Dissertation

Submitted to the

Combined Faculties for the Natural Sciences and for Mathematics

of the Ruperto-Carola University of Heidelberg, Germany

for the degree of

Doctor of Natural Sciences

presented by

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born in: Chaohu city, Anhui, PRC

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Title

The serine/threonine kinase AKT switches between functional modes of the vesicle release machinery

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Declaration

I hereby declare that this thesis is my own original research work. This thesis has not been used for the award of any degree or its equivalent in any other university or institution.

The work was done at the Institute for Neurobiology of the Ruperto-Carola University of Heidelberg under the guidance of Prof. Dr. Christoph M. Schuster from April 2008 to December 2013.

Date:.....

Signed:.....

(Lihao Ge)

谨以此文献给：

我远方的奶奶

及亲爱的爸爸妈妈和妹妹

For my Grandma in heaven,

and my beloved Parents & Sister

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1 Summary

Neurotransmitter release is a fundamental process in the nervous system by which neurons are enabled to communicate with postsynaptic cells in a highly controlled manner. This communication is mediated by three types of neurotransmitter release: synchronous, asynchronous and spontaneous vesicle releases, of which each has its own functional importance. A misbalance of these forms of transmitter release is therefore likely associated with improper information processing and may hence lead to network malfunction or even psychiatric conditions. While the fundamental principles of synaptic neurotransmission have been well clarified, questions remain whether for example the different release modes are based on distinct identities of vesicular release machineries or whether they reflect different functional states of the same vesicle release machinery. In this thesis we approached these and related questions at the larval (neuromuscular junction) NMJ of *Drosophila melanogaster*.

We found that the membrane-associated serine/threonine kinase AKT, a nodal part of the phosphatidylinositol-3-kinase (PI3K) signaling pathway, is required to maintain the release machineries of presynaptic vesicles in a tightly clamped and calcium-sensitive status. In this status vesicle release can be evoked synchronously or asynchronously by action potentials and associated calcium influxes. Lost or reduced AKT activity switches the release machinery into a calcium-insensitive and fusogenic status resulting in spontaneous vesicle release that is independent of action potentials or the presence of calcium. AKT-mediated switching of the functional status of vesicle release machineries is a rapid process that acts acutely on readily releasable vesicles and hence can dynamically influence synaptic communication. Mechanistically, we show that the calcium sensor Synaptotagmin 1 that is also part of the release machineries' fusion clamp mediates the AKT effects and hence may be a direct target of AKT phosphorylation. We further show that the clustered and functionally intact voltage gated calcium channel Cacophony is required for the AKT interaction with the release machinery, suggesting that only docked and primed vesicles are accessible to and regulated by AKT. These results demonstrate that AKT is an essential direct regulator of the mode by which synaptic vesicles can be released.

In chapter II, with an attempt to identify potential synaptic regulators of AKT we assessed whether the recently identified ATP-dependent on-off switch of AKT might play a role in the regulation of its synaptic function. We found that treatment with

oligomycin resulted in enhanced spontaneous vesicle release that was similar to that elicited by AKT blockade. Short trains of nerve stimulation during oligomycin incubation triggered an instantaneous increase in the rate of spontaneous vesicle release whereas a similar stimulation without oligomycin was without effect. These data indicate that nerve stimulation strongly enhances the ATP consumption in nerve terminals resulting in ATP-depletion and perhaps in AKT-inactivation. To test this hypothesis we made use of an alternative ATP-depletion strategy that allowed us to make postsynaptic recordings during periods of intense nerve stimulation. We found that stimulation-induced ATP-depletion triggered a strong enhancement of spontaneous vesicle release that was indeed AKT- and PI3K-dependent, which was mediated by Synaptotagmin 1 and depended on the functional presence of the Cacophony. These results confirmed the functional existence of a Cacophony/AKT/release machinery complex and they suggest that AKT is regulated by ATP. They further suggest that AKT may serve at vesicle release sites as a local energy sensor that depending on the availability of ATP switches individual vesicle release machinery either into a tightly clamped mode for evoked release or at low ATP levels into a loosely clamped mode generating spontaneous release. This novel and evolutionarily conserved synaptic role of AKT could shed new light onto the pathogenesis of Schizophrenia or Autism Spectrum Disorders in which AKT activities seem to be reduced. It also needs to be considered in recent approaches to treat several forms of cancer with AKT-inhibitors.

In parallel to the above work, I was involved in a collaborative project that aimed at establishing a three-dimensional computational model of the glutamatergic synapses of larval NMJs. Chapter III summarizes the experimental data that formed the basis of the computational model and showed that high frequency nerve stimulation leads to a disproportional decay of evoked EJP amplitudes due to limited vesicle supply. The model is described in the discussion.

2 Zusammenfassung

Die Freisetzung von Neurotransmittern ist ein fundamentaler Prozess des Nervensystems, der es ermöglicht, dass Neurone mit postsynaptischen Zellen in einer kontrollierten Art und Weise kommunizieren können. Dabei werden Neurotransmitter auf drei verschiedene Arten freigesetzt: synchron, asynchron und spontan, wobei jede ihre eigene funktionelle Bedeutung hat. Ein Ungleichgewicht der verschiedenen Arten von Vesikel-Freisetzungen führt deshalb möglicherweise zu Problemen in der neuronalen Informationsverarbeitung und könnte zu Fehlfunktionen von Netzwerken und sogar zu psychiatrischen Erkrankungen führen. Während die grundlegenden Prinzipien der synaptischen Signalübertragung gut erforscht sind, bleibt zum Beispiel die Frage unbeantwortet, ob die verschiedenen Freisetzungsformen von Vesikeln auf unterschiedlichen Identitäten vesikulärer Freisetzungsmaschinerien oder auf verschiedenen funktionellen Zuständen derselben Vesikel-Freisetzungsmaschinerien beruhen. In dieser Arbeit untersuchten wir diese Fragen an der neuromuskulären Endplatte von Drosophila melanogaster Larven.

Wir fanden heraus, dass die membranassoziierte Serin/Threonin-Kinase AKT, ein Knotenpunkt des Phosphatidylinositol-3-Kinase(PI3K)-Signalwegs, erforderlich ist, um die Vesikel-Freisetzungsmaschinerie in einem sicher „geklemmten“ und Kalzium-sensitiven Zustand zu erhalten. In diesem Zustand kann ein Vesikel synchron oder asynchron durch Aktionspotentiale und den damit verbundenen Einstrom von Kalzium freigesetzt werden. Reduzierte AKT-Aktivität schaltet die Freisetzungs-maschinerie in einen Kalzium-insensitiven und „fusogenen“ Status, was zu spontaner Vesikelfreisetzung führt, die unabhängig von Aktionspotentialen oder Kalzium ist. Das AKT-vermittelte Umschalten des funktionellen Zustands der Vesikelfreisetzungs-maschinierie ist ein schneller Prozess, der akut auf Vesikel des „readily releasable vesicle pools“ wirkt und daher dynamisch synaptische Kommunikation beeinflussen kann. Mechanistisch zeigen wir, dass der Kalziumsensor Synaptotagmin1, der Teil der Fusionsklemme der Vesikel-Freisetzungsmaschinierie ist, die Wirkungen von AKT vermittelt und somit ein direktes Ziel von AKT-Phosphorylierung sein kann. Wir zeigen weiterhin, dass der funktionell intakte spannungsgesteuerte Kalziumkanal Cacophony für die AKT-Interaktion mit der Freisetzungsmaschinierie erforderlich ist. Dies deutet darauf hin, dass nur „gedockte und geprimte“ Vesikel für AKT zugänglich sind und reguliert werden können. Diese Ergebnisse zeigen, dass AKT ein essentieller und direkter

Regulator des Modus vesikulärer Freisetztung ist.

In Kapitel II haben wir geprüft, ob der kürzlich identifizierte ATP-abhängige An- und Ausschalter von AKT eine Rolle bei der Regulation der synaptischen Funktion von AKT spielen könnte. Eine Behandlung mit Oligomycin führte zu einer verstärkten spontanen Vesikelfreisetzung, die der ähnelt, die durch AKT-Inhibierung ausgelöst wird. In Anwesenheit von Oligomycin riefen bereits kurze Episoden von Nervstimulationen eine sofortige Erhöhung der Rate an spontan freigesetzten Vesikeln hervor, während eine ähnliche Stimulation ohne Oligomycin keine Veränderung bewirkte. Diese Daten deuten an, dass Nervstimulation zu einem verstärkten ATP-Verbrauch in Nervenendigungen führen und der Mangel an ATP eine Inaktivierung von AKT hervorrufen könnte. Um diese Hypothese zu testen, bedienten wir uns einer alternativen Methode, ATP-Mangel hervorzurufen und mit der postsynaptische Aufnahmen während intensiver Nervenstimulation möglich sind. Ein auf diese Weise induzierter ATP-Mangel bewirkte eine starke Erhöhung der spontanen Vesikelfreisetzung, die auch AKT- und PI3K-abhängig war, durch Synaptotagmin 1 vermittelt wurde und funktionsfähige Cacophony-Kanäle benötigte. Diese Ergebnisse bestätigten die Existenz eines funktionellen Komplexes aus Cacophony, AKT und der Vesikel-Freisetzungsmaschinerie und dass AKT durch ATP reguliert werden kann. Desweiteren weisen sie darauf hin, dass AKT an „Aktiven Zonen“ als lokaler Energiesensor dienen könnte. Dieser versetzt in Abhängigkeit von der Verfügbarkeit von ATP einzelne Vesikel-Freisetzungsmaschinerien entweder in einen „geklemmten“ Modus zur evozierten Freisetzung oder bei niedrigem ATP-Spiegel in einen „fusogenen“ Modus, der zu spontaner Freisetzung führt. Diese neue und evolutionär konservierte synaptische Rolle von AKT könnte neue Hinweise zur Pathogenese von Schizophrenie und Autismus-Spektrum-Störungen liefern, bei denen AKT reduziert zu sein scheint. Vor diesem Hintergrund sind aktuelle Studien in der Krebstherapie, bei denen AKT-Blocker eingesetzt werden bedenklich.

Parallel zu den oben genannten Arbeiten, war ich in einem kollaborativen Projekt beteiligt, das darauf abzielte ein dreidimensionales Computermodell der glutamatergen Synapsen der larvalen NMJs zu entwickeln. Kapitel III fasst die experimentellen Daten, die die Grundlage des Rechenmodells bilden zusammen und zeigt, dass hochfrequente Nervenstimulation aufgrund der begrenzten Versorgung mit Vesikeln zu einem überproportionalen Abfall der Amplituden evozierter EJPs führt. Das Modell wird in der Diskussion beschrieben.

3 Introduction

How can people see, listen, think and move? The interest about the nervous system has begun more than 2000 years ago. Over the last decades, a wealth of knowledge about the nervous system has been acquired in both vertebrate and invertebrate. In general, neuronal signals are transmitted by electrical and chemical synapses. In electrical synapses, electrical signals can directly pass two adjacent neurons through a low-resistance pathway for small molecules (like low-molecular-mass dyes and ions) - gap junction channel (Waranabe, 1958; Fushpan and Potter, 1959). Some studies have shown that gap junction conducted electrical synapses are crucial in the synchronization of inter-neuronal networks in the mammalian brain (Galarreta and Hestrin 2001, 2002; Bennett and Zukin, 2004). Other studies have suggested that the formation of local connectivity in the developing neocortex requires gap-junction-mediated neuronal communication (Yuste et al., 1992; Peinado et al., 1993; Yuste et al., 1995). A recent work also indicated that transient electrical coupling can regulate lineage-dependent assembly of precise excitatory neuron microcircuits in the neocortex (Yu et al., 2012).

