spontaneous NT release (Tafoya *et al.*, 2006, Bronk *et al.*, 2007).

Synaptobrevin 2 is one of the most abundant vesicle proteins (Takamori *et al.*, 2006). Loss of neuronal-synaptobrevin – the syb2 homolog in *drosophila* – abolishes evoked release entirely and reduces spontaneous release significantly (Deitcher *et al.*, 1998). In the nematode there are two v-SNARE isoforms, namely snb-1 and snt-1. Both, single knockout of snb-1 and double knockout of snb-1 and snt-1 lead to severe defects in synaptic transmission (Nonet *et al.*, 1998). In mouse the syb2 knockout has been studied in high-density cultures of hippocampal neurons (Schoch *et al.*, 2001). Even though Ca^{2+}-mediated evoked release is virtually absent without syb2, the pool of readily-releasable vesicles (RRP) is only reduced to around 10% of wildtype levels and spontaneous fusion of SVs can still be observed. The additional depletion of cellubrevin (VAMP3) in these cells revealed no aggravated phenotype, which speaks against a

compensatory effect of this v-SNARE in the syb2 KO background (Deak *et al.*, 2006). A possible explanation for the aggravated effect on evoked release compared to spontaneous release was recently introduced by Revelo and colleagues: They showed that vesicles fusing spontaneously carried less syb2 and instead more VAMP4 compared to vesicles released after stimulation (Revelo *et al.*, 2014). The strength of evoked neurotransmission and its balance with spontaneous neurotransmission depends largely on the linker region between the SNARE domain and the transmembrane region of syb2. Inserting only a few extra residues in this region abolishes evoked release completely, whereas spontaneous release is still maintained (Deak *et al.*, 2006).

1.8 Neurotoxins and SNAREs

A different approach to study the role of SNAREs in neurotransmission is the use of neurotoxins. The bacteria *Clostridium tetani* and *Clostridium botulinum* produce toxins that specifically cleave the SNARE proteins and thereby abolish NT release. The light chain of tetanus toxin cleaves syb2 (Schiavo *et al.*, 1992). This impairs evoked neurotransmission while decreasing but not eliminating spontaneous neurotransmission (Herreros *et al.*, 1995, Sweeney *et al.*, 1995). Botulinum neurotoxin A cleaves SNAP-25 (Blasi *et al.*, 1993I). Studies in organotypic slices from hippocampus showed a reduction of both action potential-dependent and spontaneous neurotransmission (Capogna *et al.*, 1997). Syntaxin is cleaved by botulinum neurotoxin C, which can also cleave SNAP25 at a much lower efficiency (Foran *et al.*, 1996). Application of the toxin to squid axons leads to complete inhibition of neurotransmission (O'Connor *et al.*, 1997). Experiments carried out using these toxins have the disadvantage that the blocking is rarely complete and residual release might be caused by uncleaved SNARE proteins.

1.9 Whole-cell voltage clamp recording

The primary method used in this work to assess changes in synaptic release parameters is the patch-clamp technique: A glass micropipette with a tip diameter of approximately 2-4 μm is gently pressed against a membrane patch of the neuron. By additional suction the rim of the pipette tip and the plasma membrane come into close proximity and form a high resistance electric barrier of several GΩ (called a "giga seal"). A short and strong pulse of negative pressure applied through a mouth piece connected to the micropipette ruptures the plasma membrane patch

under the pipette, providing access to the neuron. The pipette contains an Ag/Ag^+-electrode and is filled with a solution, having a similar composition like the neuronal cytoplasm (the Cl^- concentration is an exception, explained below). Electric current can pass from the microelectrode through the internal solution into the cell. Through open membrane channels it is relayed to the external buffer and finally to another Ag/Ag^+-electrode placed in the buffer. Both electrodes are connected to a feedback amplifier, which "clamps" the voltage at a set value by injecting current into the cell. The injected current is measured, amplified and can be monitored on a computer.

1.10 The autaptic cell culture system

A big part of the experiments done in this work take advantage of the autaptic cell culture system developed by Bekkers and Stevens, 1991: After dissection of the mouse brain a low concentration of neurons is plated onto small islands of astrocytes in a culture dish, in a manner that approximately one neuron grows on an island. The neuron continues its development in the incubator and – lacking any partner neurons – will start making synapses onto itself (so-called autapses). By "patching" onto these neurons pre- and postsynaptic function of a neuron can be

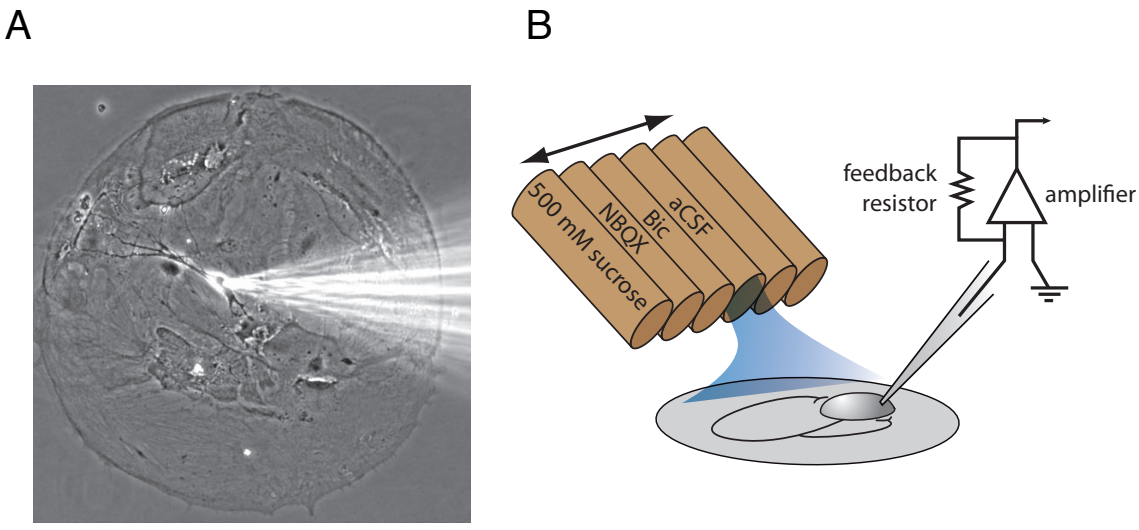

Figure 4: The autaptic cell culture system.
A: Brightfield image of an autaptic neuron (center) on an island of astrocytes (grey cirlce) patched with a glass pipette (bright triangular shape on the right). B: Cartoon of our fast flow system (brown) and the experimental setup with an autaptic neuron on an astrocyte island and a patch electrode (grey). The position of the tubes can be moved and opening of the corresponding valve leads to application of aCSF, 500 mM sucrose, or various other drugs directly onto the cell. Scheme of the feedback amplifier in the top right.

monitored by a single recording micropipette and the experimenter has control over both the electrical in- and output of the neuron (Figure 4). Since all synapses can be measured at the same time and individual neurons can be addressed, the method is highly quantitative. In combination with the usage of neurons from genetically modified animals and rescue experiments with lentiviral constructs it is an important technique for structure-function analysis. Another advantage of the autaptic culture (and other cell culture systems in general) is the accessibility of the neurons. We use a fast-flow system to apply different drugs directly onto the neuron (Figure 4B). The solution exchange takes only milliseconds and enables studies of kinetics and synaptic properties like the readily-releasable pool (explained below). Disadvantages are the systems inherent lack of neuronal network activity and hence the possibility of reporting artifacts being caused by the solitude of the neuron rather by other external factors (see for example Liu *et al.*, 2009).

1.11 Electrophysiological protocols used in this study

Recordings with a micropipette from an unstimulated neuron show recurring small deflections from baseline – so called "minis". They are caused by the spontaneous and Ca^{2+}-independent fusion of synaptic vesicles with the plasma membrane. The NT content binds to post-synaptic receptors, generating the recorded currents. The charge that is relayed by a single vesicle is called the quantal size *q* (Del Castillo and Katz, 1954). Its size (and the kinetics of the signal) depends on the amount and type of NT in a single vesicle and the number and type of postsynaptic receptors. The inter-event interval (usually measured in its reciprocal form, the frequency) depends on the number of synapses and the release probability of the vesicles in the terminals (P_{vr}).

If an action potential reaches the synaptic terminal (e.g., by depolarizing the neuron from -70 mV to 0 mV for 2 ms with a patch pipette) voltage-gated sodium and potassium channels open, which in turn leads to the opening of voltage-gated calcium channels at the active zone. Calcium rushes into the terminals promoting the synchronous fusion of multiple vesicles at various synapses (for mechanistic details, see 1.2). The size of the recorded postsynaptic current (PSC) depends on the quantal size, the total number of vesicles ready to be released and their release probability.

The number of readily-releasable vesicles (the so-called "readily-releasable pool" (RRP)) can be assessed with a protocol developed in our laboratory (Rosenmund and Stevens, 1996). Using the fast-flow system (Figure 4B) the neuron is exposed to a hypertonic solution containing 500 mM sucrose for several seconds. This osmotic shock leads to the release of all fusion-competent vesicles and the RRP size can easily be determined. Dividing the charge of the evoked response by the total charge of all fusion-competent vesicles results in the P_{vr}.

Another method used in this thesis to compare release probabilities between neurons and to assess changes in short-term plasticity is the paired-pulse protocol. The cell is depolarized twice with an interstimulus interval of 25 ms (for glutamatergic neurons) or 50-100 ms (for GABAergic neurons, respectively). Comparing the size of the first to the second pulse provides insights into the release parameters: A neuron with a first pulse being bigger than the second pulse (paired-pulse ratio (PPR) < 1) is called depressing. A high P_{vr} leads to release of a great number of vesicles during the first stimulus, reducing the amount of fusion-competent vesicles available for the second pulse. Contrarily, if the P_{vr} is low, fewer vesicles are released during the first pulse. Calcium accumulates at the terminal and thus increases the P_{vr} for the second pulse. This results in facilitation and a PPR > 1.

To measure changes in short-term plasticity I also exposed the patched cells to high frequency trains. Using a stimulation frequency of 10 Hz for 5 s (= 50 pulses) one can easily compare the rate of depression or facilitation between neurons.

1.12 Inhibitory and excitatory synapses

A synapse is called excitatory, if an AP in the presynaptic terminal increases the likelihood of an AP in the postsynaptic neuron. This is the case if binding of NTs to postsynaptic receptors results in a reversal potential that is higher than the AP threshold in the postsynaptic cell. Conversely, if binding of NTs results in a reversal potential that is below the threshold of AP firing, it will make firing less likely and is thus inhibitory (Purves *et al.*, 2001). The NT glutamate generally activates receptors that are non-selectively permeable to cations, resulting in an increased likelihood for AP firing. Glutamate is thus often called an "excitatory NT", even though according to the definition mentioned above this is technically incorrect. In the case of GABA (often titled as "inhibitory NT") this subtle discrimination becomes more relevant: During development hippocampal neurons have a high intracellular chloride concentration.

Binding of GABA to postsynaptic $GABA_A$ receptors opens the chloride conductance of these channels. Due to the high internal chloride concentration, chloride will leave the cell, leading to a more positive resting potential and increasing the likelihood for AP firing. Thus, in these neurons GABA acts excitatory. Later in development the intracellular chloride concentration is lower than the outside concentration and binding of GABA to $GABA_A$ receptors is inhibitory (reviewed in Ben-Ari, 2002).

Excitatory and inhibitory synapses are usually morphologically distinguishable. Type I synapses (also termed "asymmetric synapses") are often glutamatergic. They have a wider synaptic cleft, prominent presynaptic dense projections and the post-synapse is denser than in type II ("symmetric") synapses. Whereas type I synapses are often located to the spines of dendrites, type II synapses are more frequently found on the neuronal cell body (Kandel *et al.*, 2000).

1.13 Recordings with high chloride internal solution

The hippocampal and striatal neurons I recorded from at DIV 11-16 have a low intracellular chloride concentration. As mentioned above, under these conditions GABA is inhibitory. By filling the patch pipette with a solution containing a high chloride concentration, chloride ions diffuse along their concentration gradient into the cell once the pipette has broken into the neuron. If GABA binds to $GABA_A$ receptors of this cell, their opening leads to an eflux of chloride rather than an influx like in the naive state. This has the advantage that $GABA_A$ receptor-mediated currents can robustly be recorded as negative currents at a holding potential of -70 mV.

1.14 Aims of this work

Our goal was to investigate the effect of different protein paralogs on synaptic neurotransmitter release. The mammalian brain usually operates with more than one version of a protein, to cope with the various demands to regulate neurotransmission. We focused on two essential vesicular proteins – synaptobrevin and VGLUT – and investigated, how different paralogs of these proteins change properties of neurotransmitter release.

Previous findings that vesicle fusion persists in the absence of the v-SNARE synaptobrevin 2 were puzzling and we wanted to test if another synaptobrevin isoform might be responsible for remaining release. We decided to study the syb2 KO in the autaptic cell culture system, in order to assess SV fusion in individual neurons. Furthermore, we did immunoblots from neuronal mass cultures to test for the presence of the v-SNARE paralog syb1 in these neurons. To compare the release properties of syb1 and syb2 we expressed both isoforms individually in autaptic syb2 KO neurons and performed patch-clamp experiments on them. Lastly, we knocked down syb1 levels in syb2 KO mass culture to determine if the residual release was caused by this v-SNARE.

In the second part of this work we wanted to find out if VGLUT3 – the least-expressed VGLUT paralog throughout the brain – can induce glutamate co-release in GABAergic neurons. We expressed the glutamate transporter in cultured GABAergic neurons of the striatum to induce GABA/glutamate co-release. Using glutamate and GABA receptor antagonists we analyzed the decay kinetics of miniature events, thus determining if the two NTs are stored in the same vesicles. Next, we were wondering if co-release of glutamate increases the amount and clustering of AMPA receptors in GABAergic neurons. In a last set of experiments we compared glutamate/GABA co-release in different culture systems to determine if the neuronal network influences the capabilities to detect co-release.

2. Material and Methods

2.1 Neuronal culture

All experiments involving animals were performed according to the regulations of Berlin-animal experiment authorities and the animal welfare committee of the Charité Berlin.

The majority of experiments described in this thesis take advantage of the autaptic cell culture system developed after a protocol described by Bekkers and Stevens, 1991. The required steps are summarized in the following section:

2.1.1 Preparation of plates

Glass coverslips of 30 mm diameter were cleaned in 1 M HCl over night on a shaker, rinsed several times with water and finally stored in 95 % ethanol. Before placing the coverslips into 6-well plates, they were briefly flamed to remove the alcohol. They were then coated with liquefied 0.15 % agarose type IIa (Sigma-Aldrich, Germany), a substrate upon which cells are reluctant to attach. Next, a collagen/poly-D-lysine (Gibco Life Technologies, Germany and Sigma Aldrich, Germany) coating mixture was applied using a custom-built stamping device to make microislands of uniform size (~200 μm diameter, 500 μm space between spots). For high density "continental" cultures the entire plate was coated with collagen/poly-D-lysine substrate. Plates were sterilized using UV light and stored at room temperature until further use.

2.1.2 Preparation of astrocyte feeder layers

Newborn C57/BL6-N mice were decapitated, the brains removed and quickly placed into 4 °C cooled Hank's Balanced Salt Solution (HBSS, Gibco Life technologies, Germany). Cortices were dissected out and digested with 0.05 % trypsin-EDTA (ethylenediaminetetraacetic acid) for 20 minutes at 37 °C in a shaker at 800 rounds per minute (rpm). The digested cortices were then placed in fresh full medium (see Table 1) and slowly tritruated to obtain a solution containing only single cells. 400 μl cell suspension was transfered into a flask containing 13 ml pre-warmed full medium. After 1-2 weeks at 37 °C in an incubator the astrocyte flasks were vor-

texed for approximately 1 minute, washed with phosphate-buffered saline (PBS) and detached from the flask by adding 4 ml of 0.05 % trypsin-EDTA. To stop the digestion process 10 ml full medium was added, the cells were tritruated and transferred into tubes for centrifugation. After centrifugation for 5 min. at room temperature and 800 rpm the cells were counted and ready for plating.

For neuronal autaptic culture approximately 50 000 astrocyte cells from cortex tissue of postnatal day 0-2 (P0-2) mice were plated per individual well of 6-well plates. Only astrocytes falling onto the coated microislands can survive and form the substrate for the neurons. After growing for one week in Dulbecco's Modified Eagle's Medium (DMEM (Gibco Life Technologies, Germany)) cell division was stopped with 5-fluoro-2-deoxyuridine (FUDR)-containing solution (81 μM FUDR, 204 μM uridine (Sigma-Aldrich, Germany)).

2.1.3 Seeding of neurons

For syb2 KO cultures male and female mouse embryos (18.5 days old (E18.5), C57/BL6 background) were delivered by cesarean section. WT mice and mice carrying non-lethal mutations were sacrificed at P0-2. For the striatal culture newborn WT C57/BL6N mice were sacrificed. Brains were dissected out and placed in 4°C cooled HBSS. Hippocampus or striatum, respectively, were removed and incubated in a papain-containing solution (Table 1; for pH adjustment carbogen gas was applied to the solution for 5 min.) to dissociate the cells. After 45 min. the reaction was stopped by gently exchanging the solution to an inactivating solution containing 2.5 mg albumin and 2.5 mg trypsin-inhibitor (Sigma-Aldrich, Germany) in 5 % fetal calf serum (FCS). After 5 min. the medium was replaced by our neuronal growth medium (neuronal basal A (NBA), supplemented with B27, Glutamax (Gibco Life Technologies, Germany), and penicillin/streptavidin (Roche, Germany)) and the cells were counted in a Neubauer counting chamber. For neuronal autaptic cultures 2 500 hippocampal or 3 000 striatal neurons were seeded. For immunocytochemical analyses 50 000 - 100 000 neurons were seeded per well on astrocyte-layered 12-well (22 mm diameter) or 6-well plates, respectively. Neurons were grown for 9-16 days *in vitro* (DIVs) at 37 °C in 5 % CO_2. Lentiviral infections were performed on DIV 1 (see section 2.3).