However, electrical connections have only been demonstrated in a limited number of mammalian brain regions, and neuronal communication is mediated largely by chemical synapses (Galarreta and Hestrin 2001). In chemical synapses, neuronal communication is mediated by the release of neurotransmitters like gamma-aminobutyric acid (GABA), glycine and glutamate from the presynaptic terminals, their binding to corresponding receptors in postsynaptic neurons and ion-channel opening. After the finding of the chemical synapses, three types of neurotransmission are widely investigated, including action potential (AP) triggered Ca^{2+}-influx that evokes synchronous and asynchronous release of presynaptic vesicles, as well as AP independent spontaneous vesicle release. For a long time, the action potential and voltage-gated calcium channel (VGCCs) triggered vesicular neurotransmitter release have been in the spotlight of neuroscience research (Neher and Sakaba, 2008; Südhof, 2012). Recently, substantial studies aimed in asynchronous and spontane-ous release (Hefft and Jonas, 2005; Peters et al., 2010; Yang et al., 2010; Yao et al., 2011). What's the difference between various forms of vesicle release? Do they share the same vesicles or same release machineries? How can they be affected by calcium or other regulatory mechanisms? In the following part, I will focus on addressing these questions.

3.1 Synaptic vesicle pools

In chemical synapses, neurotransmitters are stored in small membrane compartment - synaptic vesicle, typically 40 ~ 50 nm in size (Alabi and Tsien, 2012). At rest, synaptic vesicles can collapse into the plasma membrane spontaneously in a relatively slow rate of 0.01 - 0.02 Hz per release site (Fatt and Katz, 1952); upon electrical stimulation, vesicles fuse rapidly and synchronously at many synapses in a narrow time window. In both synchronous and spontaneous release, questions about whether the same synaptic vesicles are used and whether these vesicles belong to the same vesicle pool have been asked and debated for decades. Here, the recent research work concerning the above two questions will be compared, and our own evidence from *Drosophila* NMJ will be shown in results part.

3.1.1 Classification of synaptic vesicle pools

The earliest observation about vesicle pools goes back to the work on acetylcholine release from cat sympathetic ganglia by Birk and MacIntosch in 1961, which ignited the concept about “readily releasable”. This was further confirmed by Elmqvist and Quastel in 1965. There were bewildering list of names for vesicle pools, terms like total recycling pool, reluctant/resting pool and super pool are also very frequently used. How to distinct the vesicles into different pools? With the help of a list of tools and methods: from the earlier FM dyes to the recent genetically encoded ph-sensitive pHluorin, widely used electrophysiology, photoconversion combining EM, the vesicle pools can be classified into three canonical vesicle pools according to their properties (Fig. 1): readily releasable pool (RRP), recycling pool/exo-endo recycling and reserve pool (RP), each has its own function and properties in providing synaptic vesicles for synaptic communication (Rizzoli and Betz, 2005; Alabi and Tsien, 2012).

RRP (~ 1% of total vesicle number): normally indicates the pool of vesicles which are immediately releasable upon stimulation. RRP vesicles are thought to be docked to a specialized presynaptic membrane region opposite to the postsynaptic density - active zone (Couteaux and Pecot-Dechavassine, 1970) and primed for release. However, we should be aware that docked vesicles are not all immediately releasable (Rettig and Neher, 2002).

Recycling pool (5 ~ 20% of total vesicle number), is proposed to repopulate vacancies within the RRP and can be defined as the pool of vesicles that sustain

release on moderate stimulation. It can recycle continuously and refilling rapidly under physiological frequencies of stimulation (Richards et al., 2003; Kuromi and Kidokoro 2003; de Lange et al., 2003).

Reserve pool (typically ~ 80 – 90% of total vesicle number) is a reservoir of synaptic vesicles from which release is only possible during intense stimulation and rarely or never recruited during physiological activity (Kuromi and Kidokoro, 1998). Vesicles in reserve pool are less mobile, which was hypothesized to be glued to the cytoskeleton by synapsin. Phosphorylation of synapsin by calcium/calmodulin-dependent protein kinase II (CaMKII) reduces vesicle binding-affinity and releases the vesicle from the reserve pool (Benfenati et al., 1992; Pieribone et al., 1995; Takei et al., 1995; Hilfiker et al., 1999). To recruit reserve pool vesicles, the required stimulation frequencies are at minimum 5–10 Hz in frog NMJ (Heuser and Reese, 1973; Richards et al., 2000), 30 Hz in *Drosophila* larval NMJ (Kuromi and Kidokoro, 2000) or prolonged high potassium application at the calyx of Held (de Lange et al., 2003). In addition to the above three classical vesicle pools, other pools like resting pool and super pool are also occasionally mentioned. Resting pool (~ 50 – 80% of total vesicle number), which can be understood as a set of vesicles that remain unreleased even after

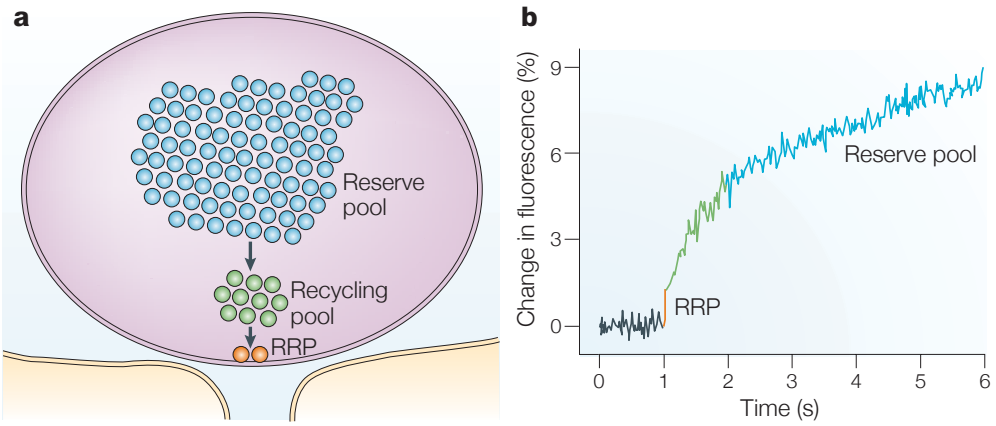

Figure 1. Three types of vesicle pools.

a. The classic three-pool model. The reserve pool makes up ~80–90% of the total pool, and the recycling pool is significantly smaller (~10–15%). The readily releasable pool (RRP) consists of a few vesicles (~1%) that seem to be docked and primed for release. **b.** Three kinetic components of release (indicating release of three vesicle pools) on depolarization of goldfish bipolar cells. The cell was stimulated in the presence of the styryl dye FM 1-43, and the increase in fluorescence gives a direct measure of exocytosis. Reproduced from Rizzoli and Betz, 2005.

prolonged stimulation, is most likely the source of spontaneous release (Fernandez-Alfonso and Ryan, 2008; Fredj et al., 2009; Denker et al., 2011). Notably, resting pool and reserve pool share a large population of vesicles albeit the different name. They

contribute to the superpool, which transits rapidly between synapses, as seen in neuronal culture (Krueger et al., 2003), native brain tissue (Staras et al., 2010), and *in vivo* (Herzog et al., 2011).

3.1.2 Identity of evoked and spontaneously releasing vesicles

Except that different vesicle pools possess various recruiting kinetics by electrical stimulation, one important question concerning vesicle pools is whether evoked and spontaneous release use the same vesicles with the same molecular composition. Recently, accumulating data provided evidence in two directions. On the one hand, spontaneous release employs apparently a separate pool of vesicles from evoked release (Sara et al., 2005; Fredj et al., 2009). In rat hippocampal neurons, using fluorescent styryl dyes and antibodies against synaptotagmin 1 (Syt1) or horseradish peroxidase to label the synaptic vesicles, which shows that synaptic vesicles recycle at rest and populate a pool of limited size which are more likely to refuse spontaneously. Blocking vesicle refilling with folimycin at rest specifically depleted spontaneously released vesicles without significantly affecting evoked transmission. In the absence of synaptobrevin, both spontaneous and evoked vesicles could mix more, suggesting function of synaptobrevin in separating the two pools of vesicles (Sara et al., 2005). Also in rat hippocampal cultures, similar results were obtained with a genetically encoded biosyn which labels presynaptic vesicle proteins by combining *in vivo* biotinylation of transmembrane proteins with irreversible tagging by fluorescent streptavidin (Fredj et al., 2009). On the other hand, evidence also shows that the same group of synaptic vesicles can participate in both types of releases. Similarly in rat hippocampal neurons, studies indicate releasing probabilities for both release are well correlated with the vesicle pool size at single synapse (Prange and Murphy, 1999). In addition, using spectrally separable styryl dyes and a later study with genetic probe synapto-pHluorin (spH, a fusion construct of synaptobrevin with a pH-sensitive GFP variant) and αSyt1-cypHer, spontaneous and evoked release were proved to use the same recycling synaptic vesicle pools (Groemer and Klingauf, 2007; Hua et al., 2010). A conclusive work, combining the above-mentioned experimental techniques (pHluorin imaging, antibody-labeling of Syt and FM dye imaging), strengthened the point that the same pool of vesicles drives both synchronous and spontaneous release in *Drosophila*, frog and mouse. Nevertheless, the discrepancy remains to be clarified (Wilhelm et al., 2010).

What could distinct a vesicle from the other vesicles? Recently, molecular markers distinguishing evoked and spontaneously releasing vesicles have been identified. By the expression of tetanus toxin-insensitive vesicle-associated membrane protein (VAMP7) and glutamate vesicular transporter 1 (VGLUT1) in primary hippocampal neurons, spontaneously released vesicles and recycling pool of vesicles can be distinguished by their reluctancy to intensive stimulation recruitment. VAMP7 expression mostly labels the spontaneously releasing vesicles through the interaction of longin domain and vesicles. By contrast, VGLUT1 is more expressed on recycling vesicles, although both recycling and resting pools undergo spontaneous release (Hua et al., 2011). In addition, a noncanonical SNARE molecule, Vps10p-tail-interactor-1a (vti1a) has been identified to selectively maintain spontaneous neurotransmitter release (Ramirez et al., 2011). The knowledge of molecular marker offers a better chance to understand vesicle populations and synaptic transmission.

3.1.3 Fate of vesicular protein after fusion

Another interesting issue is, after fusion, will the vesicular proteins disperse into the plasma membrane, or remain clustered and undergo concerted retrieval? In rat hippocampal neurons, by introducing a tobacco etch virus (TEV) protease cleavage site between synaptobrevin and GFP on the basis of spH, a spH-TEV construct can be formed and used to investigate membrane retrieval (Wienisch and Klingauf, 2006). The newly exocytosed vesicle proteins diffuse into the plasma-membrane and mix with the plasma membrane–stranded proteins, the freshly endocytosed vesicles during and after stimulation contain majorly the membrane-stranded components. The degree of nonidentity of vesicular proteins exo- and endocytosed depended on stimulus duration, with the stronger stimulation the higher nonidentity (Wienisch and Klingauf, 2006). Consistently, recent work on the same tissue also indicated that moderate stimulation (20 Hz for 2 s, releasing ~ 19% of all vesicles) barely causes a diffusion of Syt out of synaptic boutons, while strong stimulation (20 Hz for 30 s, releasing the whole recycling pool) induces a stronger diffusion of Syt. However, it was pointed out that native proteins could diffuse and mix only in a limited level upon vesicle recycling, and appeared in clear clustered distribution while the tagged proteins are diffusing in the membrane under subdiffraction resolution stimulated emission depletion microscopy (STED) (Opazo et al., 2010). The controversy here

might simply due to the much higher resolution in STED over confocal microscopy. It is very likely that the vesicular protein stays together in vesicle release and recycling which keeps the efficiency of neurotransmission.