2.1.4 Culture media

Full medium

reagents	volume in ml
fetal bovine serum	50
penicillin-streptomycin	1
MITO + serum extender	1
DMEM + GlutaMAX-1	500

FUDR medium

reagents	amount
5-Fluor-2-deoxyuridine	50 mg
uridine	125 mg
DMEM	25 ml

Enzyme solution

reagents	amount
cysteine	2 mg
DMEM	10 ml
$CaCl_2$ (100 mM)	0.1 ml
EDTA (50 mM)	0.1 ml
papain	20-25 units/ml

Inactivation solution

reagents	amount
albumin	2.5 mg
trypsin-inhibitor	2.5 mg
5 % FCS medium	1 ml

Neurobasal-A medium

reagents	amount
neurobasal-A	100 ml
B27 supplement	2 ml
GlutaMAX-1	1ml
penicillin/streptomycin	200 μl

Table 1: Media and solutions

2.2 Polymerase chain reaction

To determine the genotype of genetically modified newborn mice a part of the brain tissue was lysed for 5 min. in an enzyme solution (100 μg/ml Proteinase K (Roche, Germany), 10 mM Tris-HCl, 100 mM NaCl (Carl Roth, Germany)) at 55 °C. This destroys cell and nuclear membranes and gets the DNA into solution. The enzymatic reaction was stopped by heating the tube to 99 °C for 10 min. Next, the tube was centrifuged at 14 000 rpm for 2 min. to spin down cell debris. 1 μl of this DNA-containing supernatant was used in the PCR reactions described in Table 2 and Table 3. The PCR was carried out with a MastercyclerPro PCR machine (Eppendorf, Germany).

PCR results were loaded onto a 100 ml 2% agarose gel containing 10 ml of GelStar (Biozym Diagnostics, Germany) to visualize DNA fragments and imaged in a UV chamber (Biometra, Germany).

reagents	volume in μl
ddH_2O	15.3
DMSO	1.25
10x NEB buffer	2.5
2.5 mM dNTPs	1
10 µM syb2 WT-F	1.25
10 µM syb2 WT-R	1.25
10 µM syb2 KO-R	1.25
NEB Taq polymerase	0.2
DNA	1
total volume	25

primers

syb2 WT-F
GCCCACGCCGCAGTACCCGGATG

syb2 WT-R
GCGAGAAGGCCACCCGATGGGAG

syb2 KO-R
GTGGCCGGCTGGGTGTGGCGGAC

Cycling parameters

step	temperature in °C	time
1	94	2 min
2	94	30 s
3	60	30 s
4	72	30 s
	go to step 2 and repeat 30x	
5	72	2 min
6	12	end

PCR products

WT: 500 bp

KO: 300 bp

Table 2: syb2 PCR protocol

reagents	**volume in μl**
ddH_2O	16.8
25 mM $MgCl_2$	1.5
10x NEB buffer	2.5
2.5 mM dNTPs	1
10 µM V3-GFP-FW	1
10 µM V3-GFP-RV	1
NEB Taq polymerase	0.2
DNA	1
total volume	25

primers

V3-GFP-FW
GAGAAGGCGGATCACATGGT

V3-GFP-RV
AGGCTCCAGAAACAGTCTAACG

Cycling parameters

step	**temperature in °C**	**time**
1	94	2 min
2	94	20 s
3	62	15 s
4	72	30 s
	go to step 2 and repeat 30x	
5	72	1 min
6	12	end

PCR products

GFP: 550 bp

Table 3: VGLUT3GFP PCR protocol

2.3 Lentivirus constructs and production

Sequences of murine synaptobrevin 1 and 2, respectively were cloned into a lentiviral shuttle vector under the control of a human synapsin-1 promotor. For co-release experiments the sequence of murine VGLUT3 was cloned in a shuttle vector. To enable identification of infected cells the expression cassette of the desired protein was fused to a nuclear localization sequence-tagged green or red fluorescent protein (NLS-GFP or NLS-RFP) via a self-cleaving P2A peptide (Kim *et al.*, 2011). For syb1 KD a murine syb1 specific short interfering RNA (siRNA) target sequence (5' – CAG GCG GTT ACA GCA GAC C – 3') was obtained using Genscript siRNA Target Finder (https://www.genscript.com/ssl-bin/app/rnai) and cloned as short hairpin RNA (shRNA) into a lentiviral shuttle vector under the control of a U6 promoter. To identify infected neurons, the shuttle vector contained a human synapsin-1 promoter, which drives the expression of a nuclear-targeted red fluorescent protein (NLS-RFP).

Lentiviral particles were prepared as described in Lois *et al.*, 2002. HEK293T cells were cotransfected with 10 µg shuttle vector and the helper plasmids pCMVdR8.9 and pVSV.G (5 µg each) with X-tremeGENE 9 DNA transfection reagent (Roche Diagnostic, Germany). After 72h the virus containing cell culture supernatant was collected and purified by filtration. Aliquots were flash-frozen in liquid nitrogen and stored at -80 °C. Viruses were titrated with mice WT hippocampal mass-cultured neurons. For infection, about $1x10^6$ infectious particles were pipetted onto 1 DIV hippocampal neurons per 35 mm-diameter well.

2.4 Electrophysiology

Whole-cell patch clamp recordings were performed between DIV 9 and 16 under conditions and with protocols described in the following paragraphs.

2.4.1 Solutions and drugs

The experiments were carried out in a standard extracellular solution at room temperature containing the following (in mM): 140 NaCl, 2.4 KCl, 10 HEPES (Merck, Darmstadt, Germany), 10 glucose (Carl Roth, Karlsruhe, Germany), 2 $CaCl_2$, (Sigma-Aldrich, St. Louis, USA), 4 $MgCl_2$ (Carl Roth, Karlsruhe, Germany); 300 mOsm; pH 7.4. To block glutamatergic or

GABAergic responses 10 µM 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) (Tocris Bioscience, Bristol, UK) and 30 µM bicuculline (Tocris Bioscience, Bristol, UK), respectively, were added to the extracellular solution. To block both glutamatergic and GABAergic responses both 10 µM NBQX and 30 µM bicuculline were added to the external solution. In synaptobrevin mass culture experiments voltage gated sodium channels were blocked by adding 0.5 µM tetrodotoxin (TTX) (Tocris Bioscience, Bristol, UK). To assess the size of the readily-releasable pool (RRP, see below) the extracellular solution was made hypertonic by adding 500 mM sucrose (Sigma-Aldrich, St. Louis, USA) (described in Rosenmund and Stevens (1996)). Internal solution contained the following (in mM): 136 KCl, 17.8 HEPES, 1 EGTA (Carl Roth, Karlsruhe, Germany), 4.6 $MgCl_2$, 4 Na_2ATP , 0.3 Na_2GTP (Sigma-Aldrich, St. Louis, USA), 12 creatine phosphate (Calbiochem, Darmstadt, Germany), and 50 U/ml phosphocreatine kinase (Sigma-Aldrich, St. Louis, USA); 300 mOsm; pH 7.4. In the experiments, where I identified the patched cells *post-hoc*, 5 mM fixable Cascade Blue (Life Technologies, Darmstadt, Germany) was added to the internal solution.

2.4.2 Patch pipettes and experimental set-up

Borosilicate glass pipettes (Science Products, Hofheim, Germany) had a resistance of 2 - 3.5 MΩ. All recordings were performed with a Multiclamp 700B amplifier and a Digidata 1440A digitizer under control of Clampex 10.0 (Molecular Devices, Sunnyvale, USA). Data was acquired at 10 kHz and filtered at 3 kHz. In most of the experiments, membrane capacitance and 70 % of the series resistance were compensated while changes in series resistance were monitored frequently throughout the experiments. Only cells with a series resistance <10 mΩ were used for analysis. Paired recordings were carried out on a standard electrophysiology microscope equipped with two electrode holders.

2.4.3 Electrophysiological protocols

Excitatory (EPSC) and inhibitory (IPSC) postsynaptic currents were recorded after a 2 ms somatic depolarization from -70 mV to 0 mV, which resulted in an unclamped action potential. Responses were baselined to measure the amplitudes. The charge was determined by integrating the response from where it first hit baseline until it reached baseline again. Rosenmund and

Stevens, 1996, defined the pool of readily-releasible vesicles by applying a hypertonic sucrose solution to autaptic neurons. All docked fusion-competent SVs release their content into the synaptic cleft by a yet unknown mechanism. Knowledge of the RRP provides valuable insights into the presynaptic fusion machinery. It is quantified by first setting the baseline to the steady state current, which is established 2-3 seconds after sucrose application, and then integrating the charge of the transient synaptic current. The number of fusogenic vesicles (n_{RRP}) can be determined by dividing the RRP charge (Q_{RRP}) by the charge of the average miniature postsynaptic current (Q_{mPSC}).

(1) $$n_{RRP} = Q_{RRP} / Q_{mPSC}$$

By dividing the charge of a single EPSC (Q_{EPSC}) (or IPSC, respectively) by the charge of the RRP (Q_{RRP}) we can determine the vesicular release probability (P_{vr}), e.g. the probability of a release event upon action potential stimulus.

(2) $$P_{vr} = Q_{EPSC} / Q_{RRP}$$

I used two different protocols to assess short-term plasticity: The 10 Hz protocol consisted of 50 depolarization stimuli within 5 s and the responses were normalized to the first EPSC. The second protocol was a so-called paired-pulse protocol: 2 depolarization stimuli were given within a time window of 25 ms or 100 ms for excitatory neurons and inhibitory neurons, respectively. Dividing the amplitude of the second pulse by the amplitude of the first pulse results in the paired-pulse ratio (PPR). Neurons with PPRs > 1 are called facilitating, whereas depressing neurons have a PPR<1. There is also a direct correlation between PPR and P_{vr}. If the release probability is high more vesicles are being released in response to the first stimulus. This in turn leads to a smaller response to the second stimulus because of a reduced RRP. If the P_{vr} is low, the response to the first stimulus is small, but Ca^{2+} can accumulate, thus leading to a bigger second response.

2.5 Detection and analysis of spontaneous events

Miniature postsynaptic currents were recorded in presence and absence of 10 μM NBQX and 30 μM bicuculline, respectively, for background noise subtraction. In co-release experiments both drugs were also mixed and applied simultaneously. Spontaneous events were analyzed after filtering data at 1 kHz and then running a template-based detection algorithm implemented in AxoGraph X 1.5.4 (AxoGraph, Berkeley, USA).

To analyze the decay kinetics of spontaneous events for the co-release experiments I used two different methods, both resulting in a similar outcome: (1) After running the mini recognition template in AxoGraph the decays of detected mPSCs were fitted in AxoGraph with a single exponential or a double exponential from the peak until the decay reached baseline. The fits were judged by eye and the single exponential fit was discarded, when it was clearly not representing the signal. (2) Individual traces (from peak to baseline) were loaded into GraphPad Prism and the software's internal fitting algorithm was used to determine if a single or a double exponential was preferential.

In order to plot decay times from single and double exponentials into a single graph I calculated the weighted decay. The double exponential function is represented as follows:

$$(3) \quad I(t) = A_1 e^{-\tau_1 t} + A_2 e^{-\tau_2 t}$$

where I(t) is the PSC at the time t, A_1 and A_2 are the amplitudes of the fast and slow components, respectively, and τ_1 and τ_2 are the decay constants of the fast and slow components. Then the weighted decay T can be calculated with equation (4):

$$(4) \quad T = (A_1 \tau_1 + A_2 \tau_2) / (A_1 + A_2)$$

Fit of Gaussian's and sums of Gaussian's were also performed in GraphPad Prism and judged by the software's internal comparison algorithm.

2.6 Immunocytochemistry

Cells were washed with PBS and then fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, USA) for 10 minutes at RT after 14 DIV or directly after patch-clamp recordings. To permeabilize membranes the cells were treated with 0.02 % Tween20 in PBS for 20 min. Unspecific binding of antibodies was minimized by 1 h incubation in 4 % normal goat serum. Primary antibodies were used in concentrations described below (Table 4). Cells were incubated with primary antibodies at 4° C over night. Fluorescently labeled secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, USA). Images were acquired on an Olympus (Tokyo, Japan) IX81 epifluorescent microscope with a UPLSAPO 60x water immersion objective and MetaMorph software (Molecular Devices, Sunnyvale, USA).

To correlate electrophysiological data with immunolabeling I recorded only one cell per coverslip and filled it with fixable Cascade Blue dye. Thus, recorded cells could be easily identified by fluorescence in the 405 nm channel. Ratiometric imaging was performed with an imageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, USA) macro by choosing synaptophysin 1 positive synapses and dividing the fluorescence intensities of syb1 by those of synaptophysin 1.

AMPAR clustering was measured as follows: Lines of approximately 20 μm were drawn on dendritic structures positive for synaptophysin 1. These lines were used as masks to measure the intensity histogram of the GLUR2 signal. The line scans were loaded into AxoGraph and clusters were detected by using the software's macro for measuring peaks and shapes.

ICC and western blot antibodies

antibody	manufacturer	concentration
anti-syb2	Synaptic Systems, Germany	1:1000
anti-syb1	abcam, UK	1:200
anti-syp1	Synaptic Systems, Germany	1:1000
anti-tubulin III	Sigma-Aldrich, Germany	1:1000
anti-VGLUT3	Synaptic Systems, Germany	1:1000
anti-VGAT	Synaptic Systems, Germany	1:1000
anti-GLUR2	UC Davis	1:200

Table 4: List of antibodies used in immunocytochemistry and western blots

2.7 Western blot

Hippocampal neurons were plated at 30000 cm^{-2} on astrocyte-free coverslips and lysed after 14 DIV using 50 mM Tris/HCl (Merck, Darmstadt, Germany), pH 7.9, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 250 µM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, USA), 1% Non-idet P-40, and a tablet of Complete Protease Inhibitor (Roche Diagnostics, Berlin, Germany). After separation by SDS-PAGE proteins were transferred to nitrocellulose membranes and incubated with primary and secondary antibodies (primary antibodies: anti-syb1 and anti-syb2 see above, anti-tubulin III (catalogue number T8660) from Sigma-Aldrich (St. Louis, USA); secondary antibodies were horseradish peroxidase-conjugated (Jackson ImmunoResearch West Grove, USA). Secondary antibodies were detected with ECL Plus Western Blotting Detection Reagents (GE Healthcare Biosciences, Freiburg, Germany) in a Vilber (Eberhardzell, Germany) Lourmat Fusion FX7 detection system.

2.8 Statistics

Data acquired during experiments were combined from multiple cultures. If not stated otherwise mean ± standard error of the mean (SEM) are displayed. Differences between two sample populations were tested with a two-tailed Student's t-test. For three or more groups two-way ANOVA (analysis of variance) was used, followed by Tukey's multiple comparisons test.

The null hypothesis was rejected for $p \leq 0.05$ and indicated with asterisks (*). For the correlation analysis (Figure 9B) Pearson's correlation coefficient was calculated. Data from syb1 overexpression (depicted in Figure 9B) was not included in the calculation for the correlation, because they represent averages over all recorded cells. Data analysis and statistical tests was performed with GraphPad Prism version 6.0d for Mac (GraphPad Software, USA).

3. Results

Part I

The SNARE proteins play a fundamental role in SV fusion. In this first part of my thesis I investigated the function of synaptobrevin in NT exocytosis. The data revaluates the necessity for v-SNAREs in evoked and spontaneous synaptic release and suggests different release properties for synaptobrevin 1 and 2. The following results have recently been published in the Journal of Neurophysiology (Zimmermann *et al.*, 2014).

3.1 Characterization of the syb2 knockout in autapses

Previous studies of murine syb2 KO neurons in high-density culture had shown that in the absence of this major v-SNARE, the cells can still maintain some basal forms of SV release. Even though evoked release was virtually absent, the pool of readily-releasable vesicles was only reduced to ~10 % of WT levels and spontaneous fusion of SVs could still be observed (Schoch *et al.*, 2001). In order to determine the cause of this residual release in syb2-deficient neurons, hippocampal cells from E18.5 syb2 KO mice and WT littermates were grown on astrocyte microislands. In this autaptic culture system the neurons can only form synapses with themselves, which provides a great tool to measure in- and output of single cells. After 9-14 DIVs the neurons were transferred into an aCSF-containing chamber for electrophysiological analysis.

Only 1 out of 59 syb2 KO neurons showed a measurable postsynaptic response to an unclamped action potential, evoked by a 2 ms depolarization pulse in patch-clamp recordings (Figure 5A). The single EPSC that was measured in a syb2 KO neuron had an amplitude of 0.64 nA. Cells from WT littermates had robust responses in 54 out of 55 cases, with an average amplitude size of 3.4 ± 0.5 nA (Figure 5B). These findings were congruent with a previous report in autaptic culture by Guzman *et al.*, 2010 and reflect also the lack of evoked responses observed in syb2 KO mass culture (Schoch *et al.*, 2001).

Vesicles being released after evoked influx of Ca^{2+} originate from the readily-releasable pool of vesicles (RRP). Interestingly, in high density cultures of syb2 KOs this pool was still measurable (yet smaller than WT), indicating a specific problem in Ca^{2+}-mediated release, rather than a complete malfunctioning of the release machinery. I assessed the size of the RRP in syb2 KO

autapses using a protocol first described by Rosenmund and Stevens, 1996: Hypertonic solution, containing additional 500 mM sucrose, was applied for 5 s to release all fusion competent vesicles. Surprisingly, only 7 out of 54 cells had a measurable pool (Figure 6A). In WT all but one out of 55 cells did respond to sucrose application. Compared to WT the RRP sizes of the 7 KO cells was very small. The average charge was 56 ± 35 pC in syb2 KOs and 366 ± 50 pC in WT (Figure 6B). However, it should be noted that one of the KO neurons had a WT-like response (302 pC). In contrast to studies in mass culture we demonstrate a heterogeneous population of neurons: Complete absence of fusogenic vesicles in the majority of syb2 KOs and a reduced pool size in a subdivision of cells.

The significantly reduced pool size in the KO should also be reflected in a reduced number of spontaneous fusion events ("minis"). These events presumably originate from the same pool of vesicles, which is stimulated during evoked release. However, spontaneous release is supposedly Ca^{2+}-independent (for a detailed discussion, see Kavalali, 2015). In contrast to previous studies in mass culture (Schoch *et al.*, 2001) I found the majority of syb2 KO cells to be devoid

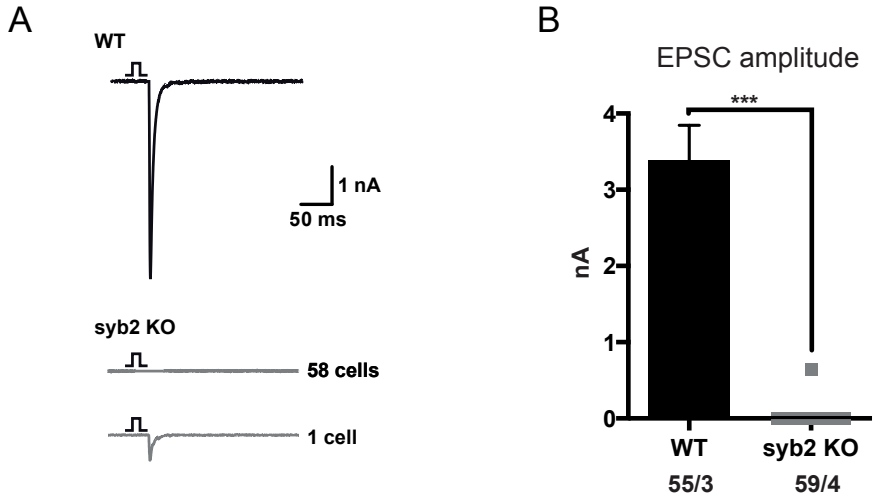

Figure 5: Evoked responses are mostly absent in autaptic syb2 KO neurons.
A: Example traces of a WT EPSC (top) and two KO EPSCs (bottom) after a 2 ms depolarization. Stimulations are indicated by an open square; artifacts and action potentials are blanked B: Plot of average excitatory postsynaptic current (EPSC) amplitudes in WT and syb2 KO autaptic neurons. Gray squares represent individual KO neurons. Note that there was only a single evoked glutamatergic response in the KO cultures (bottom trace in A). Bar graphs show mean ± SEM; *** indicates p<0.001. Cell number per number of independent cultures are indicated in graphs.

of any spontaneous release. 44 out of 47 cells were completely silent and the mini frequency in the three responding cells was drastically reduced compared to WT (Figure 7A and B). The mean frequency in WT neurons was 6.1 ± 0.9 Hz, whereas the three KO neurons had frequencies of 0.3 Hz, 0.7 Hz and 1.2 Hz, respectively. Interestingly, the amplitude of these fewer minis was also reduced. The average WT mEPSC size was 24.7 ± 1.0 pA compared to 12.5 ± 1.7 pA in KO neurons (Figure 7C).

Since the majority of hippocampal cells is excitatory, I focused my study on glutamatergic cells. It should be noted, however, that there were two GABAergic neurons in the culture with considerable evoked current responses (6.8 and 0.9 nA) (Figure 7D). The numbers were too low to include them into any statistical analysis but a follow-up study on inhibitory neurons could potentially be interesting. A recent publication by Imig and colleagues also suggests that a syb2 paralog is present in inhibitory neurons in the hippocampus (Imig *et al.*, 2014).

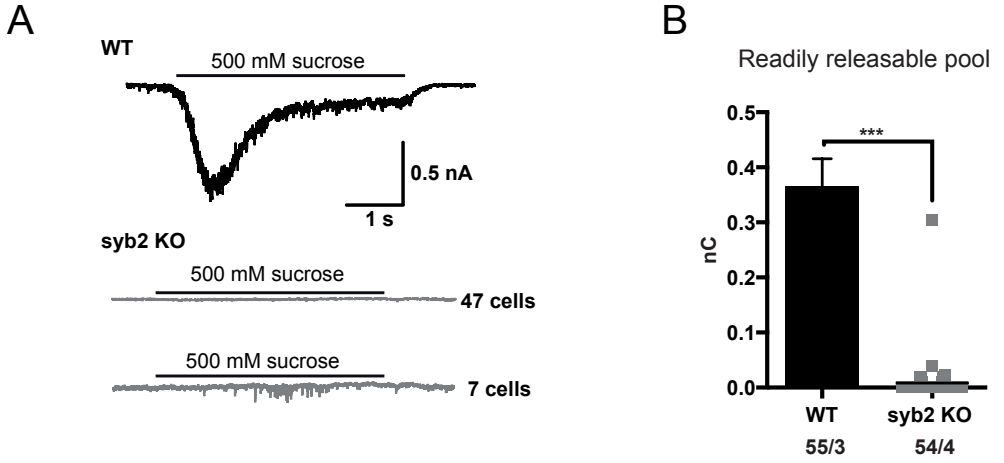

Figure 6: Readily-releasable pool is significantly reduced in autaptic syb2 KO neurons.
A: Example traces of a WT response (top) and two syb2 KO neurons (bottom) to 5 s of 500 mM sucrose application. The majority of cells (47 of 54) had no measurable RRP. B: Quantification of average RRP sizes in WT and syb2 KO autaptic neurons. Bar graphs show mean ± SEM; *** indicates p<0.001. Cell number per number of independent cultures are indicated in graphs.

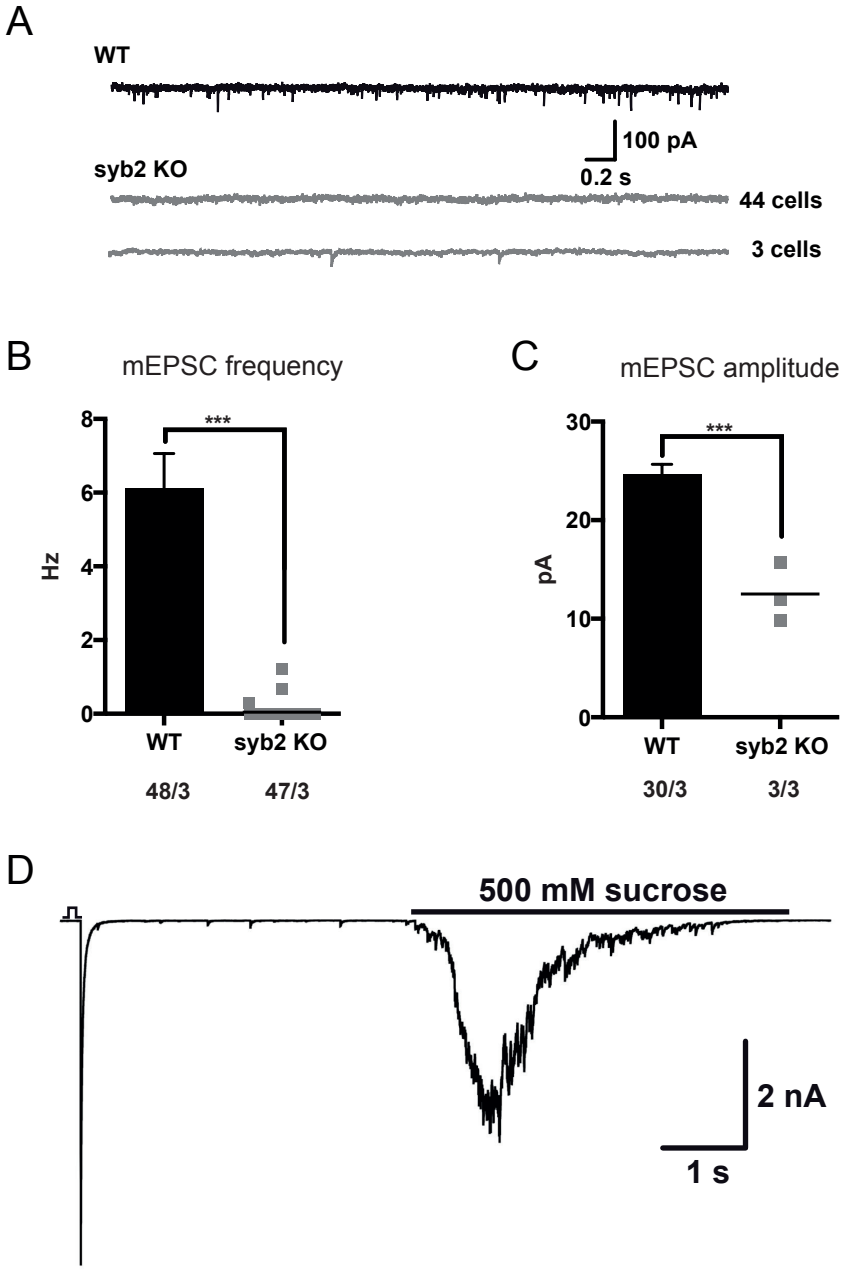

Figure 7: Spontaneous release in syb2 KO neurons is reduced.
A: Example traces of mEPSCs recorded in a WT and two syb2 KO autaptic neurons. Note that only 3 out of 47 neurons showed spontaneous release. B: Plot of mEPSC frequencies and (C) amplitudes. Gray squares represent individual KO neurons. Bar graphs show mean ± SEM; *** indicates $p<0.001$. Cell number per number of independent cultures are indicated in graphs. D: trace of a GABAergic neuron with WT-sized IPSC and RRP found in the syb2KO culture.

3.2 Identification of another syb isoform

The finding that a subpopulation of autaptic syb2-deficient neurons was still capable of releasing NTs, whereas the majority of cells was completely silent made us look for another syb isoform. In the previous study by Schoch and colleagues, 2001, the researchers used whole brain lysates for western blots to detect the major syb2 paralogs synaptobrevin 1 (syb1, VAMP1) and cellubrevin (ceb, VAMP3). At that time, the antibodies did not show any signals, which led Schoch *et al.* to the conclusion that syb2 is the only isoform present and remaining release would potentially be SNARE-independent.

We retested the presence of syb1 in our hippocampal culture. Since the cell density in a microisland culture is too low to produce enough material, we did immunoblots from syb2-KO mass culture lysates. We incubated with a syb1 antibody and found considerable amounts of this syb2 paralog in the neurons (Figure 8A). The amount of syb1 protein was comparable between WT and syb2 KO neurons, indicating that the absence of syb2 does not lead to an upregulation of syb1 protein levels.

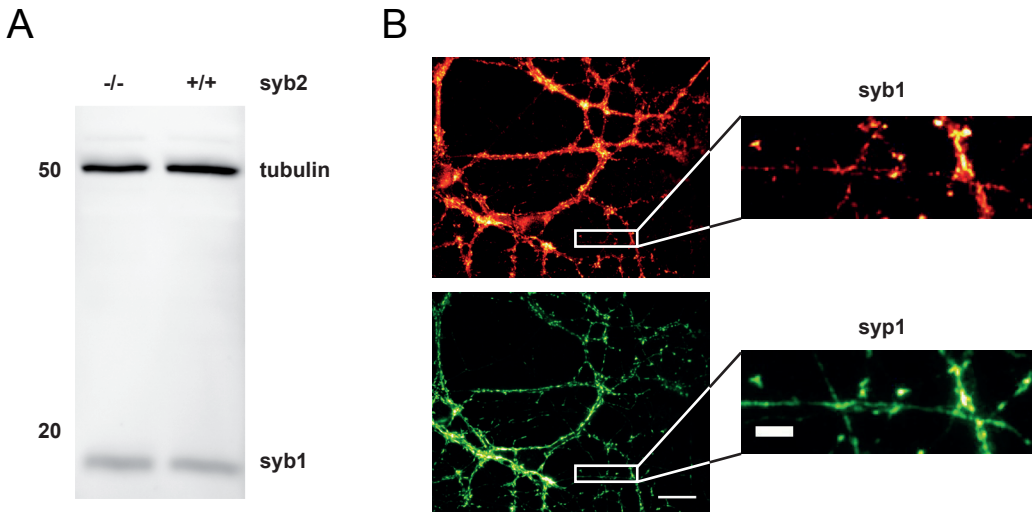

Figure 8: Syb1 is expressed in hippocampal neurons.
A: Western blot of WT (+/+) and syb2 KO (-/-) hippocampal high-density protein lysates after 14 DIVs incubated with antibodies against tubulin and syb1. B: Immunocytochemistry of hippocampal high-density culture. Antibodies against syb1 (red) and syp1 (green). Scale bar 20 μm (left). Inset: Magnification of the area indicated by a white square (scale bar 5 μm).

Most membrane fusion processes in a cell are mediated by a variety of SNARE proteins. Thus, detecting a syb1 signal in western blots does not prove an involvement in SV release. To test if the expression of syb1 was synaptic, I performed immunocytochemical stainings in PFA-fixed neurons. In hippocampal mass culture I could detect synaptic staining of syb1, co-localized with synaptophysin 1 (syp1), an abundant vesicular protein, which is used as a synaptic marker (Figure 8B). However, the distribution of the syb1 signal was not uniform between synapses. Some had a strong syb1 signal, whereas others were very weak. Due to the high density of cells it was not possible to assign the signal strength to individual neurons. For this reason we wanted to test if syb1 expression can be correlated to remaining release in the autaptic culture.

3.3 Syb1 expression correlates with RRP size

The presence of syb1 in hippocampal mass culture neurons led to the question if the remaining release seen in a few autaptic neurons was also mediated by syb1. To answer this, I patched onto single autaptic neurons with a dye-filled patch pipette and assessed the size of the readily-releasable pool by application of 500 mM sucrose solution. After recording, the cells

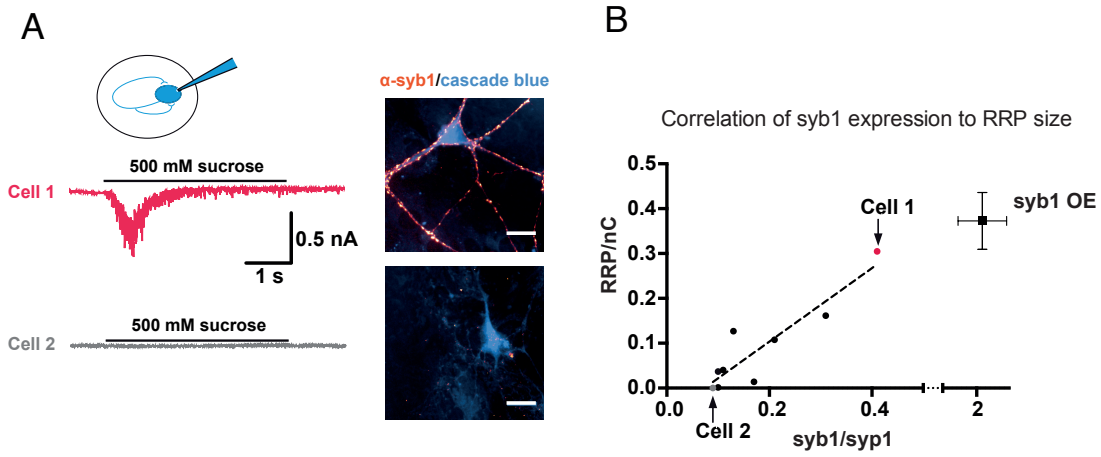

Figure 9: RRP size is correlated to syb1 expression levels in syb2-deficient neurons.
A: RRP traces of 2 exemplary syb2 KO autaptic neurons that have been filled with Cascade Blue dye during recordings (left) and their corresponding *post hoc* immunostainings for syb1 (right). Evoked response of cell 1 is shown in Figure 5A. Scale bars, 20 μm. B: plot of RRP size against the intensity of syb1 fluorescence normalized to the fluorescence intensity of synaptic marker synaptophysin 1 (syp1). Cells 1 and 2 shown in A are indicated in red and gray, respectively. Mean RRP size and syb1 expression levels of the syb1 rescue are labeled with "syb1 OE" (error bars show SEM). Pearson's correlation coefficient r=0.91, p<0.001(data point for syb1 OE was excluded from the correlation analysis; see materials and methods).

were fixed and incubated with antibodies against syb1 and the synaptic marker syp1. Since the cells were filled with the fixation-stable dye they could later be identified under the fluorescence microscope and syb1 levels (as well as syp1 levels) were determined by fluorescence measurements. I plotted the recorded pool size against the levels of syb1 protein levels (normalized to syp1 levels) (Figure 9). The correlation between the two measured variables was very high, with a Pearson's correlation coefficient of 0.91. The majority of neurons did not respond to the hypertonic solution at all and had no or low syb1 signal in the *post hoc* stainings. However, the few cells responding to sucrose showed all robust syb1 signals in ICC. It seems feasible that the observed release is mediated by syb1, however we first had to show that syb1 can actually function in SV exocytosis in hippocampal neurons.

3.4 Syb1 can rescue loss of syb2 but has different release properties

To provide evidence that syb1 expression is capable of mediating vesicle priming in hippocampal neurons I used a lentiviral shuttle vector to express syb1 in a syb2-deficient autaptic culture. To compare release parameters a vector expressing syb2 was used as a control. Furthermore, I compared both groups to WT neurons to test for effects that might be caused by viral infection or by over- or underexpression of the constructs.

Both, expression of syb1 and syb2 in syb2 KO neurons robustly rescued evoked responses. However, the mean EPSC amplitude was reduced in the syb1 rescue (2.2 ± 0.3 nA) compared to syb2 (3.6 ± 0.6 nA) and WT (3.5 ± 0.4 nA) (Figure 10A and B). In contrast, there was no significant difference in the readily-releasable pool size between the three groups (Figure 10C and D). This finding indicates that expression levels of both constructs are in principal sufficiently high but the release probability (P_{vr}) of neurons rescued with syb1 seems to be lower compared to syb2. Dividing the charge of the EPSC by the charge of the sucrose response I found a decrease in P_{vr} from 6.4 ± 0.6 % in the syb2 rescue to 2.6 ± 0.6 % in the syb1 rescue (Figure 11A). The release probability of WT neurons was slightly bigger compared to the syb2 rescue but the difference was not significant.