3.1.4 *Drosophila* vesicle pools

Coming back to *Drosophila*, the total synaptic vesicle number in the NMJ has been estimated to be around 87,000 vesicles dispersing among 400 active zones. Therefore, the vesicle number per active zone is about 217 (Atwood et al., 1993). How are the vesicle pools located in *Drosophila* NMJ? Studies with high potassium and cyclosporin A/tetanic stimulaiton treatment showed the absence of 'center-periphery pattern' that recycling pool localizes at the periphery of the boutons while the reserve pool vesicles occupies the center of the boutons. And also the recycling and reserve pool intermixed thoroughly at the EM level. (Denker et al., 2009). How are these vesicle pools regulated in physiological conditions? In *rutabaga* mutant, which encodes a calmodulin dependent adenylate cyclase that converts ATP to cyclic AMP (Levin et al., 1992), vesicle mobilization from reserve pool during tetanic stimulation was suppressed, while it was enhanced in *dunce* which is defective in cyclic AMP degradation due to decreased or absent phosphodiesterase (Dudai et al., 1976). Furthermore, induction of AP-1, a heterodimer of Fos and Jun, induces cAMP- and cAMP response element-binding protein (CREB)-dependent presynaptic enhancement, accompanied by recruiting vesicles from the reserve pool which results in an increase in the recycling vesicle pool (Kim et al., 2009). In hippocampal terminals, inhibition of cyclin-dependent kinase 5 (CDK5) mobilizes resting synaptic vesicle pool, which can be achieved by prolonged neuronal silencing. Restoration of the resting pool involves an opposing action by the calcium-dependent phosphatase calcineurin. Therefore, phosphorylation - dephoshorylation may tilt the balance between recycling and nonrecycling vesicles to adaptively alter synaptic strength without changing total vesicle number or bouton size (Kim and Ryan 2010). In the present work, we expanded the knowledge about the vesicle pools for evoked and spontaneous release in *Drosophila* NMJ.

3.2 The release machinery: SNARE complex and its regulatory proteins

3.2.1 SNARE complex and SM proteins

How are the different vesicles released, is there any common molecular mechanism mediating vesicle release? At most of the chemical synapses, neurotransmitter release relies on the orderly execution of membrane fusion by a group of protein components forming the release machinery. In the past 30 years, SNARE protein (soluble N-ethylmaleimide-sensitive factor attachment protein receptor protein) and its regulatory proteins like SM (Sec1/Munc18-like) protein, complexin (cpx) and synaptotagmin were quite intensively investigated in the exquisite control and compartmental specificity of the fusion process. NSF (N-ethylmaleimide–sensitive factor), a hexamer ATPase, and SNAP (soluble NSF attachment protein) were first purified in a cell-free system for their necessity in transport vesicle fusion in Rothman lab (Malhotra et al., 1988; Wilson et al., 1989; Clary et al., 1990). Later on, SNARE proteins, including v-SNARE/R-SNARE (synaptobrevin/VAMP) on the synaptic vesicles and t-SNAREs/Q-SNAREs (syntaxin-1 and SNAP-25) on the plasma membrane, were identified to be receptors for SNAP and NSF (Söllner et al., 1993). These SNARE proteins can spontaneously assemble into an energy stable four-helix bundle, which spans vesicle and plasma membrane (Fig. 2) named “SNAREpin/trans-SNARE complex”, and it catalyzes fusion by zippering up the two membranes (Hanson et al., 1997; Weber et al., 1998). After fusion, the zippered SNARE complex is in turn called “cis-SNARE complex”. SNARE association and dissociation are ATP-dependent, with exergonic membrane merging and SNARE proteins folding, whereas endergonic unfolding and returning to their original status by NSF (Weber et al., 1998). Before the merger of the bilayer, the SNAREpin is maintained or clasped in a ready-to-go status by SM protein (Munc18-1). SM proteins have an “arc-shaped” structure with a conserved ~ 600 amino acid sequence, which clasps SNARE complexes by binding with both v - and t - SNAREs (Fig. 2). The interaction between the SNARE proteins and SM proteins can be modeled in two steps, first in a “closed” conformation, SM protein exclusively binds to syntaxin-1 and disabling the SNAER complex formation (Dulubova et al., 1999; Misura et al., 2000). Then in an “open” conformation, SM protein is anchored by its N-terminal lobe to a specific N-terminal peptide sequence of the syntaxin, this configuration gives SM

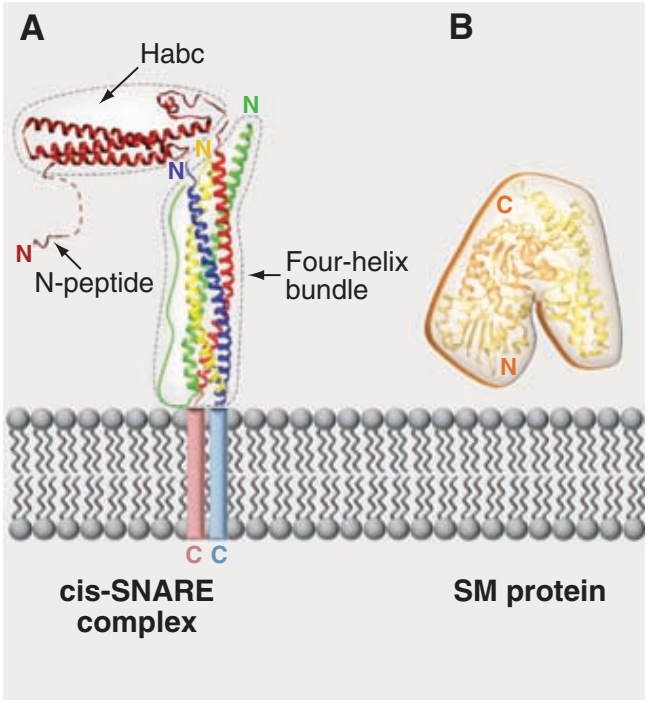

Figure 2. Structure of SNARE and SM proteins.

A. SNARE complex (also called cis-SNARE complex) of VAMP/synaptobrevin (blue helix), syntaxin (red helix), and SNAP-25 [green and yellow helices for the N- and C-terminal domains, respectively; adapted from (Sutton et al., 1998)]. The Habc domain of syntaxin-1A [brown helices, adapted from (Fernandez et al., 1998)] is positioned arbitrarily. **B.** An SM protein, highlighting its arch-like structure [Munc18-1, adapted from (Misura et al., 2000)]. Modified from Südhof and Rothman, 2009.

protein more space to clasp the remaining SNARE proteins and form four-helix bundle between the membrane, which can be further zippered by regulatory proteins like synaptotagmin and complexin, thus accomplishes vesicle release (Richmoud et al., 2001; Yamaguchi et al., 2002; Dulubova et al., 2002).

3.2.2 Complexin clamps the SNARE complex

Before vesicle fusion, release machinery is organized as SNAREpin, which has a thermo-dynamical tendency to fuse spontaneously and results in neurotransmission with less accuracy and short of vesicle supply due to a rampant release of vesicles. However, despite the huge variety of the species, neurotransmission is universally regulated in a precise timing and with limited aimless releasing in physiological condition. Two molecules, complexin (cpx) and synaptotagmin (syt; will be described later), are intensively studied in the clamping and fast activation of the fusion machinery. Cpxs are neuron-specific cytosolic proteins that interact with assembled SNARE complexes with a 1:1 stoichiometry which gives rise to their name, containing approximately 120 residues that are evolutionarily conserved in all

animals (McMahon et al., 1995; Chen et al., 2002). Crystal structure of Cpx-SNARE complex indicated that Cpx possesses a central short ~20-residue α-helix that nestles into the groove formed by the synaptobrevin-2 and syntaxin-1 SNARE motifs in the SNARE complex in an antiparallel fashion and an accessory α helix that does not contact the SNARE complex in the middle of the protein, N- and C-terminal sequences that are probably largely unstructured (Fig. 3). In physiological conditions,

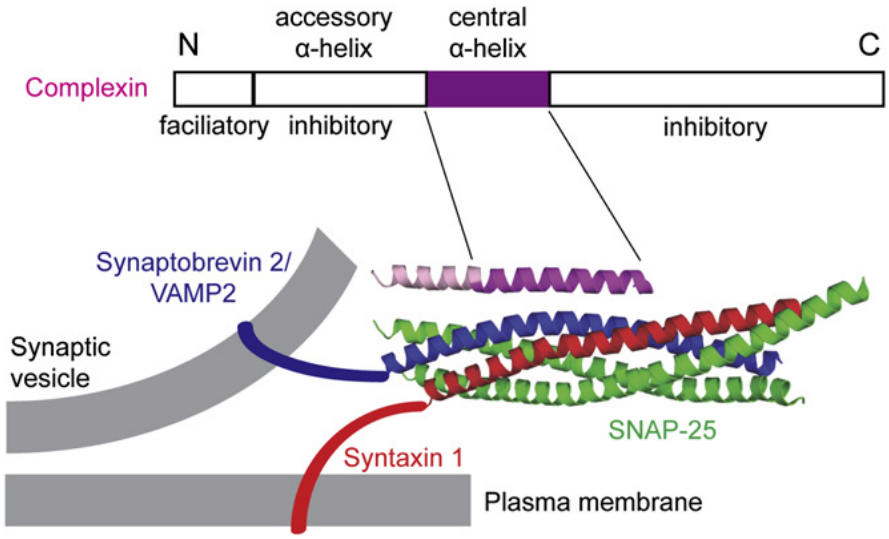

Figure 3. Domain Organization of Complexins.

The effect of each domain on vesicle release is indicated. The bottom part shows the crystal structure of the central helix of complexin bound to the neuronal SNARE complex (structure model based on PDB ID: 1KIL [Chen et al., 2002]), depicted here as tight trans-complex. Figure obtained from Stein and Jahn 2009.

Cpx binds to t-SNAREs with different affinities, with higher affinity to fully assembled SNARE complexes comparing to their heterodimers (McMahon et al.,1995; Pabst et al., 2000; Chen et al., 2002; Bracher et al., 2002). In mammals, four Cpx isoforms are expressed, Cpx I and II are widely distributed in the body and abundant in brain (McMahon et al. 1995), Cpx III and IV are enriched in retina ribbon synapses. Different from Cpxs I and II, Cpxs III and IV contain a CAAX-box motif at their carboxy-terminals, which represents a consensus sequence for posttranslational prenylation and likely attaches Cpx III and IV to the plasma membrane (Reim et al., 2005). By comparison in *Drosophila*, only a single gene encoding the Cpx protein (Tokumaru et al., 2001) can be found. Like its name, the role of Cpx in regulating vesicle fusion is quite controversial over long time, although it becomes more clarified recently. When Cpx I and II are removed in mice, evoked release is reduced in

hippocampal autaptic cultures and similarly when *Drosophila* Cpx is knocked out (Reim et al., 2001; Huntwork and Littleton, 2007), suggesting an activating role in synaptic vesicle fusion. However, spontaneous release is dramatically increased