Another way to examine release probability is by exposing the neuron to a high-frequeny train of depolarizations and measure the short-term plasticity. Neurons with high initial P_{vr} tend to depress much stronger than neurons with a low P_{vr}. Applying 50 depolarization stimuli at

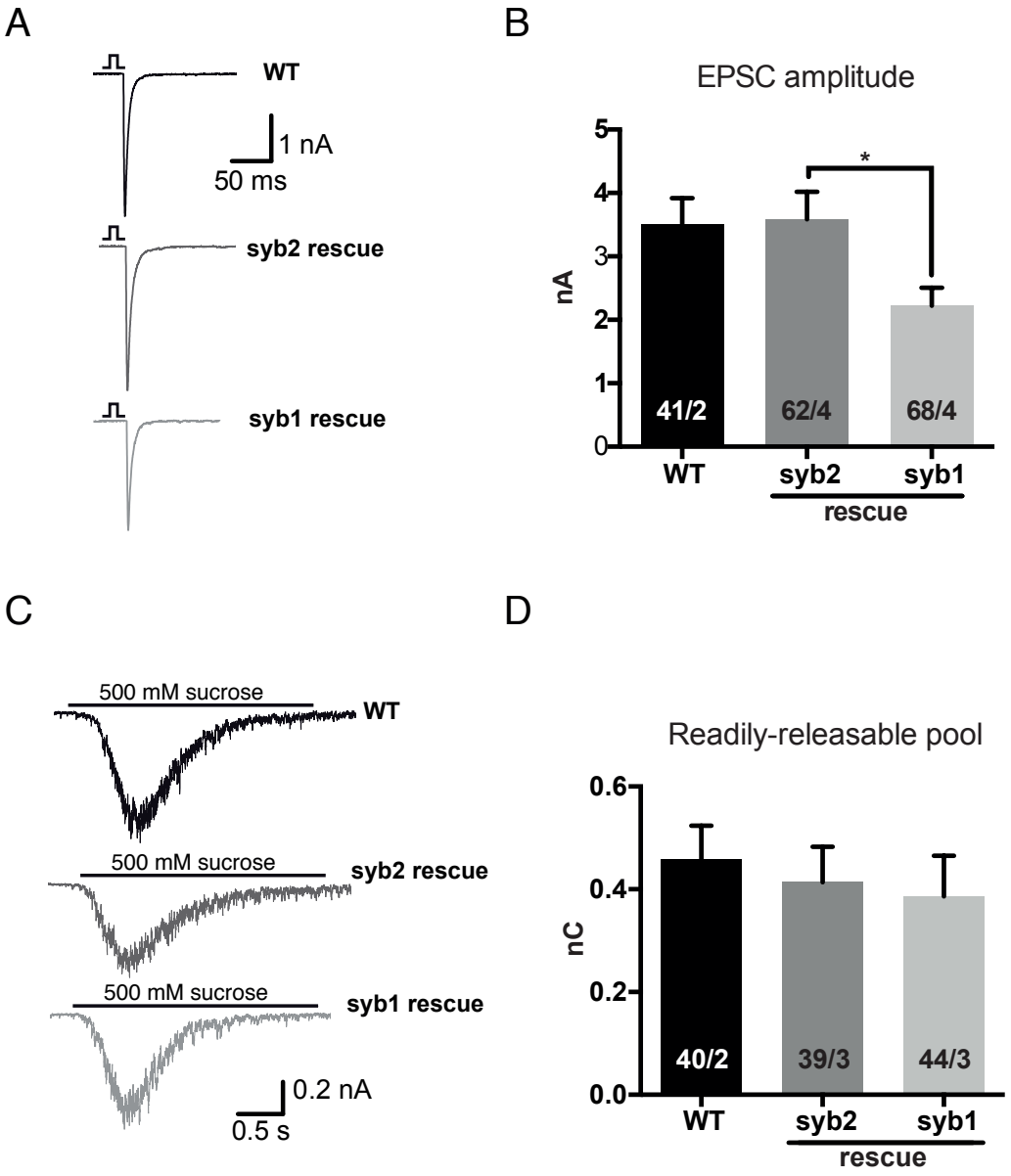

Figure 10: Syb1 rescues release in syb2 KO neurons.
A: Example traces of a WT (top) and two syb2 KO responses (one rescued with syb2 (center), the other one rescued with syb1 (bottom)) to an unclamped AP. Stimulations are indicated by an open square; artifacts and action potentials are blanked. B: Plot of the average EPSC amplitudes in the three conditions. C: Example traces of WT (top), syb2 rescue (center) and syb1 rescue (bottom) in response to 500 mM sucrose. D: Plot of the average pool charge. Note that the average EPSC amplitude for the syb1 rescue in B is significantly smaller than the other two conditions, whereas the RRP size is unaltered. Bar graphs show mean ± SEM; * indicates $p<0.05$. Cell number per number of independent cultures are indicated in graphs.

10 Hz I found the syb1 rescue to depress significantly less compared to the syb2 rescue and WT (Figure 11B). This confirmed my previous findings that syb1 mediates release less efficiently than syb2.

Even though the pool size of the two constructs reached WT levels (Figure 10C) I detected a significant decrease in mEPSC frequency in the syb1 rescue compared to syb2 (Figure 12A and B). The frequency was reduced from 4.5 ± 0.7 Hz in the syb2 rescue to 1.6 ± 0.2 Hz in the syb1 rescue. This is interesting because usually the mini frequency correlates with the pool size. It indicates a higher energy barrier for fusion-competent vesicles. Mini amplitude and charge on the other hand were unaltered in the syb1 rescue (Figure 12C and D).

Taken together, the rescue experiments demonstrate that syb1 can substitute for loss of syb2 in terms of maintenance of neurotransmitter release in central synapses. However, syb1 promotes evoked and spontaneous release less efficiently compared to syb2. While it is possible that the change in release probability is caused by a change in relative expression levels of the two synaptobrevin paralogs, we consider it unlikely as both constructs rescue vesicle priming equally well.

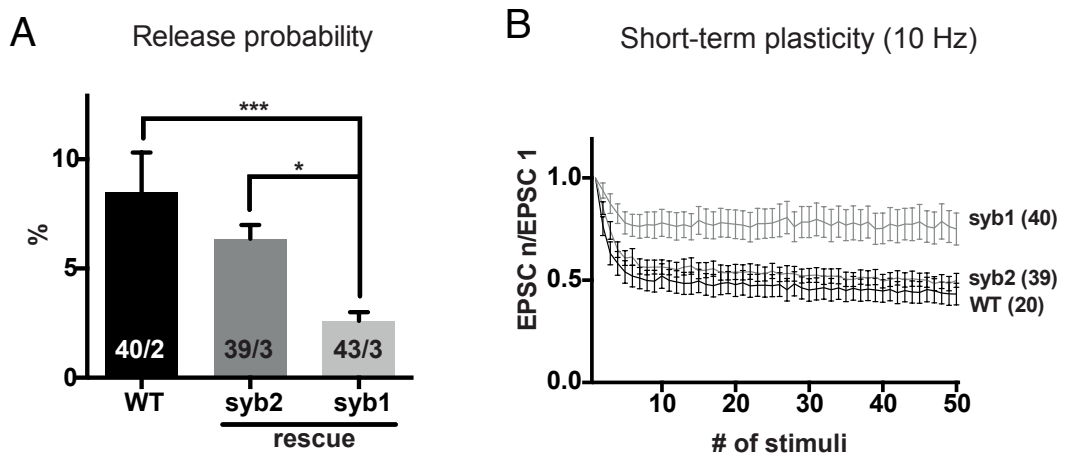

Figure 11: Release probability reduced in the syb1 rescue.
A: Plot of vesicular release probability in WT neurons and syb2 KO neurons rescued either with syb2 or syb1, respectively. Values are calculated from the results depicted in Figure 10. B: Plot of average amplitudes in response to a 10-Hz stimulation protocol. Amplitudes are normalized to the first response. "syb1" and "syb2" indicates rescues of syb1 and -2 in the syb2 KO background, respectively. Number of cells in parenthesis. Bar graph shows mean ± SEM; * indicates $p<0.05$, *** $p<0.001$. Cell number per number of independent cultures are indicated in graph.

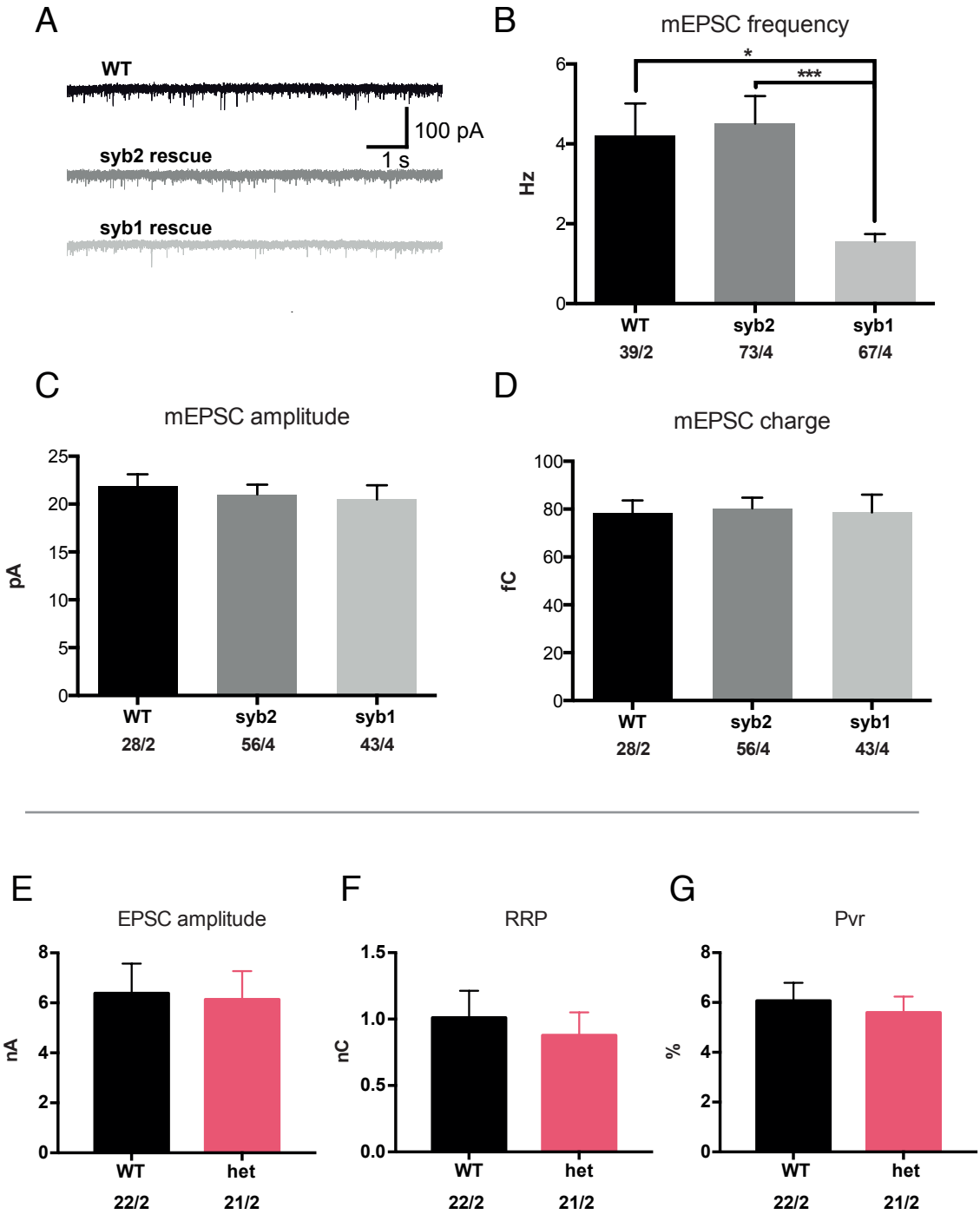

Figure 12: mEPSC frequency significantly reduced in syb1 rescue.
A: Exemplary traces of spontaneous NT release of autaptic neurons in WT (top), syb2 rescue (center) and syb1 rescue (bottom). Traces filtered at 1 kHz. B: Plot of average mEPSC frequency in the three conditions. "syb2" and "syb1" indicate the respective rescues in a syb2 KO background. C: Plot of average mEPSC amplitudes and (D) charges. E: Plot of average EPSC amplitude in WT autaptic neurons and syb2 +/- heterozygotes (het). F: Plot of average readily-releasable pool (RRP) size. G: Plot of average vesicular release probability (Pvr). Bar graphs show mean ± SEM; * indicates $p<0.05$, *** $p<0.001$. Cell number per number of independent cultures are indicated in graphs.

Furthermore, synaptic function in our system appears relatively insensitive to changes in expression levels. I compared EPSC amplitudes, RRP sizes and P_{vr} of WT cells with neurons containing only a single syb2 gene (from syb2 heterozygous (het) mice). The neurons did not display significant changes in vesicle release, priming or release probability (Figure 12E-G).

3.5 Residual release in syb2 KO mass culture

Thus far, we could show that syb1 is capable of mediating release in hippocampal neurons and it is responsible for the remaining release in syb2-deficient autaptic cells. Next, we wanted to find out if the remaining release observed in syb2 KO mass culture (Schoch *et al.*, 2001) is also caused by syb1 expression in these cells.

First, I repeated the experiments in mass culture and compared the rate of spontaneous release between WT neurons and neurons lacking syb2 (Figure 13). In congruence with previous results I also observed significant levels of "mini" events in the syb2 KO. The frequency in the KO was even higher than reported in Schoch *et al.*, 2001. However, our data includes inhibitory events, whereas the previous experiment was performed with low chloride concentration in the internal solution, so mIPSCs were not visible. I also assessed the size of the RRP and could confirm that in syb2 KO mass culture the number of fusogenic vesicles is reduced (Figure 15A and B).

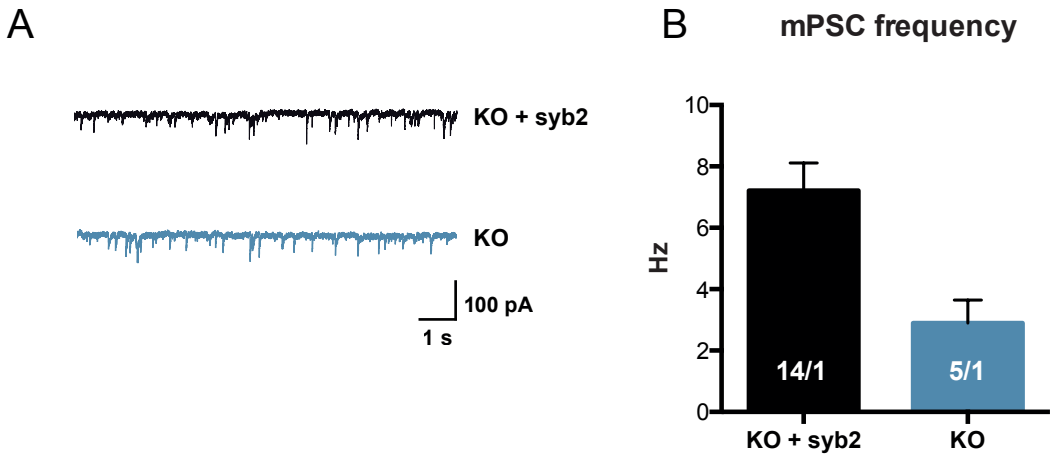

Figure 13: Spontaneous release in syb2 KO mass culture.
A: Example traces of mPSCs in syb2 KO neurons with (top) and without (bottom) a syb2 rescue in the presence of 0.5 μM TTX, filtered at 1 kHz. B: Plot of average mPSC frequency. Bar graph shows mean ± SEM; Cell number per number of independent cultures are indicated in graph.

3.6 Creation of a syb1 knockdown

Having shown that remaining release in autapses is correlated to syb1 expression levels, we wanted to test, if a reduction of syb1 levels in syb2 KO mass culture would reduce the residual release. We created a viral construct containing a shRNA to genetically manipulate syb1 expression in syb2 KO neurons. The efficiency of the knockdown was quantified by western blot and by immunocytochemistry (Figure 14). Since the expression of syb1 in WT hippocampal neurons is already quite low I used the syb1 construct described in 3.4 to overexpress the protein for immunocytochemistry. The shRNA significantly reduced both the overall levels of syb1 (quantified by western blot (Figure 14C)) as well as the protein levels in synapses (quantified by ratiometric imaging (Figure 14A and B)).

3.7 Knockdown of syb1 reduces RRP size in syb2 KO mass culture

After establishing a working knockdown construct, I assessed the size of the readily-releasable pool in three different groups of hippocampal mass culture neurons: WT, syb2KO and syb2KO plus syb1KD. Since in mass culture the input to a neuron can be both glutamatergic and GABAergic I applied either bicuculline or NBQX to our bath solution to determine the impact of this manipulation on both NT systems. I found that the remaining RRP, which can be observed in syb2KOs, was significantly reduced in cells where the levels of syb1 had been knocked down (Figure 15A and B). The effect was stronger for GABAergic cells (0.3 ± 0.1 % of WT RRP) than for glutamatergic cells (0.7 ± 0.2 % of WT RRP). Surprisingly, the frequency of spontaneous fusion events in the syb2KO + syb1KD was only slightly more reduced compared to syb2KO (Figure 15C and D).

The results suggest that residual release in the syb2 KO is indeed caused by syb1, a v-SNARE paralog that promotes SV release with different properties compared to syb2. The causes for remaining spontaneous release is a subject for future studies.

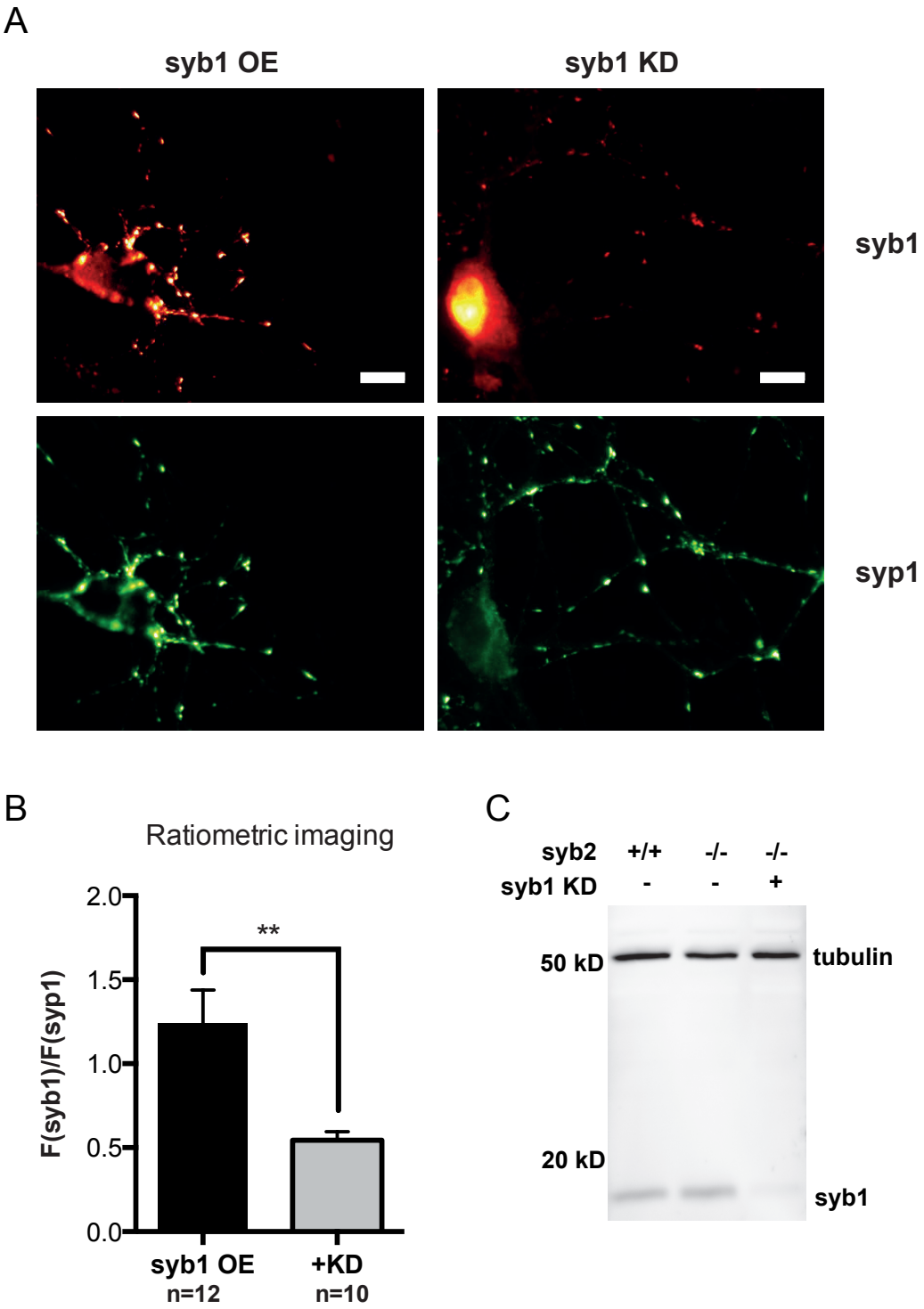

Figure 14: Syb1 knockdown construct reduces syb1 levels

A: Immunocytochemistry of syb1 (red) and syp1 (green) in mass culture neurons. Under both conditions syb1 had been overexpressed with a lentiviral construct. "syb1 OE" indicates only overexpression, whereas "syb1 KD" indicates both overexpression and knockdown of syb1. Scale bar 20 μm. B: Ratiometric imaging of syb1 levels divided by syp1 levels. n equals the number of cells. Bar graph shows mean ± SEM; ** indicates $p<0.01$. C: Immunoblot of protein lysates from WT (left lane) and syb2 KO (center and right lane) hippocampal mass cultures after 14 DIV. Antibodies against syb1 and tubulin. Note that syb1 levels were drastically reduced in the syb1 knockdown (right lane).