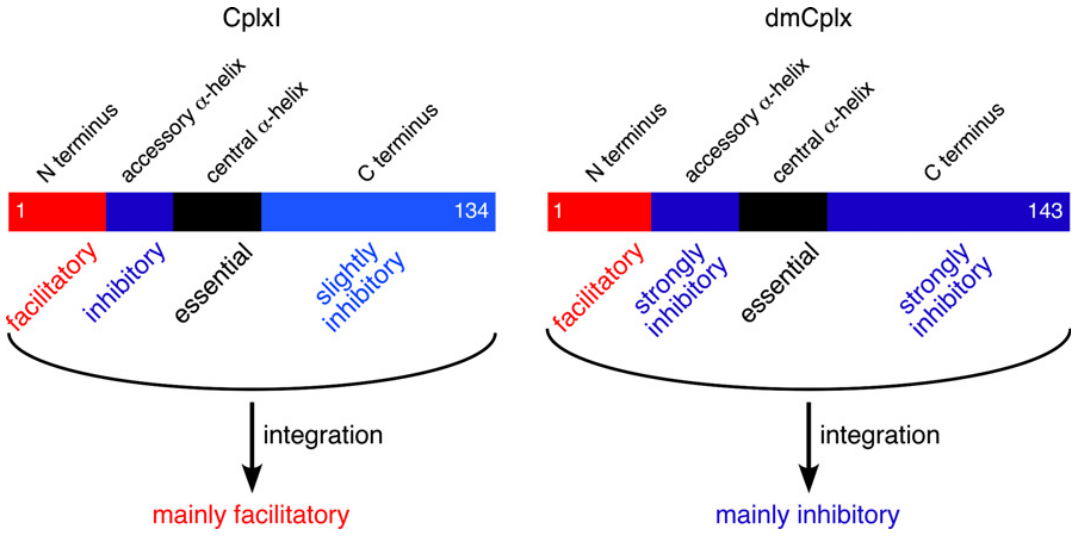

Figure 4. Distinct Functional Domains of CplxI and dmCplx Domains are indicated above the schematic diagrams and the corresponding functions are indicated below for CplxI and dmCplx.
The integration of facilitatory and inhibitory functions associated with distinct domains leads to apparently differential effects on neurotransmitter release by CplxI and dmCplx. Reproduced from Xue et al., 2009.

when Cpx is ablated in *Drosophila*, Caenorhabditis elegans or knockdown of Cpx I and II by RNAi in mouse cortical neurons (Huntwork and Littleton, 2007; Maximov et al, 2009; Hobson et al., 2011), which indicates a clamp function in neurotransmitter release. The discrepancy in synchronous and spontaneous neurotransmitter release can be better understood by a recent *in vitro* studies, which show that Cpx blocks membrane fusion by arresting vesicles at a docked stage, which in turn supplies the syt1 mediated synchronous release with sufficient synaptic vesicles (Malsam et al., 2012). In addition, the double or triple knockout of Cpx I/II or Cpx I/II/III in mice showed a decrease in the spontaneous neurotransmitter release in the excitatory synapses and in some inhibitory synapses in the respiratory brainstem slices (Xue et al., 2008). The above controversy can be reconciled by the strength of each inhibitory or facilitatory function differs significantly between *Drosophila* and murine complexins (Fig. 4). In the present work, the function of Cpx and its interaction with Syt as a regulator of SNAREs and downstream signaling of AKT pathway are further investigated.

3.3 The role of Ca^{2+} in triggering different mode of release

How is the vesicle release machinery generally triggered? Ca^{2+}, a second messenger, plays an essential role in many biological processes, including muscle contraction, synchronizing neural activities in the brain, fertilization of the oocyte, cell survival and neural apoptosis and triggering secretion in neuroendocrine chromaffin cells. In nervous system, the activation and regulation of neurotransmitter release recapitulates the key function of calcium.

3.3.1 Structure and function of voltage gated calcium channel

Calcium can enter into the cytosol through voltage-gated calcium channels (VGCCs), including L-, N-, T-, P/Q-, or R-type VACCs (Tsien et al. 1988; Wheeler et al., 1994; Jun et al., 1999; Catterall, 2011), according to their physiological and pharmacological property. L-type and T-type Ca^{2+} currents are recorded in a variety of cell types (Nowycky et al. 1985), N-, P-, Q-, and R-type Ca^{2+} currents are most prominent in neurons (Llina's and Yarom, 1981; Randall and Tsien, 1995; Nowycky et al., 1985). In general purified Ca^{2+} channels consist of α1, α2, ß,γ and δ subunits (Takahashi et al., 1987; Catterall, 2000), as shown in figure 5. As the major and largest component, α1 subunit contains ~2000 amino acid structured in four homologous domains (I - IV) forming the conduction pore and harboring the voltage sensors in the fourth transmembrane domain (Yu et al., 2005). The other four auxiliary protein subunits are more or less functionally associated with the α1 subunits, e.g. ß and α2δ subunits strongly increase α1 subunits cell surface expression and the former shifts voltage dependence and the kinetics of activation and inactivation, however, the functional role of the γ subunits is less well-known (Hofmann et al., 1999; Dolphin, 2003; Davies et al., 2007).

Beside the crucial function of providing channel for Ca^{2+} influx, the large intracellular domains of Ca^{2+} channels also serve as a signaling platform for Ca^{2+}-dependent regulation of neurotransmission (Fig. 5), including molecules of the SNARE-complex, (Sheng et al., 1994; Rettig et al., 1996), the presynaptic Ca^{2+}-sensor Syt (Sheng et al., 1997), Gßγ-subunits (Herlitze et al., 1996), protein kinase C, CaMKII, CaM (Lee et al., 1999) and further calcium sensor proteins (Lee et al., 2002). At *Drosophila* NMJs, the major calcium channel cacophony is a N-type-VGCC that is the primary mediator of action potential triggered Ca^{2+}-influx (Littleton and Ganetzky, 2000;

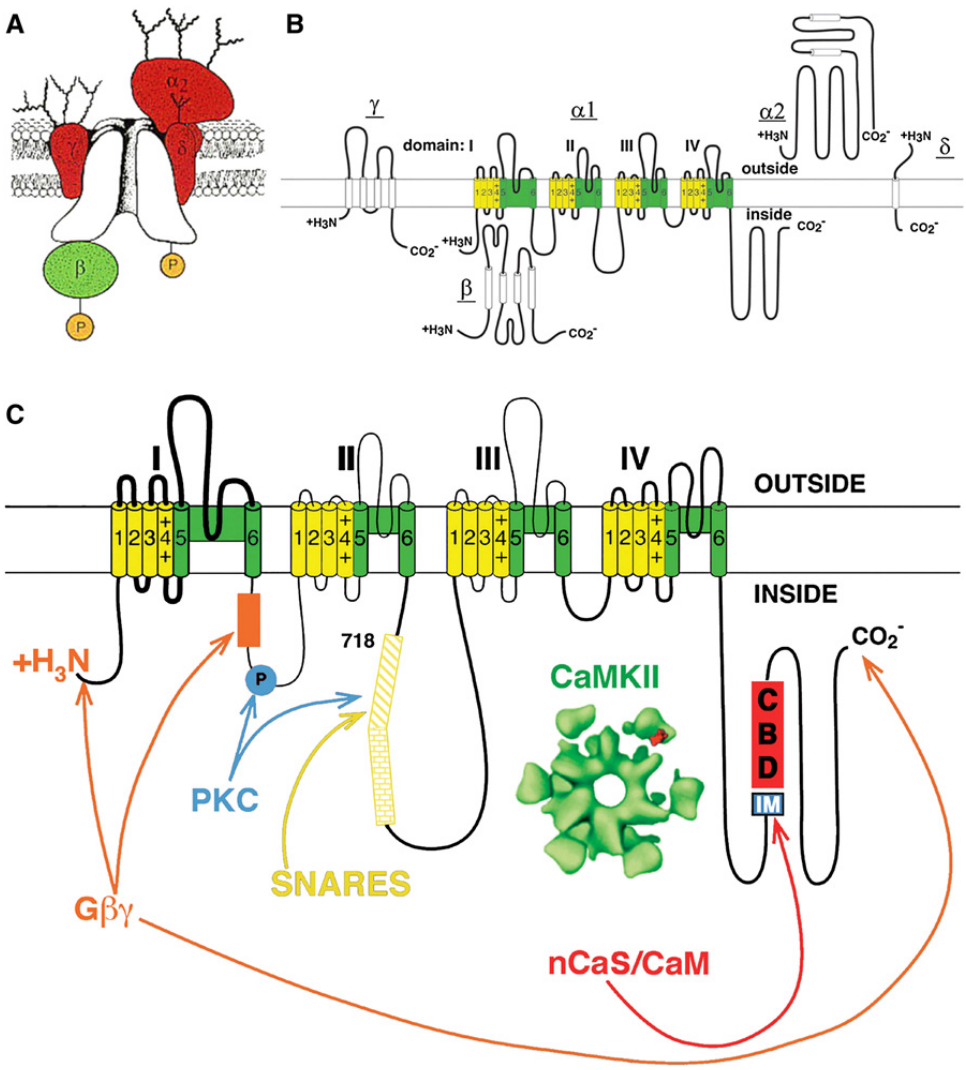

Figure 5. Subunit structure of Ca^{2+} channels.

A and B. The subunit composition and structure of high-voltage-activated Ca^{2+} channels are illustrated. B. Predicted helices are depicted as cylinders. The lengths of lines correspond approximately to the lengths of the polypeptide segments represented. The voltage-sensing module is illustrated in yellow and the pore-forming module in green.

C. The sites of interaction of different regulatory proteins on the intracellular surface of the α1 subunit of Ca_v2 channels are illustrated. Reproduced from Catterall and Few. 2008.

Kawasaki et al, 2000; Macleod et al., 2006). The function of cacophony in addition to the pore for calcium entry will be further described in the results part.

3.3.2 Basic properties of synchronous release

Ca^{2+} enters the presynaptic terminals via VGCCs when an action potential invades a nerve terminal, which consequently initiates synaptic transmission. The increase of intracellular calcium in the so-called micro- or nanodomain triggers the release of neurotransmitter containing vesicle as we named "synchronous release" (Adler et al., 1991; Llinas et al., 1992; Stanley, 1993; Neher, 1998; Bucurenciu et al., 2008).

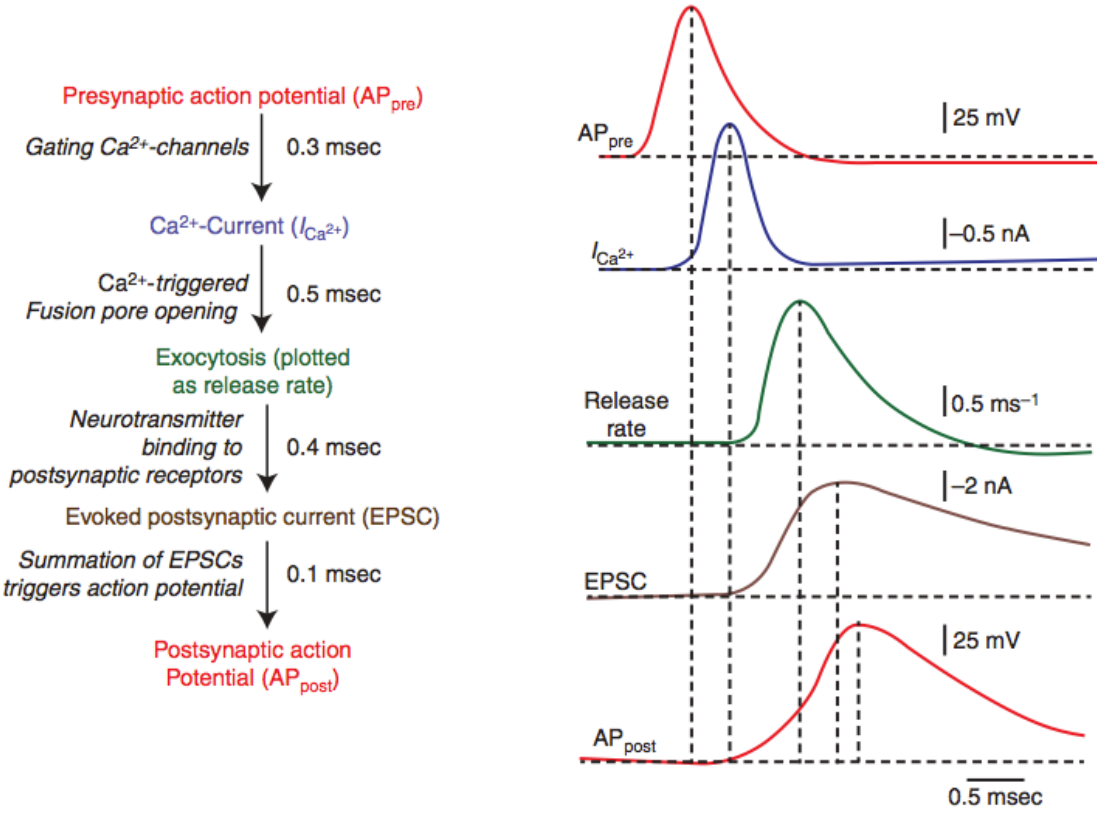

Figure 6. Principle and time course of Ca^{2+}-triggered synaptic transmission.
Schematic illustration of the sequence and time course of synaptic transmission as measured by simultaneous pre- and postsynaptic patch-clamp recordings at the calyx of Held synapse. Note that Ca^{2+} currents and EPSC are shown inverted. Image is obtained from Südhof 2012.

The term nanodomain represents the immediate vicinity of some calcium channels, a typical 10 - 100 nm regions, where the local Ca^{2+} is not removed by the fast calcium chelators like BAPTA. Microdomain includes subcellular areas of elevated Ca^{2+}, usually 0.1 to 1 µm, excluding nanodomain (Naraghi and Neher, 1997). When the release of the neurotransmitter is sufficient to change the postsynaptic membrane potential and surpasses a certain threshold, an action potential can be generated and propagated in the neural circuit. This fundamental discovery was made by Katz and Miledi in 1967. Later on, a variety of 'model' systems were studied to acquire the precise time course of fast neurotransmission, e.g. giant squid axon synapses

(Augustine et al., 1985), and rat cerebellar parallel fiber synapses (Sabatini and Regehr, 1996) and also in the calyx of Held synapses in the midbrain (Fig. 6). By accurately modulating the presynaptic Ca^{2+} concentration with Ca^{2+}-uncaging at calyx of Held, the effects of Ca^{2+} on vesicle release can be investigated and simulated.

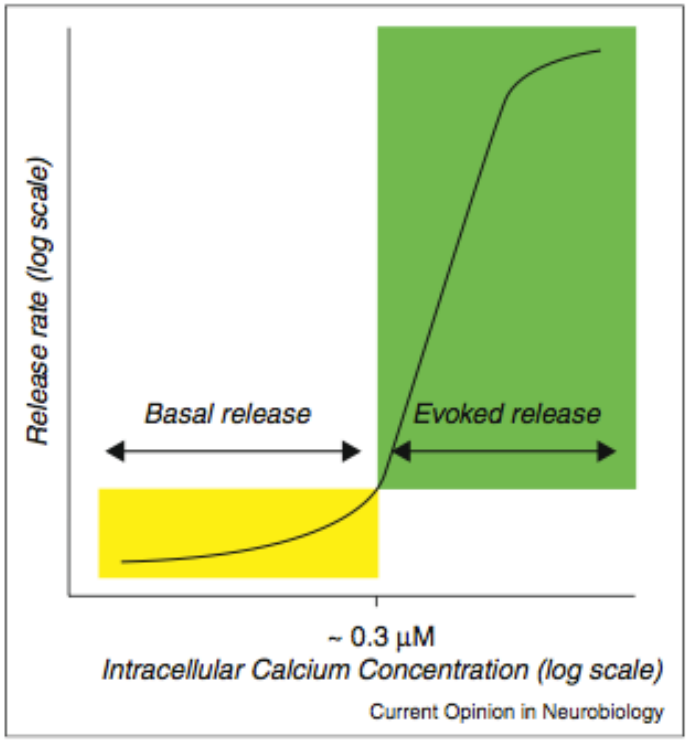

Figure 7. Ca^{2+}-dependence of spontaneous and evoked neurotransmitter release.
Diagram depicts the calcium-dependent increase in the rate of vesicle fusion (Lou et al., 2005, Sun et al., 2007). At low intracellular Ca2+ concentrations indicated by the yellow zone, small changes in Ca2+ signaling may selectively alter spontaneous release without significantly modifying evoked release properties. Image is obtained from Ramirez and Kavalali, 2011.

Relationships between Ca^{2+} and release rates were approximated by power laws with exponents between 3 and 6. At the calyx of Held, this relationship was extended to very low calcium (Lou et al., 2005; Sun et al., 2007). At a Ca^{2+} concentration of more than 0.3 µM, the Ca^{2+} cooperativity increases steeply (> 4) and the transmitter is mostly released in a synchronous way, while the Ca^{2+} concentration is reduced from 0.3 µM to basal level (~ 20 - 30 nM), the Ca^{2+} cooperativity will also be lowered (< 1) (Fig. 6 and 7), where the vesicles tend to fuse in a spontaneous way (Lou et al., 2005; Ramirez and Kavalali, 2011).

3.3.3 Basic properties of asynchronous release

Ca^{2+}-triggered synchronous neurotransmitter release is well understood, however the

very existence of asynchronous release remains enigmatic. Asynchronous release is very often described as a concomitant following strong synchronous component, a delayed part of vesicle release “asynchronous release” that is only loosely locked to an action potential (timescale: msec). Asynchronous release is built up during trains of action potentials, particularly in some of inhibitory neurons (Lu and Trussell, 2000; Hefft and Jonas, 2005; Best and Regehr, 2009; Daw et al., 2009). Usually, the slowly releasing vesicles may contribute to asynchronous release during and after high-frequency firing, while the fast releasing ones are recruited for synchronous release (Sakaba, 2006). In these synapses with predominantly asynchronous release, asynchronous release outcompetes synchronous release during the action-potential train (Atluri & Regehr, 1998; Lu and Trussell, 2000; Hagler & Goda, 2001; Otsu et al. 2004; Hefft and Jonas, 2005). In the fast CNS synapses of the avian nucleus magnocellularis, at low frequency stimulation (< 10 Hz), evoked release was mostly synchronized, however at higher frequency (> 20 Hz), quantal release starts to merge into a continuous and desynchronized manner (Lu and Trussell, 2000). EGTA-AM, a membrane permeable slow intracellular calcium chelator can largely suppress the asynchronous part of release, which indicated the delayed asynchronous release is a result of accumulation of presynaptic calcium (Lu and Trussell, 2000). Similarly, a comparison between cholecystokinin (CCK)-expressing interneurons in rat dentate gyrus and parvalbumin (PV) interneurons shows that a burst of 10 APs, the ratio of asynchornous to synchronous release is 3:1 and 1:5 separately and EGTA-AM can strongly suppress the asynchronous release of CCK interneurons but has less effect on PV interneurons. This difference can be explained by the existing of different Ca^{2+} channel types: N-type channels in CCK interneuron and P/Q type channels in PV interneuron synapses. Also the effects of EGTA-AM indicate a long-lasting presynaptic residual Ca^{2+} and a long distance between Ca^{2+} source and sensor of release machinery in CCK interneuron synapses (Hefft and Jonas, 2005). Another explanation for the ability of asynchronous release to out-compete synchronous release during and after high-frequency stimulus trains could be that asynchronous release has a higher apparent Ca^{2+} affinity although its Ca^{2+} cooperativity is the same as synchronous release (Goda & Stevens, 1994; Ravin et al., 1997; Lou et al., 2005; Sun et al., 2007), thus the rising residual Ca^{2+} during the train would trigger asynchronous release in between action potentials at Ca^{2+} levels which synchronous release can hardly be evoked. Consistently, this can also explain why EGTA can block asynchronous release during high-frequency AP trains but have less effect on

synchronous release (Lu and Trussell, 2000; Hefft and Jonas, 2005). By comparison, synapses without predominantly asynchronous release during high-frequency trains, such as the calyx synapse and PV interneurons, might lack asynchronous release, or have a tight coupling between the Ca^{2+} channels and the Ca^{2+} sensor of exocytosis, or even have a more efficient Ca^{2+} buffering and Ca^{2+} extrusion mechanism that prevents residual Ca^{2+} accumulation (Chuhma and Ohmori, 2002; Hefft and Jonas, 2005; Schneggenburger and ForSythe, 2006). Moreover, the similar apparent Ca^{2+} cooperativity of asynchronous and synchronous release suggested that asynchronous release could be in essence a modification of synchronous release (Lou et al., 2005). Thus, the slow and complex decay of asynchronous release would reflect a further diffusional collapse of microdomain following the collapse of nanodomain after the closure of Ca^{2+} channel by Ca^{2+} binding to slow buffers (Müller et al., 2007), and Ca^{2+} extrusion and reuptake (Kim et al., 2005).

In general, the difference between asynchronous and synchronous release can be explained by two competing hypotheses:

(1) both releases share the same Ca^{2+} sensor, but differ in the coupling of vesicles to Ca^{2+} channels, and may use different allosterically regulated vesicle pools. (Lou et al., 2005; Wölfel et al., 2007);

(2) asynchronous and synchronous release are mediated by different Ca^{2+} sensors with distinct properties (Goda and Stevens, 1994; Yao et al., 2001).

3.3.4 Basic properties of spontaneous release

In addition to synchronous and asynchronous release, it has been well-known that in the absence of presynaptic action potentials, neurotransmitter release can also occur spontaneously albeit with a low probability, according to the early work of Bernard Katz and colleagues. First described as miniature end plate potentials (mEPPs) of small, subthreshold depolarizations in the postsynaptic muscle membrane at the frog neuromuscular junction (Fatt and Katz, 1950), spontaneous neurotransmission reflected stochastic fusion of single or multiple vesicles in the absence of an action potential whereas synchronous and asynchronous release represented simultaneous vesicle fusion from multiple nerve endings during and after action potential as described above. When calculated on a per-synapse basis, spontaneous release occurs only once every 2 - 3 hoursat an individual excitatory synapse of a pyramidal neuron in the CA1 region of the hippocampus, and approximately once every 3 min

at individual inhibitory synapses (Südhof 2012). Comparing to end-plate potentials, mEPPs had similar time courses and similar sensitivity to curare, but it did not propagate over the synapses after the activation of postsynaptic receptors. Dividing the amplitudes of EPPs by the mean amplitude of mEPPs revealed an underlying quantal nature of synaptic neurotransmitter release (Del-Castillo and Katz, 1954; Katz, 1966).