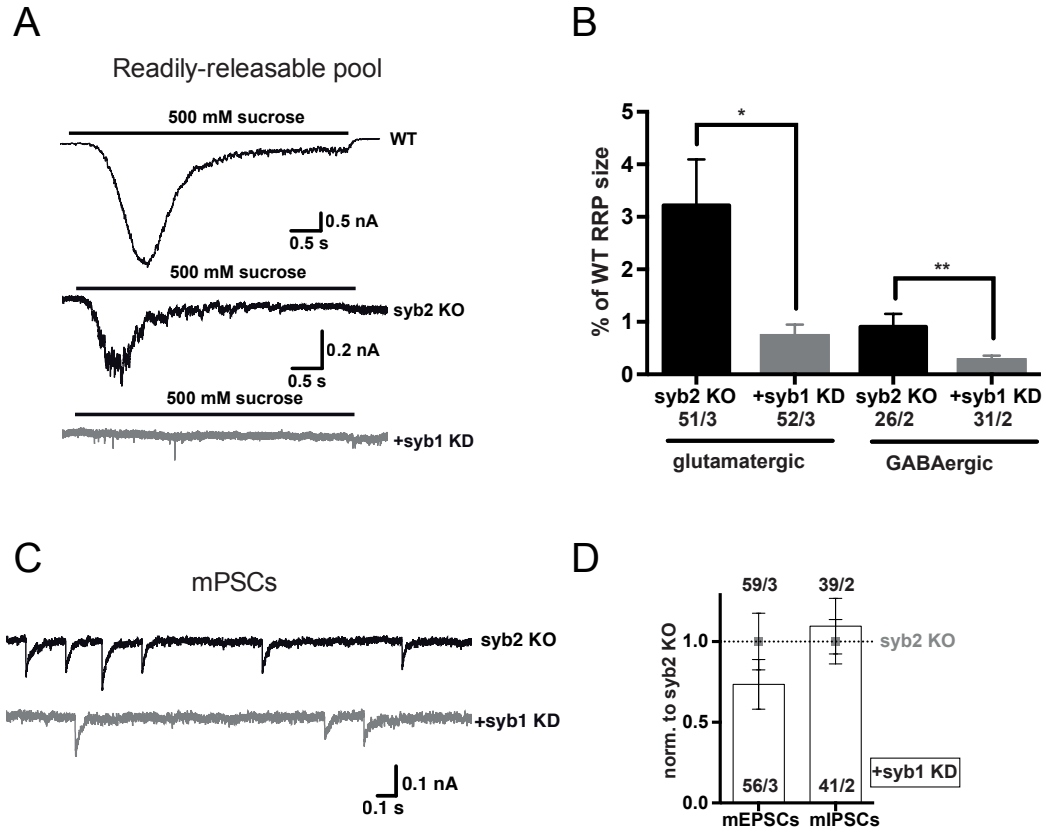

Figure 15: Syb1 expression levels determine synaptic responses in absence of syb2 in mass cultures.
A: Exemplary responses to sucrose application of WT and syb2 KO mass culture neurons infected with a scrambled shRNA (black) and syb2 KO neurons infected with a shRNA against syb1 (grey) in the presence of 30 μM bicuculline. B: Plot of glutamatergic and GABAergic RRP size in syb2 KO cultures with and without syb1 knockdown, normalized to WT RRP. C: Exemplary traces of spontaneous release in syb2 KO mass culture infected with a scrambled shRNA (black) and shRNA against syb1 (grey). D: Plot of mean mPSC frequencies normalized to syb2 KO frequencies. Bar graphs show mean ± SEM; *p<0.05, **p<0.01. Cell number per number of independent cultures are indicated in graphs.

Part II

In the second part of my thesis I investigated the co-release of the fast-acting neurotransmitters glutamate and GABA on a vesicular level. I tested if glutamate and GABA can be released from the same vesicles, and found significant divergence in the potential to detect co-release in different culture systems.

3.8 Expression of VGLUT3 in GABAergic neurons promotes glutamate release

We first wanted to test, whether expression of a vesicular glutamate transporter is sufficient to induce glutamate release in GABAergic neurons. I cultured striatal neurons from newborn mice on glial microislands (autaptic culture (Bekkers and Stevens, 1991)). The majority of neurons in the *corpus striatum* are GABAergic medium spiny neurons (MSNs) (Kemp and Powell, 1971). A lentiviral expression system was used to express VGLUT3 in these neurons. Electrophysiology experiments were performed after 13-17 DIVs. Whole-cell patch clamp re-

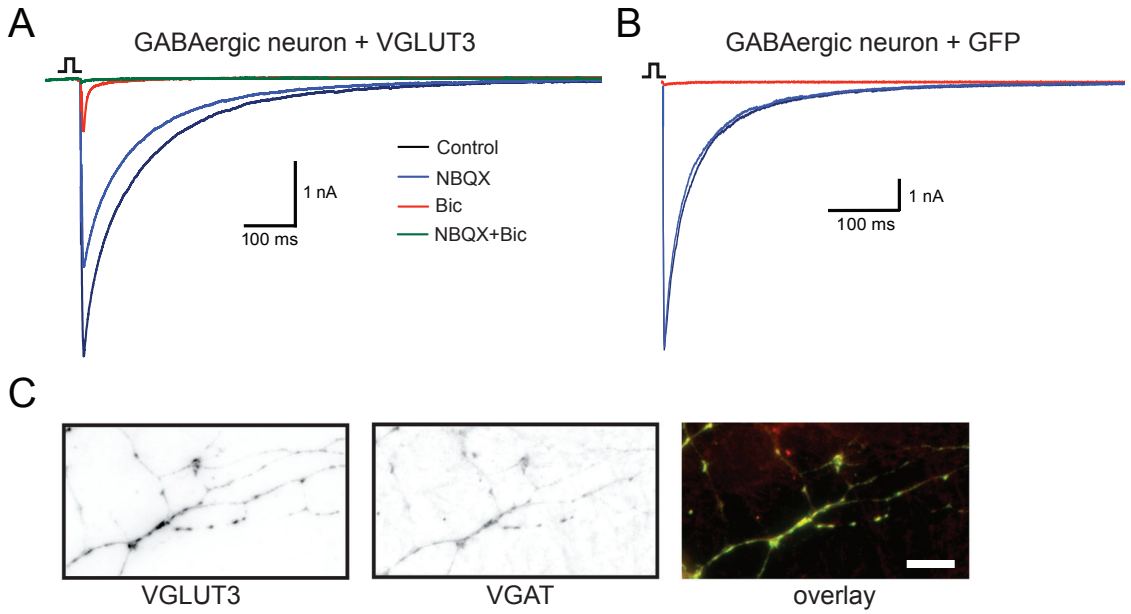

Figure 16: VGLUT3 expression promotes glutamate release in GABAergic neurons.
A: Exemplary traces of current responses to an unclamped AP of a striatal GABAergic neuron exogenously expressing VGLUT3. Application of NBQX and/or bicuculline reveals co-release of glutamate and GABA. B: Application of NBQX has no affect on control inhibitory neurons expressing GFP only. Stimulations are indicated by an open square; artifacts and action potentials are blanked. C: Immunocytochemistry stainings of autaptic striatal culture. Antibodies against VGLUT3 and VGAT show overlapping signals of the two transporters. Scale bar: 10 μm

cordings showed reliable responses after depolarization stimulus (Figure 16A and B). Addition of the GABA receptor antagonist bicuculline to the extracellular solution revealed a fast excitatory component that could be blocked with NBQX. Glutamate release was not detectable in control neurons that expressed only GFP (Figure 16B). These findings are consistent with previous results, where VGLUT1 and VGLUT2 were expressed in GABAergic neurons leading to a glutamatergic phenotype (Takamori *et al.*, 2000, Takamori *et al.*, 2001).

To test if the expression of VGLUT3 and VGAT overlap I made immunocytochemical stainings from the neuronal culture using antibodies against VGLUT3 and VGAT, respectively (Figure 16C). Both transporters largely co-localize, indicating that most terminals contain both VGLUT3 and VGAT. This supports the idea that individual neurons – and potentially individual synaptic boutons – can co-release glutamate and GABA.

3.9 No altered short-term plasticity in coreleasing neurons

To understand the physiological role of co-release of two neurotransmitters we investigated putative effects on NT release probability. Previous studies had shown that the expression of different VGLUT isoforms on SVs influences their probability to be released (Weston *et al.*, 2011). We wanted to test if the properties of GABAergic vesicles changes with co-expression of VGLUT3. Neurons stimulated twice within a short time period can have either facilitating or depressing properties, represented by a bigger or smaller secondary response in relation to the first one. I examined this paired-pulse behavior (100 ms inter-stimulus interval) in our co-release system in the presence of different receptor antagonists. I found a high correlation between the paired-pulse ratio (PPR) in control external solution compared to external solution with NBQX (Figure 17A and B). The PPR in the presence of bicuculline was slightly altered. This, however, seemed to be due to large GABAergic responses in control and NBQX-solutions. In large responses the postsynaptic current had not reached baseline before initiation of the second pulse, which might result in a perceivably bigger second amplitude (Figure 17D). If one removes the data points of large amplitudes from the graph in Figure 17B the difference between PPR in NBQX and bicuculline becomes insignificant (not shown). As expected, control cells expressing only GFP did also not show differences in the PPR in the presence of NBQX (Figure 17C). In conclusion, my results show that VGLUT3-expression does not change release probability of GABAergic vesicles.

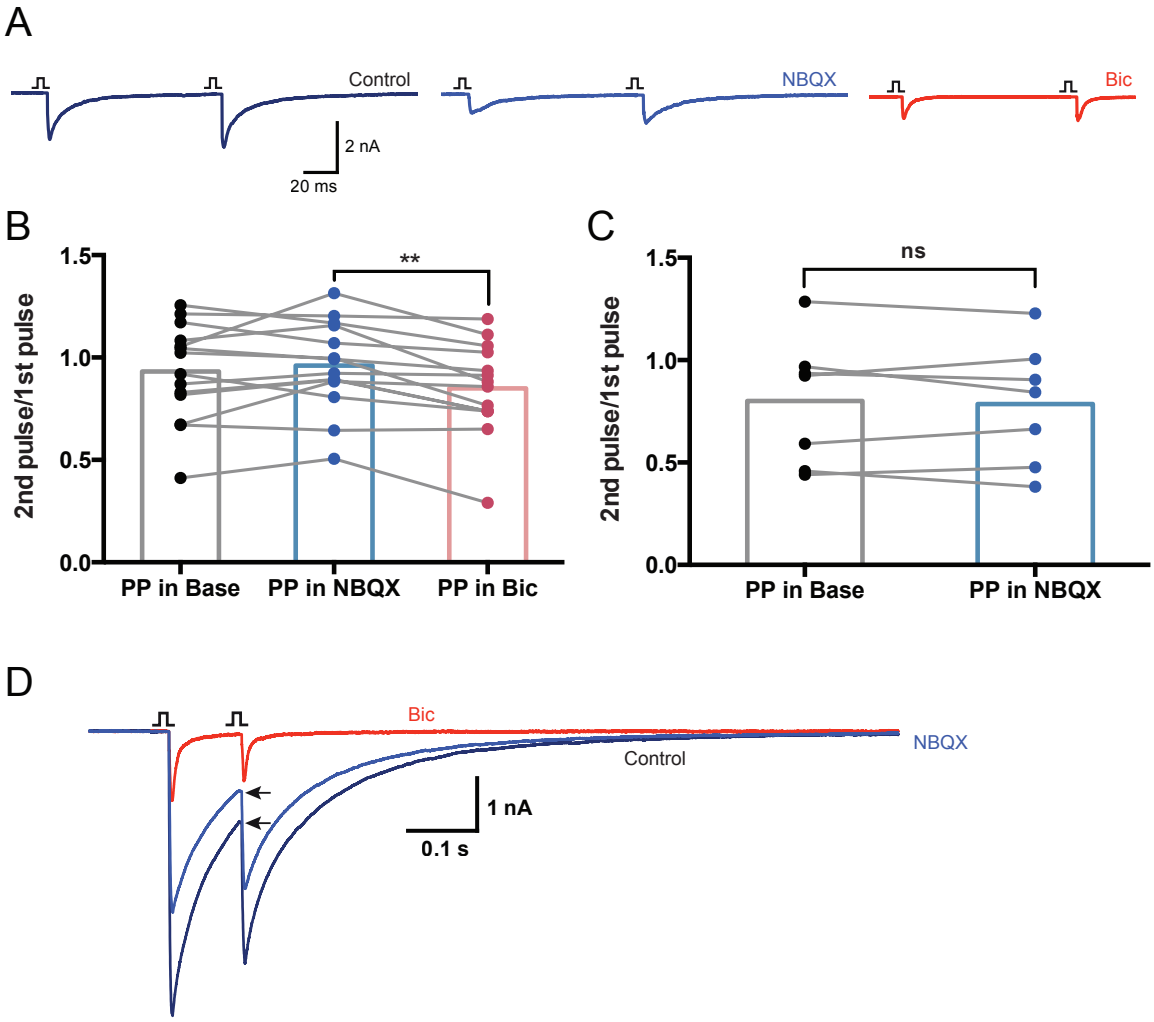

Figure 17: Paired-pulse behavior unaltered in coreleasing neurons.
A: Exemplary traces of PSCs in a striatal GABAergic neuron expressing VGLUT3 after two depolarization stimuli with 100 ms ISI. The same neuron is depicted under three different conditions: control aCSF (black), aCSF with NBQX (blue) and aCSF with bicuculline (red). B: Plot of paired-pulse ratio (PP) of coreleasing neurons under the three conditions mentioned in A. Squares represent individual cells. Gray lines connect data points from the same cell. C: Plot of paired-pulse ratio of GABAergic neurons expressing GFP only (no co-release). D: Exemplary trace of a neuron with a big GABAergic response. Note, that the PSCs have not reach baseline when the second stimulus arrives (arrows).

3.10 AMPA receptor-mediated currents are unaltered in VGLUT3-expressing cells

We next wanted to test if the expression of VGLUT3 in striatal GABAergic neurons and the resulting co-release shown in 3.8 leads to changes in AMPA-receptor (AMPAR) expression. We first compared the steady-state amplitudes induced by exogenous application of 10 μM kainate for 2 seconds between autaptic excitatory neurons from the hippocampus and autaptic inhibitory striatal neurons (Figure 18A and B). We found that even in absence of synaptic glutamate input the GABAergic neurons have on average the same kainate-mediated amplitude as glutamatergic neurons. Next, we compared the steady-state amplitude in response to 2 s kainate application between two distinct groups of inhibitory neurons from striatum. One group was infected with a construct for VGLUT3, thus coreleasing glutamate (Figure 18C and D), and the other group expressed just GFP. We found no difference between the two groups in the steady-state kainate amplitude (Figure 18D and F). The findings indicate that the total amount of AMPARs is unaltered and independent of possible glutamate release. In the next step we wanted to examine if the localization and clustering of AMPARs changes in GABAergic neurons that release glutamate and GABA.