Unlike the steep Ca^{2+} dependence of evoked transmission (Dodge and Rahamimoff, 1967; Augustine and Charlton, 1986; Borst and Sakmann, 1996; Reid et al., 1998; Rozov et al., 2001), spontaneous neurotransmission mostly displays close to linear Ca^{2+}- dependence in many synapses (Lou et al., 2005; Sun et al., 2007; Groffen et al., 2010). In the inhibitory synapses like GABAergic neurons, the fluctuation in both extracellular and intracellular Ca^{2+} greatly changes the level of spontaneous release rate. In Purkinje cells, decreasing the extracellular Ca^{2+} from 2 mM to 0.1 mM, the mIPSCs frequency and amplitude decrease to a half of the original level. By contrast, rising the extracellular Ca^{2+} from 2 mM to 5 mM, the mIPSCs frequency can be further increased while the amplitude remains the same (Llano et al., 2000). Both the frequency and the amplitude of large-amplitude miniature inhibitory postsynaptic currents (mIPSCs) can be reduced by high dose of ryanodine (10 μM), which inhibits the ryanodine receptors (RyRs) or by long-term removal of extracellular Ca^{2+} (Llano et al., 2000). However, in excitatory synapses, the effects of extracellular and intracellular Ca^{2+} on spontaneous release are somehow diverse. Some show similar Ca^{2+} dependency like inhibitory synapses (Simkus and Stricker, 2002; Emptage et al., 2001; Lou et al., 2005; Xu et al., 2009), and the others are almost not influenced (Vyleta and Smith, 2011). At glutamatergic synapses of pyramidal cells in layer II of rat barrel cortex, reducing the intracellular Ca^{2+} with 50 μM BAPTA-AM or by inhibition of a SERCA pump with cyclopiazonic acid, blocking RyRs with 20 μM ryanodine and blocking IP3 receptors with 14 mM 2-aminoethoxydiphenylborane strongly decreased the rate of mEPSCs (Simkus and Stricker, 2002). An addition of 10 mM Caffeine, which increases the intracellular calcium, enhanced the releasing probability of mEPSCs (Simkus and Stricker, 2002). Nevertheless, at excitatory neocortical synapses of mouse, spontaneous neurotransmission can be enhanced by an increasing $[Ca^{2+}]_o$, but blocking VACCs or buffering the intracellular Ca^{2+} with BAPTA and the reversal of the Na^+ - Ca^{2+} exchanger (NCX) have no effects on spontaneous release (Vyleta and Smith, 2011). Here, the calcium effect is actually due to an activation of the calcium-sensing

receptor (CaSR), a G-protein-coupled receptor present in nerve terminals (Vyleta and Smith, 2011).

Moreover, beside the pure calcium effects on spontaneous release, activation of a variety of VGCCs seems also sufficient to trigger spontaneous release. For example, at cultured mouse inhibitory neocortical neurons, VACCs are tightly coupled to the inhibitory GABA-containing vesicles, and its activation triggers spontaneous release (Williams et al. 2012). Thus, this finding sheds new light on understanding the essence of spontaneous release. In general, spontaneous vesicle release well correlates to intra- and extracellular calcium concentration, however, other regulatory mechanisms including calcium independent regulations also exist which makes spontaneous release more interesting in different local context.

3.4 Calcium sensors for neurotransmitter release

As described above, in all of the three canonical neurotransmitter release modes, Ca^{2+} may affect the amplitude, kinetic or frequency of the release. How is calcium captured after influx? How are the messages like amount, duration and diffusion velocity carried by calcium converted to intracellular signals? The molecules mediating these processes are cloned and well studied as further introduced below.

3.4.1 Type and distribution of the calcium sensors

Synaptotagmin, which was first discovered more than 20 years before (Perin et al., 1990), is nowadays well known to function as Ca^{2+} sensor for neurotransmitter release, containing a short amino-terminal noncytoplasmic sequence followed by a transmembrane region, a central linker sequence of variable length, and two Ca^{2+} binding carboxy-terminal C2-domains. In mammals like mouse, 16 mammalian canonical Syts (Syt1 - 16) have been identified and can be broadly classified into Ca^{2+} dependent (1,2,7,9 and 3,5,6,10) and Ca^{2+}-independent groups (4,8,11,12,13,14,15, and 16) (Fig. 8) (Südhof 2012). In the Ca^{2+}-independent group Syts, the C2 - domains lack canonical aspartate residues required for Ca^{2+} binding, including the C2A - domains of Syt4 and Syt11 (von Poser et al. 1997), and all C2

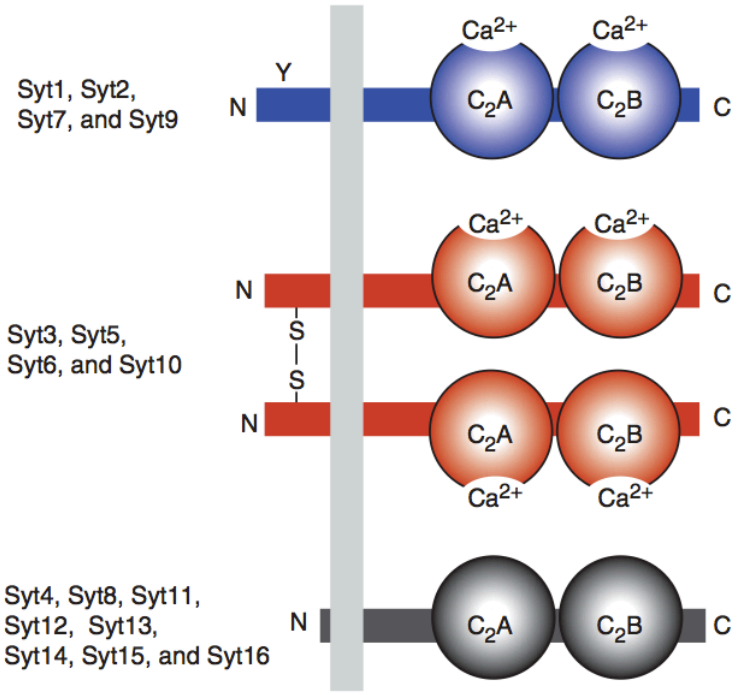

Figure 8. Structures and Ca^{2+}-binding properties of synaptotagmins.

Canonical domain structures and classification of syts. Mammals express 16 syts composed of an amino-terminal transmembrane region preceded by a short noncytoplasmic sequence and followed by a variable linker sequence and two C2-domains. Eight synaptotagmins bind Ca^{2+} (Syt1, 2, 3, 5, 6, 7, 9, and 10; blue and red); the remain- ing synaptotagmins do not (black). The eight Ca^{2+}-binding synaptotagmins fall into two broad classes that differ in the absence (Syt1, 2, 7, and 9; blue) or presence (Syt3, 5, 6, and 10; red) of disulfide-bonded cysteine residues in their amino-terminal sequences. Note that Syt1 and 2 include an N-glycosylated sequence at the amino terminus (indicated by a "Y"), and that Syt7 is extensively alternatively spliced in the linker sequence. Image is obtained from Südhof 2012.

domains of Syt8 and Syt12 - Syt16. Although in the C2B-domains of Syt4 and Syt11 which include all of the requisite Ca^{2+}-binding sequences were found to be unable to bind Ca^{2+}. This initially surprising result was explained by the atomic structure of the Syt4 C2B - domain, which showed that the canonical Ca^{2+} - binding residues in the top loops of the domain are too far apart to be able to ligate Ca^{2+} (Dai et al. 2004). Moreover, a so-called B/K protein which lacks a transmembrane region is sometimes also named Syt17. Among all of the Syts, Syt1, Syt2, Syt9, and Syt12 are present on synaptic vesicles and on secretory granules in neuroendocrine cells. Syt7 was localized to "secretory lysosomes" in nonneuronal and non - endocrine cells (Andrews and Chakrabarti 2005). Moreover, Syt10 was recently found on secretory vesicles containing IGF-1 in olfactory mitral neurons (Cao et al. 2011); and Syt4 was localized to the trans - Golgi complex, synaptic vesicles, and postsynaptic organelles, and may be widely distributed in neuronal organelles (Südhof 2004); Other Syts appear to be primarily localized to transport vesicles, although some Syt isoforms are

enriched on the plasma membrane (Butz et al. 1999), and the identity of many of the Syt - containing vesicles remains unknown.

3.4.2 How can Syt function as a Ca^{2+} sensor?

Synaptotagmins function as a Ca^{2+} sensor via C2 domains (C2A and C2B domains), which were named as sequence motifs in protein kinase C isoforms (Coussens et al. 1986), but their functional activity as autonomously folded Ca^{2+}-binding domains was first discovered in Syt1 (Davletov and Südhof 1993). Syt1 C2A and C2B domains share similar structure, taking C2A domain as example, it contains three Ca^{2+}-binding sites, of which the central two are classical for all Ca^{2+}-binding C2-domains, whereas the left Ca^{2+} site is not always present in C2-domains, like Syt1 C2B-domain (Fernandez-Chacon et al., 2001). Both of C2 domains bind to phospholipids in a Ca^{2+}-dependent manner (Davletov and Südhof 1993; Fernandez et al. 2001), and also to SNARE proteins probably by a direct interaction with syntaxin-1 that is greatly enhanced by Ca^{2+} (Bennett et al. 1992; Chapman et al. 1995; Li et al. 1995, Pang et al. 2006). In addition, the interaction of Syt1 with SNARE complexes is highly sensitive to ionic strength that it becomes Ca^{2+} independent at lower ionic strength, which may occur physiologically (Pang et al. 2006).

3.4.3 Which Syts are needed for the three types of release?

As listed above, 17 types of Syts are discovered in mammals. Among a variety of candidates, which Syts would be the ideal calcium sensors for synchronous release? Studies of hippocampal neurons cultured from homozygous Syt1 mutant mice indicate that fast neurotransmission is severely impaired (Geppert et al., 1994). The synchronous release is decreased, whereas asynchronous release and spontaneous synaptic activity is increased, and release triggered by hypertonic solution or α-latrotoxin which releases the vesicles from RRP, are unaffected (Geppert et al., 1994). Besides the key role of Syt1 as Ca^{2+} sensor, a systematic screen of Ca^{2+} binding Syts based on the ability to rescue syt1 KO phenotype indicated that Syt2 and 9 are the other Ca^{2+} sensors for fast synaptic transmission with different kinetics, with the Syt2 exhibiting the fastest kinetics and Syt9 the slowest kinetics (Xu et al., 2007).

Unlike the well-clarified Ca^{2+} sensors for synchronous release, the exact Ca^{2+}

sensor for asynchronous release still remains enigmatic, although many studies indicate the existence of such a Ca^{2+} sensor. An early work by Goda and Stevens in 1994 shows evoked release exhibits kinetics of biphasic decay which can be fitted by a double-exponential curve, revealing slow and fast components of release, and the divalent cation Sr^{2+} has differential effects on the slow and fast components of neurotransmitter release, although differential Sr^{2+} influx may also contribute to this effect, which implies the existence of two kinetically distinct Ca^{2+} sensors at most synapses, with one underlying a rapid, synchronous component of fusion, and a second mediating a slower, asynchronous component of transmitter release. Moreover, in both *Drosophila* and mice mutants lacking Syt1, the synchronous release is suppressed, whereas the asynchronous release is increased (Geppert et al., 1994; Broadie et al., 1994; Saraswati et al., 2007). Another piece of evidence appears in calyx-of-Held synapses, a knockout of Syt2 selectively blocks the synchronous release but has no influence on the asynchronous release, which provides a possibility to study the properties of asynchronous release. The research indicated at low intracellular Ca^{2+} concentration, physiological asynchronous release is dominant with a Ca^{2+} binding cooperativity of about 2 and a Ca^{2+} affinity of ~ 44 µM. In a higher Ca^{2+} concentration, synchronous release overcomes asynchronous release with a higher Ca^{2+} cooperativity around 5 and an apparent Kd of ~ 10 µM or ~ 105 µM Ca^{2+} (Sun et al., 2007), which is, indeed, to date the most direct evidence for such a separate sensor. A more recent work pointed out Doc2 is a Ca^{2+} sensor for asynchronous release. Compared to Syt1, in vitro fusion assay indicates Doc2 (α and ß) has a slower Ca^{2+} association and dissociation kinetics. Expression of Doc2α KD suppresses the asynchronous release without altering synchronous release, which for the first time convinces Doc2 as the Ca^{2+} sensor of asynchronous release (Yao et al., 2011).