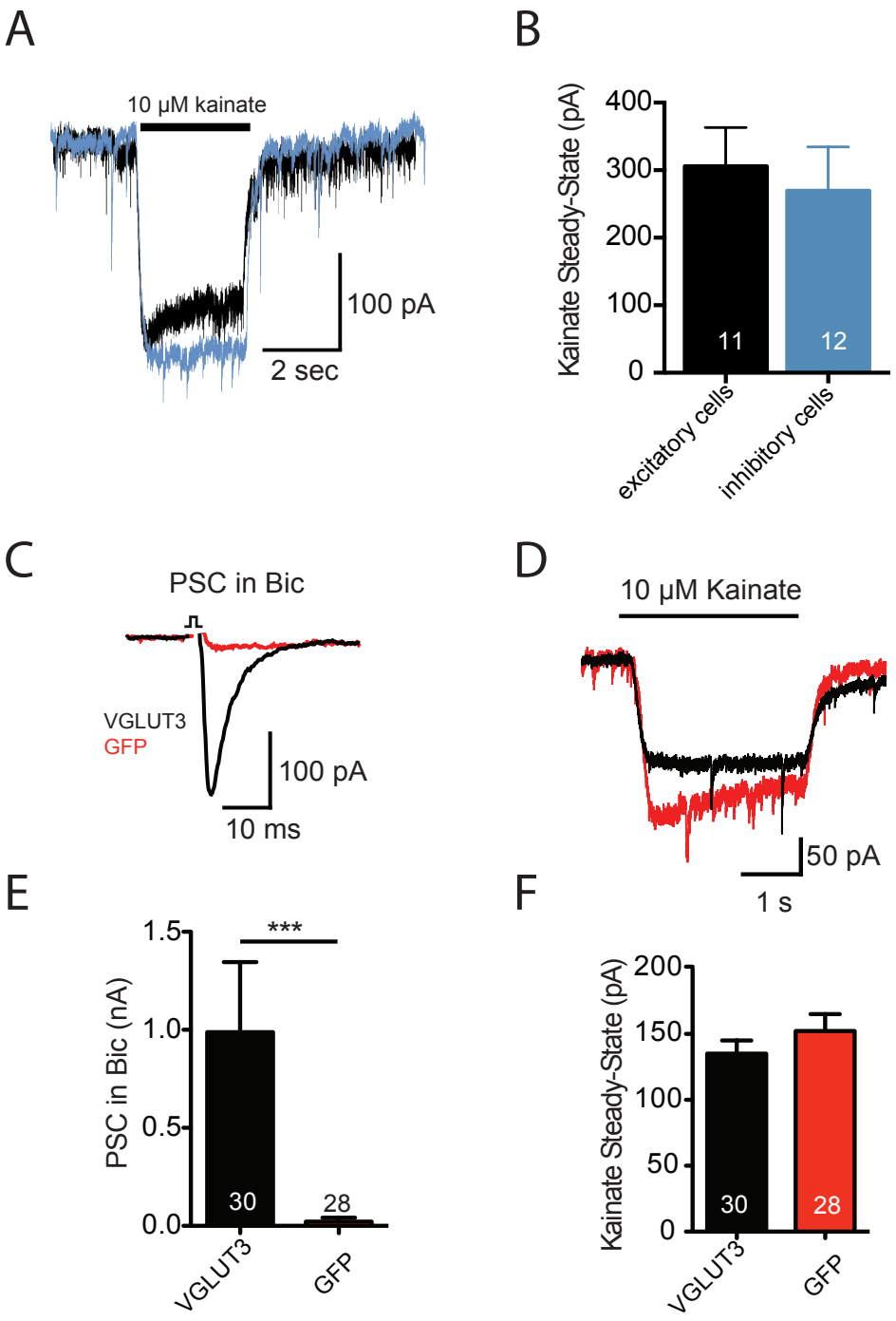

Figure 18: AMPA receptor-mediated currents unaltered in coreleasing neurons.
A: Exemplary traces of a glutamatergic neuron from hippocampus (black) and a GABAergic neuron from striatum (blue) after 2 seconds exogenous application of 10 μM kainate. B: Plot of average steady-state amplitudes in glutamatergic and GABAergic neurons after 2 s kainate application (10 μM). C: Exemplary traces of PSCs from striatal GABAergic neurons expressing VGLUT3 (black) or GFP (red) in the presence of bicuculline. D: Response to 2 s kainate application in the neurons from (C). E: Plot of average PSC size in presence of bicuculline. F: Plot of average kainate steady-state current. Number of cells indicated in graphs. (C-F experiments and figures by Melissa Herman)

3.11 Increased AMPAR clustering in autaptic coreleasing neurons

To assess if the co-release of glutamate leads to changes in the localization of AMPARs we incubated striatal autaptic cultures with fluorescent antibodies against the AMPAR subunit GLUR2 (Figure 19). One group exogenously expressed VGLUT3 and the other group expressed GFP as a control. We co-stained for synaptic marker synaptophysin 1 and used this signal to draw lines along the dendrites (Figure 19A). This mask was used to measure the clustering of GLUR2 signal. Unfortunately, the GLUR2 signal was not very strong in our autaptic culture and there was a relatively high background signal from the astrocyte islands. In two out of three cultures we observed a slight increase in AMPAR clustering in VGLUT3-expressing neurons compared to the GFP control. However, the difference was not significant. We suggest that this was due to technical problems in the staining and not necessarily because there is no increased clustering. Improvement of the ICC for AMPAR in our autaptic culture would be a goal for future experiments.

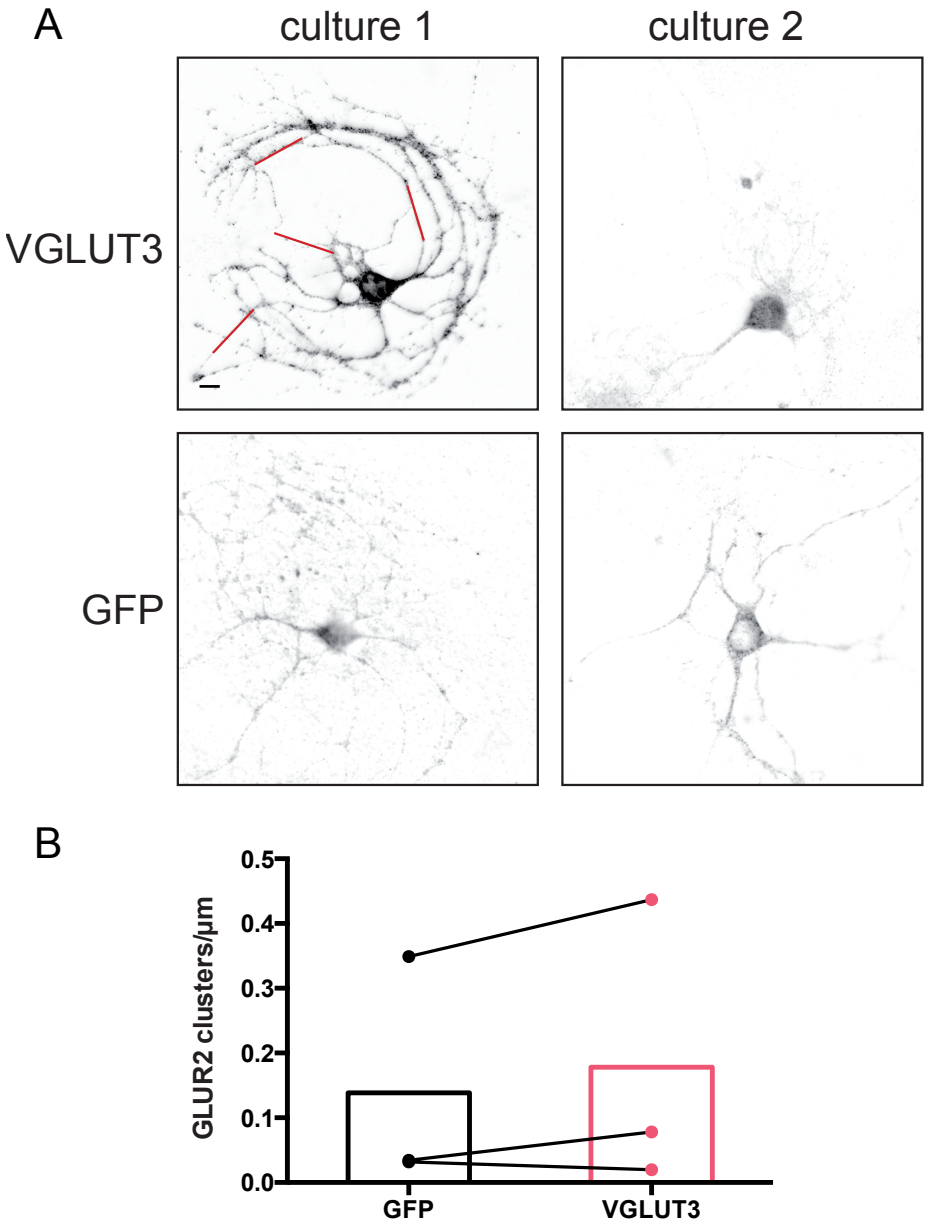

Figure 19: Slightly increased AMPAR clustering in two out of three cultures
A: Immunocytochemistry staining of striatal autaptic neurons expressing either VGLUT3 (top) or GFP (bottom). Two exemplary images from two different cultures are shown. Red lines in the top left image indicate the dendritic mask that was created with syp1 (see materials and methods). Note the differences in signal intensity between different cultures. B: Plot of average number of GLUR2 clusters per μm in striatal neurons expressing GFP or VGLUT3. Each square represents the average from one culture and is the mean value from 10 cells. (Experiments and images in (A) by Sabina Merrill, analysis by JZ)

3.12 Glutamate and GABA are stored in the same vesicles

The process of spontaneous release of single vesicles ("minis") is well suited to study if glutamate and GABA are stored in distinct vesicle pools or if they are actually being co-released from the same vesicles. I recorded miniature postsynaptic events (mPSCs) from VGLUT3-expressing autaptic striatal interneurons in control extracellular solution or in the presence of glutamate receptor antagonist NBQX or GABA receptor antagonist Bic, respectively (Figure 20A). Similar to my results where I investigated evoked release I observed fast-decaying events in the presence of Bic (Figure 20A, right trace) and slower-decaying events in the presence of NBQX (Figure 20A, center trace). To determine if the events in the control external solution simply represent the sum of the events in NBQX and Bic, respectively, or if there is also a population of mixed events, I pooled the events from each condition and analyzed the decay times. I found that distributions for all of these populations were significantly different ($p>0.0001$ for all combinations; Kolmogorov-Smirnov test). The frequency histograms for events recorded in Bic or NBQX were best fitted with a single Gaussian (Figure 20B; Bic: mean tau ± SD, 2.6 ± 1.29 ms, R2 = 0.9631; NBQX: mean tau ± SD, 18.53 ± 8.72 ms, R2 = 0.6779). The frequency histogram for events recorded in control extracellular solution was best fitted by the sum of three Gaussians (mean tau_1 ± SD_1, 3.52 ± 1.01 ms, mean tau_2 ± SD_2, 10.22 ± 2.82 ms, mean tau_3 ± SD_3, 17.83 ± 10.27 ms, R2 = 0.8617). The population with an intermediate decay time of 10.22 ms represents most likely the fraction of vesicles that contain both GABA and glutamate.

To confirm that these events recorded in the control extracellular conditions actually contained a population of mixed glutamate-GABA events, I compared the cumulative frequency distribution of the control events to an artificial population consisting of a combination of events recorded in Bic or NBQX (Figure 20C). I found that the distribution of these two populations was significantly different ($p > 0.0001$, Kolmogorov-Smirnov test), suggesting that a portion of spontaneous events recorded from VGLUT3-expressing GABAergic neurons reflect co-release of glutamate and GABA from the same synaptic vesicle.

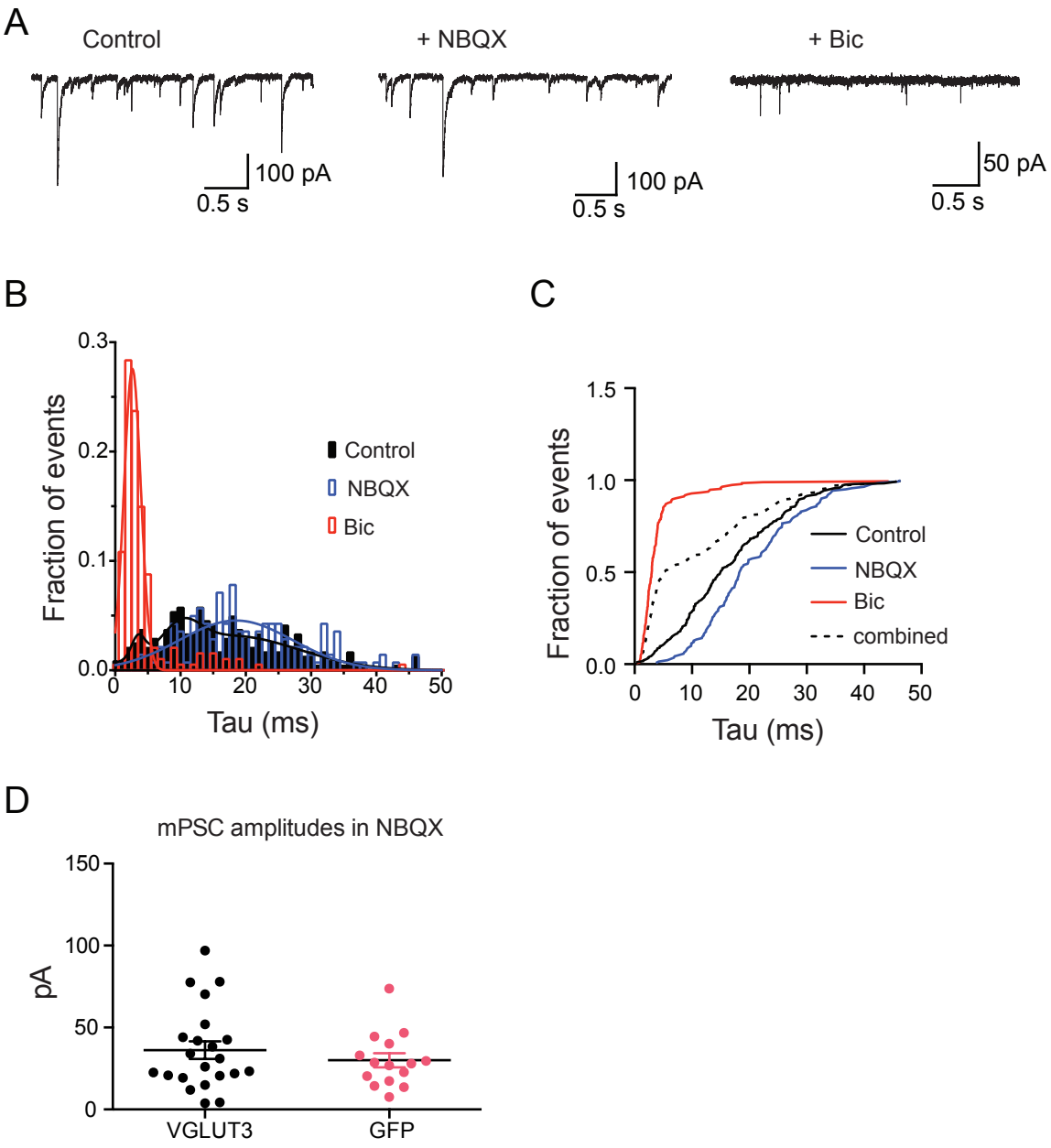

Figure 20: Glutamate and GABA are released from the same vesicles but do not synergize
A: Exemplary traces of spontaneous release in striatal autaptic neurons expressing VGLUT3 under three recording conditions: in control aCSF (left), aCSF with NBQX to block glutamate receptors (center) and aCSF with bicuculline to block $GABA_A$ receptors. B: Histogram of decay times (tau) of spontaneous events under the conditions explained in (A). The plots were fitted with single gaussians (NBQX and Bic; red and blue lines) or with the sum of three guassians (control; black line), respectively. C: Cumulative frequency plot of decay times. Values from "NBQX group" and "Bic group" were combined to create the "combined group". D: Comparison of mPSC amplitudes in presence of NBQX. Circles represent individual cells. Horizontal lines show mean ± SEM.

3.13 No vesicular synergy between GABA and glutamate

We were also wondering if co-packaging of glutamate and GABA led to an increase in GABA content of single vesicles. This synergistic effect was found in other dual-transmitter systems (Amilhon *et al.*, 2010; Hnasko *et al.*, 2010). We compared the "GABA content" of minis by measuring spontaneous event amplitudes and charge in the presence of NBQX. Striatal interneurons expressing VGLUT3 did not show altered mini amplitudes or charge compared to control interneurons expressing GFP (Figure 20D). Thus, the presence of glutamate in those vesicles does not seem to have a synergistic effect on GABA uptake.

3.14 Glutamate co-release virtually undetectable in mass culture neurons

Even though glutamate and GABA co-release is possible in autaptic neuronal cultures other studies exploring bigger networks had problems to detect glutamate release from synaptic boutons. One possible explanation would be that in networks postsynaptic AMPAR are "drawn away" to purely glutamatergic synapses. Thus, even though glutamate and GABA are co-released at certain synapses, the density of AMPAR is just not high enough to reach detection levels.

To test this hypothesis we co-cultured striatal and hippocampal neurons at a medium density of approximately 5K per well each. Neurons were infected with VGLUT3 constructs after 1 DIV and cultured for 13-16 DIVs. The culture dishes were then transferred to a microscope and we performed paired-recordings with connected neurons. Applying NBQX with our fast-flow system, we ensured that the "presynaptic cell" was GABAergic and showed a robust GABAergic response. Next, we tested if we could detect co-release of glutamate from the presynaptic cell by applying bicuculline instead of NBQX. Surprisingly, we did virtually detect no response in the presence of bicuculline, neither pre- nor postsynaptically (Figure 21A).

To confirm that the striatal neurons were *per se* capable of coreleasing glutamate we performed two types of control experiments: 1. We used autaptic neurons from the same animals that we used for the co-culture experiment and infected them with the same viral construct. Electrophysiological recordings after 13-16 DIVs showed robust co-release of glutamate and GABA. 2. We plated striatal and hippocampal neurons at low density on astrocyte islands in order to get an "intermediate-sized" network and to exclude the possibility that the micro-island system itself promotes co-release. The cells were infected with a VGLUT3 construct and paired-

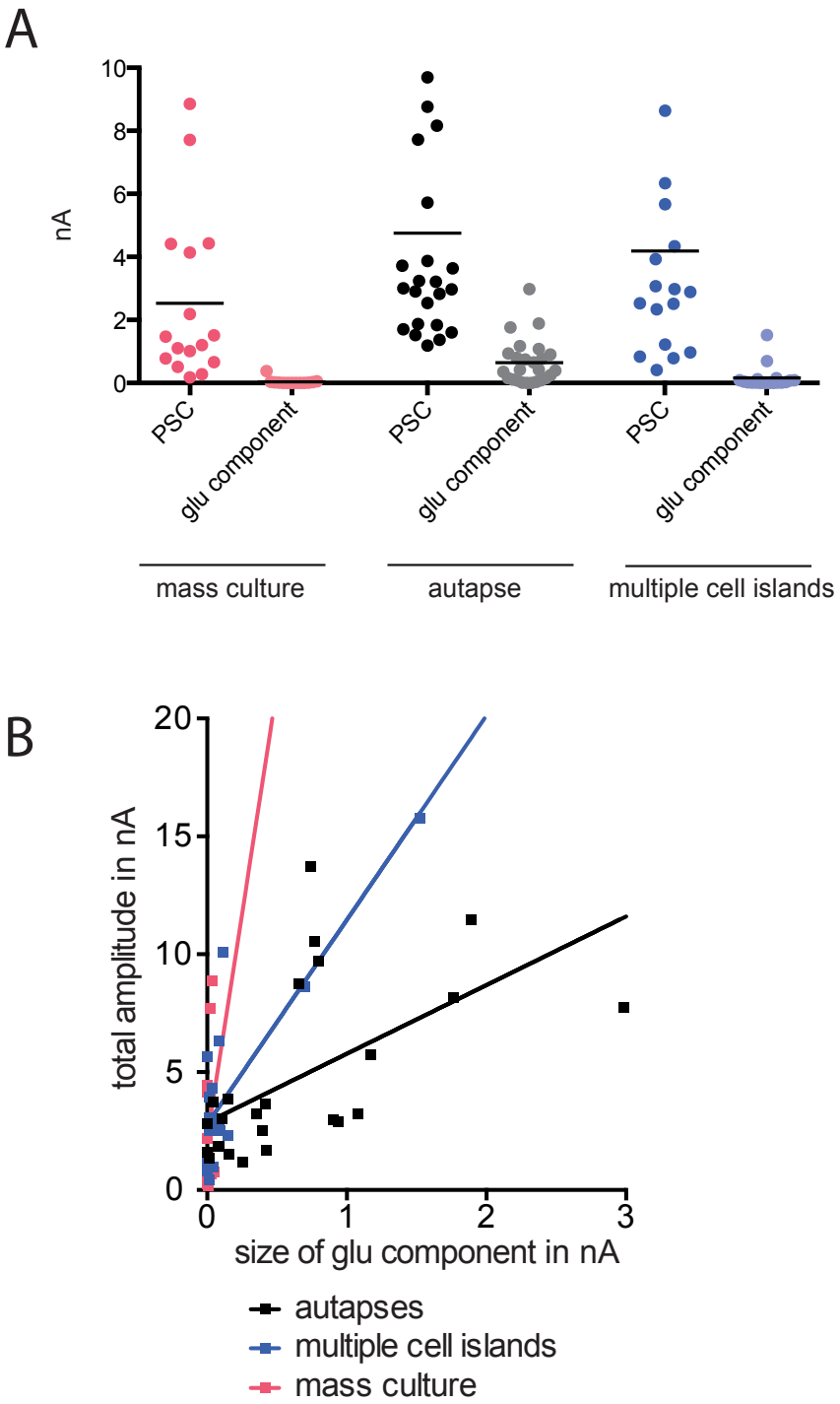

Figure 21: Amount of detectable glutamate co-release varies between different culture systems.
A: Plot of PSC amplitudes in GABAergic striatal neurons expressing VGLUT3. Recordings in 3 different culture systems: mass culture (red), autaptic culture (black/grey) and 2 or more cells on an astrocyte feeder island (blue). Depicted are the PSC sizes in aCSF (darker colors) and the glutamatergic component in the presence of bicuculline (lighter colors). Each data point represents a cell. Horizontal lines show the average response size. B: Plot of PSC amplitude in aCSF ("total amplitude") against amplitude in bicuculline ("glu component") of the same cell. Comparison of 3 different culture systems. Lines show linear regression of all data points from autapses (black), multiple cells on an island (blue) and mass culture (red).

recordings were performed after 13-16 DIVs. Detection of co-release from GABAergic neurons in these micro-island networks was possible but the glutamate-mediated currents were much smaller than in autaptic cultures. We wanted to test, if this was caused by the fact that responses in autapses generally tend to be bigger compared to mass culture (Figure 21B). We plotted the total PSC response of each cell against the corresponding response in bicuculline (Figure 21B). Linear regression revealed that the relationship between total amplitude size and glutamate co-release was different between the three culture systems: In mass culture even in the biggest PSCs only a small or no glutamate signal could be detected. The signal of the glutamate response increased from multiple cell islands to autaptic culture.

These findings indicate that synaptic terminals from purely glutamatergic neurons, which are present in mass culture, compete for AMPA receptors with the GABAergic terminals that co-release glutamate. I showed in chapter 3.10 that the amount of AMPAR is very similar between the different cell types used in this study. Apparently AMPAR clustering does preferentially take place at purely glutamatergic synapses, impeding detection of co-release in mass culture. It is conceivable that co-released glutamate exhibits other functions than binding to ionotropic receptors. By binding to metabotropic glutamate receptors it could potentially influence release parameters of GABAergic neurons. A hypothesis that needs to be validated in future studies.

4. Discussion

In my study I have looked at two classes of vesicular proteins: VGLUTs and v-SNAREs. They play a fundamental role in synaptic transmission. The former are important for vesicle filling, the latter are essential for fusion. However, they also carry out functions beyond their "classical" tasks. They can contribute to co-release as well as regulation of release probability. My goal was to investigate the role of different paralogs of these proteins in putatively non-canonical functions. Besides confirmation of expected effects I discovered previously unknown features, which were unique to specific paralogs of the studied proteins. In general my findings provide new evidence on how molecular diversity contributes to functional diversity at the synapse.

4.1 Part I: Synaptobrevin 1 mediates release in a subset of hippocampal neurons

The SNARE proteins are key players in the highly regulated process of synaptic vesicle endocytosis throughout the entire nervous system. While fusion of SV is impossible in the absence of the t-SNAREs syntaxin and SNAP25, the absolute necessity of v-SNAREs had been questioned in previous studies (Schoch *et al.*, 2001, Deak *et al.*, 2004). In the course of my PhD studies I used the advantages of the autaptic cell culture system in combination with a variety of molecular biology techniques to examine the loss of syb2, the major v-SNARE, in hippocampal neurons. In my thesis I present these major findings: 1. The majority of syb2-deficient neurons, when grown on isolated microislands, are devoid of any Ca^{2+}-mediated evoked or spontaneous release and have no measurable readily-releasable pool (Figure 5, Figure 6 and Figure 7). In a small subpopulation of neurons, however, I detected the expression of the v-SNARE paralog syb1 and found a correlation between syb1 protein levels and remaining RRP size (Figure 9). I could confirm residual release in syb2 KO mass cultures, described in earlier studies (Schoch *et al.*, 2001, Deak *et al.*, 2004, Deak *et al.*, 2006). This release could be significantly reduced by shRNA knockdown of syb1. These findings support the hypothesis that v-SNAREs are absolutely required for vesicle fusion. We interpret the remaining responses in mass cultured syb2 KO neurons to be caused by a subpopulation of neurons that express syb1. 2. In rescue experiments with syb1 and -2 I observed, that syb1 can indeed mediate evoked and spontaneous release in

hippocampal autaptic neurons. However, this SNARE paralog is less efficient in release, which is reflected in reduced EPSC amplitudes, a decreased release probability and a lower mEPSC frequency (Figure 10, Figure 11 and Figure 12).

In the following paragraphs I will discuss a number of questions that arise from my findings: (1) What are the possible reasons for the reported discrepancies between syb2 loss-of-function studies in autaptic cell culture and mass culture? (2) What are the implications of different release properties between syb1 and syb2? (3) Why does the knockdown of syb1 affect only the size of the RRP and not the "mini" frequency?

4.2 Loss-of-function studies of synaptobrevin 2

Mice lacking syb2 die shortly after birth. Consequently, most studies on SV release in absence of syb2 have been carried out in cell culture. The high-density cultures used by Schoch *et al.*, 2001 and Deak *et al.*, 2004 (and also in this study) are a great tool to investigate the consequences of protein loss in a neuronal network. Cells receive inputs from multiple neurons and entire networks of synaptic connections can be activated with a single stimulation electrode. In a heterogeneous population of cells, however, this also has some drawbacks: If a subpopulation of neurons exhibits different properties than the others, this might mask potential findings and distort the results. We suggest, that previous studies in syb2 KO mass cultures missed the presence of another v-SNARE isoform and therefore partially misinterpreted their findings.

The first study of a syb2 KO mouse by Schoch *et al.*, 2001, tested for two syb2 paralogs: syb1 (VAMP1) and cellubrevin (VAMP3). They performed immunoblots of lysates from forebrain as well as from high-density cultures. Neither showed a signal for syb1 or VAMP3. This stands in contrast to our own findings (see Figure 8). Also other studies reported expression of syb1 in the hippocampus: Trimble *et al.*, 1990, performed *in situ* hybridization with RNA probes and found much higher levels of syb2 but also low levels of syb1 in rat hippocampus. These findings were confirmed with immunohistochemical, immunofluorescence and confocal microscope techniques (Raptis *et al.*, 2005). Why the initial study could not detect syb1 expression remains unclear. However, they discuss that syb1 (and VAMP3) levels might have been below their detection levels, and could indeed be responsible for the observed residual response (Schoch *et al.*, 2001).

The autaptic cell culture system used in this work is especially suited to examine heterogeneous populations of cells. The recorded electrical in- and output comes from a single cell and can not be masked by synaptic inputs from other neurons. There is an earlier study using autaptic culture from syb2 KO mice (Guzman *et al.*, 2010). In accordance with our results they report an almost complete absence of evoked and spontaneous NT release. The average mEPSC frequency in syb2 KO neurons is between 3-4 % of WT neurons. In contrast, spontaneous release in syb2 KO mass culture reaches frequencies of 15 % of WT levels (Schoch *et al.*, 2001, Deak *et al.*, 2006). Guzman *et al.*, 2010, study the linker domain between the transmembrane domain and the SNARE motif of syb2. They use the syb2 KO neurons only as a background and therefore do not comment on the discrepancies to previous mass culture studies.

A recent study looked at hippocampal organotypic slices of syb2 KO mice using cryofixation followed by electron tomography (Imig *et al.*, 2014). They found that even though the majority of synapses had few or no docked vesicles, a subpopulation of synapses was indistinguishable from WT. Immunolabeling confirmed that approximately one third of syb2 KO synapses expresses syb1, which is most likely responsible for docking vesicles to the plasma membrane.

Another approach to disrupt synaptobrevin function is the use of neurotoxins. Tetanus toxin (TeNT), as well as several botulinum toxins (BoNT/B, BoNT/D, BoNT/F and BoNT/G) cleave synaptobrevin at specific sites (Schiavo *et al.*, 2000). This leads to loss of spontaneous as well as evoked NT release (Gansel *et al.*, 1987, Molgo *et al.*, 1990). TeNT and BoNT/B recognize the same site on syb2 (Gln 76-Phe 77) (Schiavo *et al.*, 1992). Interestingly, syb1 from rats (but not from mice) has a valine at the corresponding position and can therefore not be cleaved. It is conceivable to exploit the different properties of these toxins by exposing rat neurons to a toxin that only cleaves syb2 or adding a toxin that cleaves both syb1 and syb2, respectively. If the latter decreases residual release to a minimum, this would confirm our results that syp1 promotes fusion in absence of syb2. However, the action of neurotoxins in abolishing syb function is never as complete as a genetic knockout, which might again result in additional problems.

4.3 Release properties of synaptobrevin 1 and 2

This study is the first characterization of syb1 in the fusion of synaptic vesicles at central synapses. I showed that the release properties of syb1 and syb2 are different in several aspects. Syb2 is the dominant v-SNARE in the telencephalon and promotes a high vesicular release

probability. Syb1 vesicles, in contrast, have an intrinsically lower release probability and could be used at synapses, where release needs to be less efficient and reliable. The usage of different v-SNARE paralogs on synaptic vesicles might thus serve as a vesicle-intrinsic determinant of release probability.

Other studies support this theory, even though the results were interpreted differently at that time: Deak *et al.*, 2004, studied exo- and endocytosis in syb2 KO mass culture neurons using styryl dye and HRP uptake experiments. They found a reduced release probability in the syb2 KO cells compared to wild type neurons. Even though the authors did not attribute these findings to residual syb1 levels, it fits very well with our results that syb1 possesses an intrinsically lower release probability compared to syb2. Repeating the experiments using a syb1 knockdown might provide new insights into the distribution and action of different syb paralogs. Interestingly, the authors did not report any obvious variance between individual synapses, as would be expected if syb1 mediates release in a subpopulation of cells. The discrepancy between these uptake studies and our electrophysiological analyses remains to be studied.

At the mouse neuromuscular junction (NMJ) NT release depends partially on syb1 (Liu *et al.*, 2011). The authors compared WT NMJs to NMJs of animals where syb1 had been genetically deleted. They reported a reduction in spontaneous and evoked neurotransmitter release as well as lower initial vesicular release probability. In contrast to our findings, they did not find a decrease in pool size when syb1 levels are reduced. Instead, they report a reduced sensitivity and cooperativity to calcium in the KO neurons. Since syb1 is not the only v-SNARE isoform present at the NMJ is rather difficult to directly compare the two studies, as the number of syb proteins present is supposedly very different in the two systems. Liu and colleagues also report high expression of syb2, which influences the reported results. We find it remarkable though, that the mouse neuromuscular junction, consisting of thousands of unreliable single vesicle release sites (Meriney and Dittrich 2013), depends at least partially on syb1 – a v-SNARE that we show to mediate release with lower release probability.

Cellubrevin (VAMP3), which has also been used to rescue syb2 loss of function, can fully compensate the dysfunctional release in absence of syb2 in neurons (Deak *et al.*, 2006) as well as in chromaffin cells (Borisovska *et al.*, 2005). Comparing the amino acid sequences of syb1, syb2 and ceb shows an almost complete sequence homology in the region of the SNARE layers (Figure 22). This domain – which is essential for vesicle fusion – differs only in two amino

acids between syb1 and the other two v-SNAREs (an aspartate (D) between layers -5 and -4 is mutated to glutamate (E) and a threonine (T) between layers +6 and +7 is mutated to a serine (S)). The linker region between the SNARE motif and the transmembrane domain (TMD) is also highly conserved between the three paralogs. Several studies demonstrated that mutations in this region lead to a severe impairment in NT release (Deak *et al.*, 2006, Kesavan *et al.*, 2007, Guzman *et al.*, 2010). Overall, the sequence homology between syb1 and -2 is 76% (homology between syb2 and ceb is 74%) with the largest differences in the N-terminus. The function of the N-terminus of v-SNAREs has not been studied in great detail but an important role in NT release is unlikely: Cellubrevin, which can completely compensate the loss of syb2, does not possess a sizable N-terminus beyond the SNARE motif. It remains to be studied which part of the sequence is responsible for the reduced release efficiency of syb1.

Our findings that syb1 has a significantly lower release probability compared to syb2 might explain why the majority of syb2 KO neurons in our autaptic culture that still had a measurable RRP did not show evoked responses. Studies from syntaxin hypomorphs have shown that a reduction in the expression of the t-SNARE syntaxin 1a/1b leads to drastic reductions in both RRP size as well as release probability (Arancillo *et al.*, 2013). Combining this with the intrinsically lower release probability of syb1 would even further reduce the likelihood of Ca^{2+}-evoked fusion, causing it to drop below detection levels.

4.4 Different impact of syb1 knockdown on pool size and mini frequency

We could correlate the residual RRP size in the syb2 KO neurons to syb1 expression levels and further decreased the RRP by knocking down syb1. Interestingly, spontaneous release was not effected to the same extend (Figure 15). There are at least two different possible explanations:

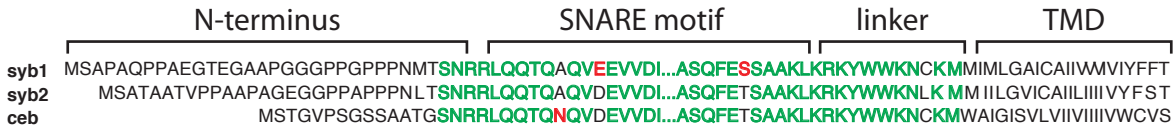

Figure 22: Comparison of amino acid sequences of syb1, syb2 and ceb.
Sequence in one-letter amino acid code of syb1 (top), syb2 (center) and ceb (bottom). Identical amino acids in the central domains of the three proteins are highlighted in green. Differences in red. Note the different size of the N-terminus and the variabilty in the transmembrane domain (TMD).

Even though spontaneous release is still observable in syb2 KO mass culture, the frequency of fusion events is quite low (0.6 to 1 Hz (compare also (Deak *et al.*, 2006; Schoch *et al.*, 2001)). It is conceivable that spontaneous fusion at this low rate can still be maintained with the reduced RRP that we saw in the syb1 knockdown. Alternatively, spontaneously fusing vesicles may originate from a different pool than the RRP (Ramirez *et al.*, 2012; Sara *et al.*, 2005). In the scenario when syb2 is absent, syb1 drives fusion of the RRP whereas an additional v-SNARE may exclusively drive spontaneous fusion. A possible candidate could be the non-canonical SNARE Vps10p-tail-interactor-1a (vti1a), which has been shown to promote spontaneous release in the absence of syb2 (Ramirez *et al.*, 2012). Similarly, in *drosophila* Ca^{2+}-evoked release is thought to be driven by the v-SNARE n-syb, whereas spontaneous release persists even in the absence of n-syb (Deitcher *et al.*, 1998; Yoshihara *et al.*, 1999). Studying a complete genetic deletion of syb1 and -2 could help to differentiate between the two explanations.

Taken together, our results support the hypothesis that v-SNAREs are absolutely required for evoked release and vesicle priming at central synapses. Furthermore, differential expression of SNARE paralogs can contribute to making vesicle release probability and short-term plasticity characteristics more diverse among different synapses.

4.5 Part II: Glutamate/GABA co-release on a vesicular level

The 80-year-old hypothesis known as Dale's principle that „neurons release only a single type of neurotransmitter at all of their synapses" (Strata and Harvey, 1999) has been disproved more than once in the last decades. Co-release of two or more classical neurotransmitters from the same neuron and even the same synapse seem to be quite common throughout the nervous system (Whittaker *et al.*, 1972; Jonas *et al.*, 1998; Higley *et al.*, 2011; Beltran and Gutierrez, 2012). VGLUT3, which was the last of the three VGLUT paralogs to be discovered, was the first to be linked to putative glutamate co-release due to its unique expression patterns in "non-glutamatergic" neurons. More recent studies, however, provided evidence that also VGLUT1 and -2 can contribute to co-release at specific types of synapses (Stuber *et al.*, 2010, Ren *et al.*, 2011). Even though this led to the conclusion that co-release of glutamate seems to be a general phenomenon in the brain rather than an exception, the processes on the vesicular level are mostly unclear: Are the two transmitters stored in distinct vesicle pools or do they share a common pool of vesicles? If they are stored in the same vesicles, does this influence the vesicular loading of NTs? And most importantly: What are the consequences of co-release of two different NTs?