In *Drosophila*, there are also evidences for non-Syt Ca^{2+} sensors for asynchronous release and short-term synaptic plasticity. In order to know whether Syt1 is the Ca^{2+} sensor for asynchronous release or paired-pulse facilitation (PPF), Syt1 was knocked out, but it doesn't abolish the PPF and asynchronous release. The other members of Syt family like Syt4, 7 were also tested, but also without an effect on these kinds of release (Saraswati et al., 2007). Interestingly, it was indicated that the Syt7 C2A and C2B domains have apparent Ca^{2+} affinities ~ 10-fold higher than the corresponding domains of Syt1, when in complex with phospholipids (Sugita et al., 2002). An ~ 10-fold lower Ca^{2+} concentration dependency was also reported for

Syt7 C2A binding to syntaxin (Li et al., 1995), suggesting that Syt7 is activated at lower Ca^{2+} concentrations than Syt1, which is a proper candidate for asynchronous Ca^{2+} sensor, however the exact identity of calcium sensor for asynchronous release in *Drosophila* is still not clear.

So what could be the calcium sensor for spontaneous release? As we know, most of spontaneous releases are Ca^{2+} dependent, which suggests the existence of a certain Ca^{2+} sensor to trigger it. In murine cortical neurons, > 95% of spontaneous release was induced by calcium binding to Syt1, and mutations that changed Ca^{2+} affinity of Syt1 altered evoked and spontaneous release correspondingly. Therefore, spontaneous and evoked releases share the same Ca^{2+} sensor. Paradoxically, Syt1 KO massively increased spontaneous release instead of inhibiting in a calcium dependent manner. This contradictory results can be explained by the existing of a second Ca^{2+} sensor, the identity of which is not known yet, with lower Ca^{2+} cooperativity and higher apparent Ca^{2+} affinity than Syt1, and deletion of Syt1 unmasked it. Comparing the point mutation of C2 domains, C2B domain mutation was less deleterious than the C2A domain mutation concerning the spontaneous release, which suggests C2A domain is more effective at clamping the second Ca^{2+} sensor than C2B domain. Thus, in addition to the function of Ca^{2+} sensor for spontaneous release, Syt1 also clamped a second more sensitive Ca^{2+} sensor for spontaneous release, the identity of which needs to be further clarified (Xu et al., 2009). One question is, in a single neuron, why two Ca^{2+} sensors for spontaneous release coexist?

In addition, mice cytosolic proteins double C2 domain (Doc2), can also act as Ca^{2+} sensor for spontaneous release in analogous way to Syt1 but with a higher Ca^{2+} sensitivity (Groffen et al., 2010). Doc2 proteins, including Doc2a and Doc2b, are soluble proteins that contain C2 domains with high similarity to Syts (Verhage et al., 1997). They are expressed in nerve terminals and interact with the secretory molecules Munc18, Munc13 and the SNARE proteins syntaxin-1 and SNAP25 (Orita et al., 1997; Friedrich et al., 2008). KO or shRNA-mediated knockdown of Doc2 proteins reduces spontaneous release significantly without effects on evoked release (Groffen et al. 2010; Pang et al. 2011). However, a continuing work shows the decrease in spontaneous release frequency induced by Doc2 knockdown can be fully rescued by expression of Doc2 proteins lacking Ca^{2+}-binding sites, indicating that Doc2 proteins do not function as Ca^{2+} sensors for spontaneous exocytosis (Pang et al. 2011). Therefore, the other Ca^{2+} sensors for spontaneous release need to be

further identified and clarified.

3.4.4 Synaptotagmins in *Drosophila*

The above knowledge about calcium sensors has been largely acquired in mouse and rat. Since the model animal used in this thesis work is *Drosophila*, therefore it is necessary to also refocus on the calcium sensors in *Drosophila*. Among the 16 known vertebrate synaptotagmins, only Syt1, 4, and 7 are also present in *Drosophila*

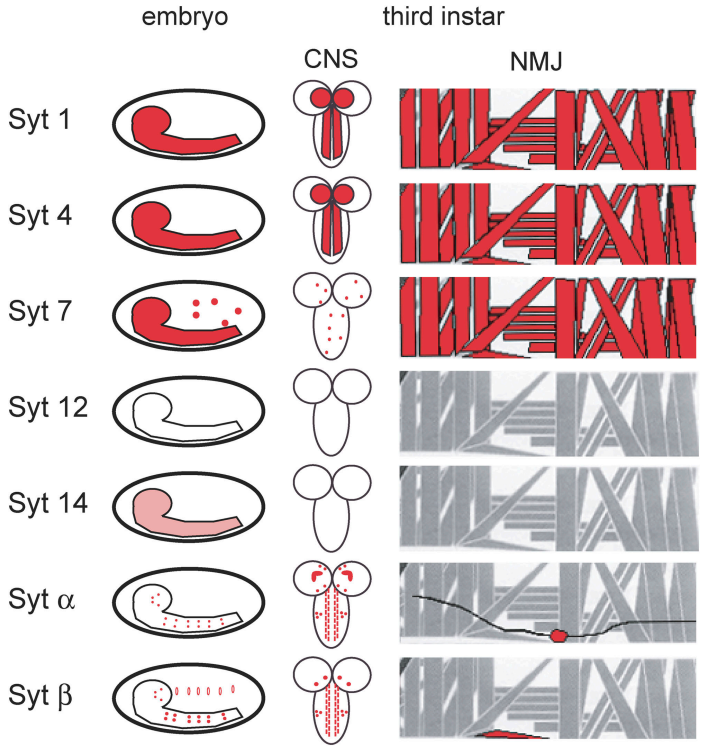

Figure 9. Summary of the expression pattern of synaptotagmin family in *Drosophila*.
The results from embryonic in situ experiments are shown in the left panel, whereas the two right panels highlight protein expression in the third instar larval CNS and periphery. The muscles labeled red indicate NMJs where presynaptic localization of Syt 1, Syt α, or Syt ß occurs, postsynaptic localization of Syt 4, and general sarcoplasmic localization of Syt 7. Figure is obtained from Adolfsen and Littleton, 2004.

and *C. elegans*, suggesting that these isoforms play especially important roles *in vivo* (Andrews and Chakrabarti, 2005). In *Drosophila*, like Syt1, Syt4 is ubiquitously present at synapses, but localizes to the postsynaptic compartment. The remaining isoforms were not found at synapses (Syt7), expressed at very low levels (Syt12 and 14), or in subsets of putative neurosecretory cells (Syt α and ß) (Fig. 9)(Adolfsen and Littleton, 2001, 2004). A mutation of *Drosophila* Syt1 strongly reduces the evoked excitatory junctional potentials (eEJPs) amplitude, accompanied by an increase in

asynchronous release and miniature frequency (Littleton et al., 1993,1994; Broadie et al., 1994), which indicates a key role of Syt1 in fast neurotransmission and a clamping effects on spontaneous release. Syt can affect synchronous release by facilitating SNARE complex formation in vitro and its mutations disrupt SNARE complex formation in vivo. Syt oligomers can bind SNARE complexes efficiently, whereas Syt binding to Ca^{2+} initiates SNARE complexes cross-linking into dimers. In *Drosophila*, mutations that diminish Ca^{2+}-triggered syt oligomerization and block Ca^{2+}-triggered conformational changes in C2B domain results in a decrease in SNARE assembly and a postdocking defect in neurotransmitter release *in vivo.* These data suggest that Ca^{2+}-driven oligomerization via the Syt C2B domain may trigger synaptic vesicle fusion through the assembly and clustering of SNARE complexes (Littleton et al., 2001). A later analysis of Syt1 indicates mutation of C2B domain Ca^{2+} sensing part has synchronous release with reduced release probability but with normal Ca^{2+} cooperativity, and synapses with a total deletion of C2B domain partially recover synchronous release, but have an abolished Ca^{2+} cooperativity, which suggests the Ca^{2+} cooperativity of neurotransmitter release is likely mediated by Syt-SNARE interactions, while phospholipid binding and oligomerization trigger rapid fusion with increased release probability (Yoshihara and Littleton, 2002).

In addition to the Syt1 functions as a calcium sensor, other Syts can modulate synaptic function in an interesting way. A *Drosophila* homologue of Syt4 contains an evolutionarily conserved substitution of aspartate to serine that abolishes its ability to bind membranes in response to Ca^{2+} influx. Its binding to Syt1 results in a Syt1-Syt4 oligomers that cannot effectively penetrate lipid bilayers and are less efficient at coupling Ca^{2+} to secretion *in vivo*: upregulation of Syt4, but not Syt1, reduces synchronous neurotransmission. These findings reveal a new molecular mechanism for synaptic plasticity (Littleton et al., 1999). When overexpressing Syt4 and 7 in the Syt1 null background, the release defects in Syt1 mutants can't be rescued, which suggests the Syt isoforms 4 and 7 don't function as a calcium sensor in the neuromuscular junction. Therefore, the identity of calcium sensors for asynchronous release and spontaneous release remains to be clarified. In the present work, the function of Syt1 as an effector of Cac and AKT signaling rather than a Ca^{2+} sensor will be elucidated.

3.5 The biological significance of various synaptic vesicle release

Due to the properties of the different modes of release and their respective Ca^{2+} sensors, their biological function can be easily interpreted and understood. Synchronous release is obviously the most important form in the nervous system, which provides fast and synchronous information transduction at many synapses. Neurons expressing Ca^{2+} sensors with different kinetic properties for synchronous release (Syt1, 2 and 9) possess distinct information processing abilities within an individual neural circuit. Except the wide expression of Syt1 which is one of the most abundant molecule in neurotransmission, Syt2 with the fastest kinetics is primarily expressed in synapses requiring very fast synaptic transmission, such as the auditory system or the neuromuscular junction, whereas Syt9, the relative slower one, primarily in the limbic system (Xu et al., 2007). By expressing different Syts, neurotransmission can be shaped with different kinetics which endow nervous systems the ability to transmit different signals.

By contrast to the clear importance of synchronous release, the significance of asynchronous release is somehow obscure, because it is more or less determined as a failure form or byproducts of synchronous release or only prominent after a stimulus train. Its function seems to be centered in modulating effects in the nervous system, e.g. asynchronous release at high frequencies may help generate a smooth inhibitory “tone” by minimizing the consequences of random timing of presynaptic action potentials (Lu and Trussell, 2000) and also it occurs at physiological temperature with behaviorally relevant stimulation patterns, thus generating long-lasting inhibition in the brain (Hefft and Jonas, 2005).

Comparing to asynchronous release, spontaneous neurotransmitter release exists more broadly, almost in all the synapses. Its function was first ignored because of its small amplitude and stochastic manner of release. Surprisingly, researchers pointed out this apparent trivial events have important roles in many biological processes: a single quantum can be sufficient to trigger postsynaptic action potential firing or regulate spike timing and postsynaptic excitability (Otmakhov et al., 1993; Carter and Regehr, 2002; Sharma and Vijayaraghavan, 2003); in addition, several types of homeostatic plasticity observed after termination of neuronal activity can be greatly modulated in magnitude and duration by spontaneous release events (Sutton et al., 2004, 2006, 2007; Aoto et al., 2008; Chung and Kavalali, 2006); furthermore,

these low probability events have been shown to inhibit dendritic local protein translation and help maintain the synaptic response stability (Sutton et al., 2006); moreover, the finding that some hippocampal neurons are connected by a single synapse suggested that communication through a single quantum, however it is triggered, must be physiologically important (Stevens and Wang, 1995). Obviously, the importance of spontaneous release was too less emphasized, it is necessary and intriguing to investigate more on this apparently stochastic event and how it is regulated.

3.6 Additional mechanisms regulating vesicle release

Ca^{2+} is a necessity in most of neurotransmission, it provides the possibility to control the timing, kinetics and duration of neural signals, which ensures efficient information transmission. However, other types of regulations like voltage and phosphorylation further increase the diversity and precision of neuronal communication.