During my PhD work I studied the co-release of glutamate and GABA in striatal interneurons, which exogenously expressed VGLUT3. I present the following findings: 1. The presence of a glutamate transporter on SVs of GABAergic neurons is enough to induce glutamate co-release. 2. Glutamate and GABA are at least partially stored in the same vesicles, however this does not result in NT synergy. GABA content is unaltered in VGLUT3-expressing vesicles 3. In a network of glutamatergic and GABAergic neurons co-release of glutamate is almost undetectable through ionotropic glutamate receptors.

In the following paragraphs I will discuss my results in the context of present published data, present potential outlooks and review several problems I encountered in the course of my study.

4.6 The VGLUTs and possible functions beyond glutamate transport

One of the prerequisites for co-release is the presence of vesicular transporters for the two NTs to be released. When VGLUT3 was discovered in 2002 it was found to be expressed mainly in cholinergic and serotoninergic neurons as well as GABAergic interneurons (Fremeau *et al.*, 2002; Gras *et al.*, 2002; Schafer *et al.*, 2002; Takamori *et al.*, 2002). With this feature it stood out against the other two paralogs VGLUT1 and VGLUT2, which exhibit a mutually exclusive expression pattern throughout the vast majority of excitatory neurons in the brain (Gras *et al.*, 2005). This difference between VGLUT1 and -2 on one side and VGLUT3 on the other side was also observed by murine knockout studies: Whereas mice lacking VGLUT1 or -2 die after birth (Wojcik *et al.*, 2004, Moechars *et al.*, 2006) VGLUT3 KO animals are viable. Since VGLUT3 is responsible for glutamate transport at inner hair cells in the auditory system these animals are deaf. Interestingly, they also exhibit seizures, indicating a role in the control of cortical excitability (Seal *et al.*, 2008).

Obviously, the primary function for VGLUTs is to pump glutamate into SVs. This solitary role of VGLUTs, however, was challenged when Weston and colleagues provided evidence that vesicles carrying VGLUT1 have intrinsically different probabilities to be released compared to vesicles carrying VGLUT2 or -3, respectively (Weston *et al.*, 2011). Due to an interaction site with endophilin A1 VGLUT1 inhibits endophilin-induced enhancement of release probability. Neurons may express the different paralogs of these transporters not only to pump glutamate into vesicles but also to increase functional diversity across synapses.

We asked the question if release probability of GABAergic vesicles could possibly be altered by co-expression of VGLUT3. By assessing the paired-pulse behavior of GABAergic neurons in presence or absence of VGLUT3 I could show that expression of VGLUT3 does not lead to changes in short-term plasticity (Figure 17). In future experiments one could repeat the assay and express VGLUT1 instead of VGLUT3 to test, if endophilin A1 interaction can also alter the release probability of GABAergic vesicles.

By transporting glutamate into “non-glutamatergic” SVs VGLUTs might also modify release properties by changing the quantal content of the other NT. These synergistic effects will be discussed in the next paragraph.

4.7 No synergistic effects between glutamate and GABA

A series of studies showed that the presence of VGLUT3 in cholinergic and serotonergic terminals stimulates the uptake of acetylcholine (ACh) and serotonin (5-HT) to synaptic vesicles (Gras *et al.*, 2008; Amilhon *et al.*, 2010). These so called "synergistic effects" have been attributed to an increase in the pH gradient (ΔpH) across the vesicle membrane due to the presence of glutamate, which then drives NT uptake (Hnasko *et al.*, 2010; El Mestikawy *et al.*, 2011). Whereas for the vesicular acetylcholine transporter (VAChT) and the vesicular monoamine transporter (VMAT2) NT transport into synaptic vesicles depends largely on ΔpH (Johnson *et al.*, 1981; Nguyen *et al.*, 1998) the situation is different for the vesicular inhibitory amino acid transporter (VIAAT). Accumulation of GABA and glycine depends both on the transmembrane potential (Δψ) and on ΔpH (Hell *et al.*, 1990). Even though there is also evidence for synergistic effects between glutamate and GABA (Zander *et al.*, 2010), my findings and a recent study by Case *et al.*, 2014, indicate that GABA content is not influenced by co-packaging of glutamate. The data by Zander and colleagues were obtained in uptake experiments using SV preparations and radioactively labelled GABA or glutamate, respectively. It is possible that this artificial system potentially produces artifacts, which can not be observed in synaptic vesicles. The discrepancy between the studies should be addressed in future experiments.

4.8 Detection of AMPAR clustering with ICC

In order to determine if co-release of glutamate leads to an increase of AMPAR clustering at the postsynapse we performed immunocyochemistry stainings (see 3.11). It has been shown that autaptic GABAergic neurons need glutamate input to create AMPAR clusters. Whereas other components of excitatory postsynaptic sites like the postsynaptic density protein 95 (PSD-95) and NMDA receptors do form clusters in absence of glutamate input, the AMPAR signal is uniformly distributed throughout the cell body and dendrites (Rao *et al.*, 2000). The striatal autaptic culture with GABAergic neurons expressing VGLUT3 would be the perfect system to determine if glutamate co-release is sufficient to induce AMPAR clustering. Unfortunately, we were unable to obtain reliable and reproducible AMPAR stainings in our autaptic culture. Due to a high background signal from the astrocytic feeder islands we had a very low signal to noise ratio, making the analysis of AMPAR clustering quite difficult. In the following I will discuss

briefly how one might improve these experiments in the future: It can be observed that the formation of dendritic spines is not complete at DIV 13-14 when we performed the analysis. Successful stainings of postsynaptic receptors in autaptic neurons were usually carried out later (DIV 15-29) when spine formation is more pronounced (Rao *et al.*, 2000, Valente *et al.*, 2015). Carrying out the staining experiments at a later time point might thus improve the detection of clustering. The above-mentioned studies used a special kind of autaptic culture, where the neurons do not grow on astrocyte islands, but were directly seeded onto a mixture of poly-D-lysine and collagen. This has the advantage that the signal to noise ratio is much higher due to the lack of astrocyte background signal. In contrast, due to the absence of an astrocyte feeder layer a high percentage of neurons does not survive this type of culture. This might potentially lead to bias, since one type of neuron could cope better with the absence of astrocytes than others.

4.9 Co-release of glutamate in autapses compared to mass culture

Even though multiple cell types in the brain have been identified, where the GABA transporter VIAAT and a glutamate transporter VGLUT are coexpressed, it has been difficult to proof co-release of GABA and glutamate at those synapses. The only two areas where co-release of the two fast-acting NTs was shown are in the lateral superior olive (LSO) (Gillespie *et al.*, 2005) and in hippocampal mossy fibers (Gutierrez, 2003, Beltran *et al.*, 2012): GABA/glycinergic synapses from the medial nucleus of the trapezoid body (MNTB) express VGLUT3 and co-release glutamate. This is important for the refinement of an inhibitory map in the auditory system (Noh *et al.*, 2010). Glutamatergic granule cells in the dentate gyrus transiently release GABA during development from single mossy fibre giant boutons (Beltran *et al.*, 2012).

Our co-release experiments in autaptic culture and mass culture provide a possible explanation for the difficulties to show glutamate co-release at GABAergic synapses expressing a VGLUT isoform: An inhibitory neuron grown in solitude on an astrocytic feeder island does not receive synaptic inputs from purely glutamatergic neurons. However, it still expresses a high amount of AMPA receptors (comparable to purely glutamatergic neurons, see Figure 18). The AMPA receptors of this inhibitory neuron can thus cluster at the “coreleasing” synapses. This would increase the likelihood to detect glutamate co-release. Mass culture neurons, in contrast, receive multiple inputs from genuine glutamatergic neurons. We suggest, that the AMPA receptors preferentially localize to these truly glutamatergic synapses, making the detection of gluta-

mate release from GABAergic terminals more difficult. To prove this theory further experiments are required. High resolution images of AMPAR clustering at the different synapses in autaptic and mass culture could provide new insights.

It should be stressed that the autaptic neuron without synaptic input from other cells is a model system, which has no equivalent in the brain. Therefore, the phenotype that we saw in mass culture (glutamate co-release is rarely detectable) is more likely to be the one found in the brain. What are possible reasons for co-release, if there are almost no AMPAR to detect glutamate? Synergistic effects, which have been shown for other NTs (El Mestikawy *et al.*, 2011) do not seem to play a role for the loading of GABA vesicles (Figure 20F, and see also Case *et al.*, 2014). However, co-released glutamate could potentially bind to metabotropic glutamate receptors (mGluRs), which can be both pre- and postsynaptically expressed (Shigemoto *et al.*, 1997). Whether glutamate binding to mGluRs leads to presynaptic inhibition or modulation of neurotransmission as seen in other systems (Sladeczek *et al.*, 1992, Bonsi *et al.*, 2005) is a very interesting question for future studies.

Summary

Release of neurotransmitters requires a fast-acting and very reliable protein machinery. At the same time it needs to be regulated for variable demands at different types of synapses. In my PhD study I investigated how different paralogs of two essential vesicular proteins influence release parameters at murine glutamatergic and GABAergic synapses.

In the first part of my thesis I studied neurotransmitter release in absence of the v-SNARE synaptobrevin 2 and compared release properties of synaptobrevin 1 and 2. The core machinery of synaptic vesicle fusion consists of three SNARE proteins, the two t-SNAREs at the plasma membrane (SNAP-25, syntaxin 1), and the vesicle bound synaptobrevin 2 (VAMP2). Formation of the trans-oriented four-α-helix bundle between these SNAREs brings vesicle and plasma membrane in close proximity and prepares the vesicle for fusion. The t-SNAREs are thought to be necessary for vesicle fusion. Whether the v-SNAREs are required for fusion is still unclear, as substantial vesicle priming and spontaneous release activity remains in mammalian mass cultured synaptobrevin-deficient neurons. Using the autaptic culture system from synaptobrevin 2 knockout neurons of mouse hippocampus, I found that the majority of cells were devoid of any evoked or spontaneous release and had no measurable readily-releasable pool. A small subpopulation of neurons, however, displayed release, and their release activity correlated with the presence and amount of v-SNARE synaptobrevin 1 expressed. Comparison of synaptobrevin 1 and 2 in rescue experiments demonstrate that synaptobrevin 1 can substitute for the other v-SNARE, but with a lower efficiency in neurotransmitter release probability. Release activity in synaptobrevin 2-deficient mass cultured neurons was massively reduced by a knockdown of synaptobrevin 1, demonstrating that synaptobrevin 1 is responsible for the remaining release activity. These data support the hypothesis that both t- and v-SNAREs are absolutely required for vesicle priming and evoked release and that differential expression of SNARE paralogs can contribute to differential synaptic coding in the brain. This work has been published elsewhere (Zimmermann *et al.*, 2014).

In the second part of my thesis I analyzed if expression of the glutamate transporter VGLUT3 induces co-release of glutamate and GABA from striatal interneurons. Co-release of two or more classical neurotransmitters from the same neuron seems to be quite common throughout the nervous system. However, the co-release of glutamate and GABA – the two

major excitatory and inhibitory neurotransmitters – has not been thoroughly analyzed on a vesicular level. I used a lentiviral construct to exogenously express VGLUT 3 in autaptic GABAergic neurons cultured from the striatum. Performing patch-clamp recordings I addressed the question whether GABA and glutamate can be released from the same vesicle by recording postsynaptic events. I found that action potentials in GABAergic neurons expressing VGLUT3 evoked mixed postsynaptic currents (PSC) mediated by both GABA and glutamate release. Using analysis of decay kinetics from miniature PSCs of spontaneous release, I determined that the quantal events underlying the evoked mixed PSC included vesicles containing both glutamate and GABA. I tested for synergistic effects of GABA loading when glutamate was present in the vesicle. Neurons expressing VGLUT3 did not exhibit an increase in vesicular GABA content, measured by the mPSC size in presence of NBQX. Glutamate release from GABAergic neurons did not alter the expression level of postsynaptic AMPA receptors, as exogenous kainate application evoked similar currents in control GABAergic cell and those expressing VGLUT3. Interestingly, synaptic input of "purely" glutamatergic neurons to striatal GABAergic neurons expressing VGLUT3 impeded detection of glutamate co-release, probably by drawing away AMPA receptors to glutamatergic synapses. The findings provide new insights into glutamate/GABA co-release and suggest that the neuronal network has a great influence on the detection of co-release.

Zusammenfassung

Die Freisetzung von Neurotransmittern erfordert eine verlässlich und schnell arbeitende Proteinmaschinerie. Gleichzeitig muss der Prozess hoch regulierbar sein, um sich an die verschiedene Anforderungen der unterschiedlichen Synapsentypen anzupassen. In meiner Doktorarbeit erforschte ich, wie verschiedene Paraloge von zwei essentiellen vesikulären Proteinen die Freisetzungseigenschaften an glutamatergen und GABAergen Synapsen der Maus beeinflussen.

Die Kernmaschinerie zur Verschmelzung von synaptischen Vesikeln mit der Plasmamembran besteht aus drei SNARE-Proteinen, den beiden t-SNAREs Syntaxin 1 und SNAP-25 in der Zellmembran und Synaptobrevin 2 (syb2), das in der Vesikelmembran verankert ist. Zur Fusion von Vesikel und Plasmamembran bilden die drei Proteine ein Vier-α-Helix-Bündel, das die beiden Membranen in direkte Nachbarschaft bringt und für die Freisetzung vorbereitet. Während die t-SNAREs absolut notwendig für die Verschmelzung von Vesikel mit Plasmamembran zu sein scheinen, ist die Situation bei v-SNAREs weniger klar. In Massenkulturen von Säugetierneuronen, bei denen syb2 genetisch ausgeschaltet wurde, konnte immer noch spontane Freisetzung und Vesikel-"Priming" beobachtet werden.

Im ersten Teil meiner Arbeit untersuchte ich Neurotransmitterfreisetzung in Abwesenheit des v-SNAREs syb2 und verglich Freisetzungsparameter zwischen Synaptobrevin 1 (syb1) und 2. Ich kultivierte Maus-Neurone, bei denen das Gen für syb2 ausgeschaltet war, im autaptischen Zellkultur-System und fand, dass die Mehrzahl der Zellen weder evozierte noch spontane Freisetzung zeigte und keinen messbaren Pool an Fusions-kompetenten Vesikeln aufwies. Allerdings konnte ich in einer kleinen Population von Neuronen Neurotransmitterfreisetzung nachweisen. Die Menge der Freisetzung korrelierte dabei mit der Proteinexpression eines anderen v-SNAREs, syb1. Ich verglich syb1 und -2 in Substitutions-Experimenten und fand, dass syb1 das andere v-SNARE ersetzen kann, aber eine geringere vesikuläre Freisetzungswahrscheinlichkeit verursacht. Die Freisetzung, die im syb2-Knockout in Massenkultur beobachtet wurde, konnte ich durch einen „Knockdown" von syb1 stark reduzieren und damit zeigen, dass syb1 für die verbleibende Freisetzung verantwortlich ist. Meine Daten unterstützen die Hypothese, dass sowohl t-SNAREs als auch v-SNAREs absolut notwendig sind für sowohl Vesikel-"Priming" als auch evozierte Freisetzung und dass die Expression verschiedener SNARE-Paraloge zur synaptischen Variabilität beiträgt.

Im zweiten Teil meiner Arbeit analysierte ich, ob die Expression des Glutamattransporters VGLUT3 in striatalen Interneuronen die Ko-Freisetzung von Glutamat und GABA induziert. Die Ko-Freisetzung von zwei oder mehr klassischen Neurotransmittern aus demselben Neuron scheint sehr verbreitet zu sein in unserem Nervensystem. Allerdings wurde die Ko-Freisetzung von Glutamat und GABA – den wichtigsten exzitatorischen und inhibitorischen Neurotransmittern – bislang nicht gründlich auf vesikulärem Level untersucht. Ich nutzte eine lentivirale Fähre um VGLUT3 exogen in autaptischen GABAergen Neuronen aus dem Striatum zu exprimieren und fand heraus, dass die Aktionspotenziale dieser Nervenzellen eine gemischte postsynaptische Antwort (engl. *postsynaptic current*, PSC) aus einer GABA- und einer Glutamatkomponente aufwiesen. Durch die Auswertung der Kinetik von Miniatur-PSCs konnte ich zeigen, dass eine Gruppe von Vesikeln sowohl Glutamat als auch GABA enthielt. Die Anwesenheit von Glutamat führte nicht zu einer Erhöhung der GABA-Konzentration, was gegen synergistische Effekte der beiden Neurotransmitter spricht. Die Ko-Freisetzung von Glutamat führte auch nicht zu einer erhöhten Expression von AMPA-Rezeptoren in GABAergen Neuronen, was durch die Applikation von Kainat gezeigt werden konnte. Interessanterweise konnte Ko-Freisetzung von Glutamat und GABA in Massenkultur, wo „echte“ glutamaterge Synapsen um die vorhandenen Rezeptoren konkurrieren, nur sehr schlecht nachgewiesen werden. Die Erkenntnisse dieser Arbeit bieten neue Einsichten in die Ko-Freisetzung von Glutamat und GABA und legen nahe, dass die neuronale Umgebung großen Einfluss auf die Detektion von Ko-Freisetzung hat.

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Statement of Contributions

If not stated differently experiments were carried out by me. Measurements of kainate application to striatal cells with and without VGLUT3 (chapter 3.10) was performed by Dr. Melissa Herman. Detection of co-release in different culture systems (chapter 3.14) was done in collaboration by Dr. Melissa Herman and me. Immunocytochemistry staining and imaging of AMPA receptors (chapter 3.11) was carried out by Sabina Merrill. Experimental designs by Prof. Dr. Christian Rosenmund, Dr. Melissa Herman (for the co-release project) and me.

Preparation of astrocyte feeder cultures and microdot stamping was performed by Metin Yenilmez and Annegret Felies. Animal genotyping for colony maintenance was done by Kathleen Grüttner and Sabine Lenz. Experimental design and production of lentiviral constructs was organized and instructed by Dr. Thorsten Trimbuch and carried out by Katja Pötschke, Bettina Brokowski and Carola Schweinoch. The above mentioned are all members of the Rosenmund laboratory. Sabina Merrill did an internship in the rosenmund lab and is a student at the University of Heidelberg.

Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version nicht veröffentlicht.

Publications

Aggarwal S, Yurlova L, Snaidero N, Reetz C, Frey S, **Zimmermann J,** Pahler G, Janshoff A, Friedrichs J, Muller DJ, Goebel C, Simons M (2011) A size barrier limits protein diffusion at the cell surface to generate lipid-rich myelin-membrane sheets. Developmental cell 21:445-456.

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Erklärung an Eides statt

Hiermit erkläre ich, Johannes Zimmermann, geboren am 23.01.1984 in Hannover, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe.

Berlin, den 07. März 2015

Johannes Zimmermann