3.6.1 Voltage regulation of vesicle release

Fast neurotransmission starts with the synchronous opening of presynaptic VGCCs induced by membrane depolarization, resulting in channel opening and calcium influx. For a long time, calcium was the main focus of investigations analyzing the trigger of vesicle release. A potential direct effect of membrane depolarization on vesicle release has largely been ignored in spite of the fact that a series of work by Parnas since 1986 resulted in the proposal of the Ca^{2+}-voltage hypothesis of neurotransmitter release (Parnas and Parnas, 1986). This hypothesis emphasized the importance of primarily G-protein-coupled receptors (GPCRs) in regulating neurotransmitter release (Parnas and Parnas, 1986 and 1988; Hochner et al., 1989). Apparently, voltage-sensitive presynaptic inhibitory GPCRs control transmitter release by directly interacting with releasing machinery (Linial et al., 1997). In the frog NMJ, application of a specific M2-muscarinic receptor (M2R) antagonist extended the time course of acetylcholine (ACh) release without changing Ca^{2+} currents (Slutsky et al., 1999). Consistently, in the NMJ of M2R-knockout (KO) mice, accelerating Ca^{2+} removal by adding BAPTA and increasing Ca^{2+} influx by repetitive firing altered the decay time course of transmitter release. In comparison, the same treatment had no effects in wild-type mice. In both M2R-KO and wild-type mice, Ca^{2+} currents were almost identical (Slutsky et al., 2003). Here, the inhibitory M_2R seems to only determine the termination of neurotransmitter release.

To further understand the function of depolarization in transmitter release, in the mouse NMJ, a depolarization can induce ACh release in a linear manner (log quantal content/log depolarization) with a slope of 7 at a fixed extracellular Ca^{2+} concentration. However, in the presence of M2R antagonist, the slope reduces to 4, indicating that depolarization can release tonic block and induce Ca^{2+} influx in the meantime (Parnas et al., 2005). A following model (Fig. 10) clarifies the above GPCRs function on transmitter release (Yusim et al., 1999): At rest, GPCRs

physically interact with the releasing machinery with high-affinity (Linial et al., 1997; Ilouz et al., 1999), therefore block the transmitter tonically (Slutsky et al., 1999; Ben-Chaim et al., 2003). Upon arrival of the AP to the presynaptic terminal, the receptor shifts to its low-affinity state (Ilouz et al., 1999; Ben-Chaim et al., 2003; Dekel et al., 2005; Ohana et al., 2006) and dissociates from the releasing machinery rapidly, then

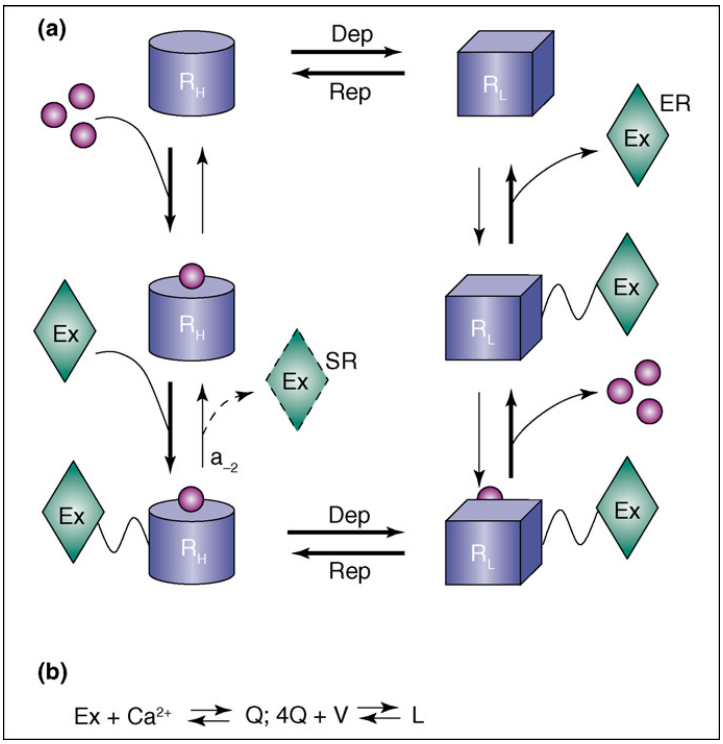

Figure 10. A simplified kinetic molecular scheme of the Ca^{2+}–voltage hypothesis (CVH). a. The inhibitory autoreceptor is present in two forms, which have either high affinity (RH) or low affinity (RL) for the transmitter. The distribution of receptors between these two states depends on the membrane potential. Depolarization (Dep) increases the fraction of RL, whereas repolarization (Rep) increases the fraction of RH. The predominant directions are denoted by thick arrows. Left: at resting potential, the receptor resides in its high-affinity state, RH. Thus, RH binds transmitter (dots), even at its low concentration in the synaptic cleft. The bound receptor associates with the exocytotic machinery (Ex; i.e. SNARE proteins and synaptotagmin) to block the release machinery. Right: when the presynaptic terminal is depolarized, RH transforms to the low-affinity state, RL, the transmitter rapidly dissociates and the exocytotic machinery detaches from the unbound receptor leading to evoked release (ER). The exocytotic machinery is now free to interact with Ca^{2+} to promote transmitter release. On repolarization, RL shifts back to RH and tonic block is reinstated. The model also accounts for spontaneous transmitter release: at resting potential (left), although the receptor is in its high-affinity state and hence bound to the transmitter, the release machinery occasionally disengages, with a low rate constant a–2 (broken arrow), leading to spontaneous release (SR). The free exocytotic machinery interacts with Ca^{2+} to promote transmitter release but, because the Ca^{2+} concentration is low (because no influx of Ca^{2+} took place), only a minimal level of transmitter is released. **b.** Phenomenological model for the joint action of intracellular Ca^{2+} and the exocytotic machinery to promote transmitter release. The combined action of the exocytotic machinery and intracellular Ca^{2+} yields a complex, Q. Cooperative action of four Q complexes with a vesicle, V, leads to release, L. Reproduced from Parnas and Parnas 2006.

the tonic block is relieved. On repolarization, the receptor returns to its high-affinity

state and reinstates the tonic block (Parnas and Parnas, 2006). A recent study shows that depolarization induced conformational changes in the orthosteric binding site of M_2R underlie the voltage-dependence of agonist binding, although the allosteric Site of M_2R is also involved in controlling the voltage-dependent agonist binding (Dekel et al., 2012). The above principles underlying the 'Ca^{2+}-voltage hypothesis' in cholinergic NMJs are also applicable to glutamatergic NMJs, as demonstrated in crayfish (Kupchik et al., 2008). In addition to the 'Ca^{2+}-voltage hypothesis', evidence suggests that Ca^{2+} independent but voltage dependent secretion also exists (Mochida et al., 1998; Zhang and Zhou, 2002). In rat superior cervical ganglion neurons, a hypertonic sucrose application enhanced transmitter release which could be further facilitated by short AP trains. This effect required the interaction between the synprint site of N-type calcium channel and the SNARE proteins (Mochida et al., 1998). Also in rat dorsal root ganglion neurons (DRG), depolarization could induce membrane capacitance increases in the absence of Ca^{2+} (Zhang and Zhou, 2002). In spite of the accumulating evidence of depolarization induced transmitter release increase, we need to know that AP or depolarization don't always increase the neurotransmitter release. In neurons of the external portion of the chicken inferior colliculus, a somatic depolarization resulted in a reduction in the spontaneous release without altering evoked release. The underlying mechanism is apparently an activation of presynaptic NMDA receptors and postsynaptic calcium dependent vesicle fusion. (Penzo and Peña, 2011). Therefore, voltage or depolarization has multifunction in neurotransmitter release although its major role of opening VGCCs.

Till now, five GPCRs have been shown to be voltage-sensitive. Two of these, the M1 and M2 muscarinic ACh receptors (Ben-Chaim et al., 2003), belong to GPCR subfamily A; The other three GPCRs are the mGlu type 1 (mGlu1) receptor, mGlu3 receptor (Ohana et al., 2006) and GABAB GPCR (Dekel et al., 2005), all belong to subfamily C. Even among the same family, the GPCRs interact with different G proteins and induce various physiological processes and exhibit voltage-dependent changes in affinity for agonists suggests that this is probably a general property of GPCRs. How is the voltage effect in *Drosophila* NMJ? In the present work, we revealed a voltage dependent and inversely calcium dependent mode of vesicle release and clarified its underlying mechanism.

3.6.2 Phosphorylation affects vesicle release

In addition to the key role of Ca^{2+} and the voltage effect in transmitter release, given the fact that the release machinery is made up of a large number of synaptic proteins (i.e., the SNAREs, SM-proteins, complexin, synaptotagmins synapsin and Rabphilin), many of which may be modulated by phosphorylation and other influences, one would expect that the release process could be subject to multiple modes of modulation. As indicated in figure 11, general information about phosphorylation of

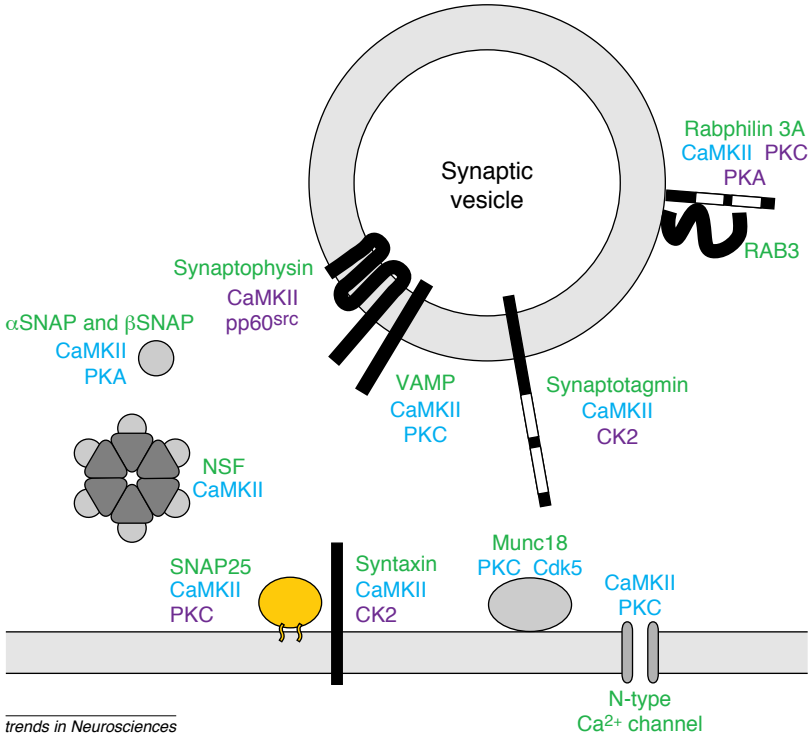

Figure 11. Phosphoproteins implicated in exocytosis at the synapse.
The kinases known to phosphorylate the various exocytotic proteins (green) on the basis of in vitro studies using purified proteins are indicated in blue. Where data come from studies using intact cells or cell extracts, the kinases are indicated in purple. Abbreviations: CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; Cdk5, cyclin-dependent protein kinase 5; CK2, casein kinase II; NSF, N-ethyl- maleimide-sensitive fusion protein; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; SNAP, soluble NSF attachment protein; SNAP25, 25 kDa synaptosomal associated protein; VAMP, vesicle associated membrane protein (or synaptobrevin). Reproduced from Turner et al., 1999.

SNARE proteins and their regulatory proteins are listed. Biochemical studies have demonstrated *in vitro* phosphorylation of syntaxin 1A, VAMP and SNAP25 by CaMKII, of VAMP and SNAP25 by PKC, and of syntaxin 1A by casein kinase II (CK2) (Bennett et al., 1993; Nielander et al., 1995; Hirling and Scheller, 1996; Shimazaki et al., 1996), although their physiological function on transmitter release

are not all clear yet.

The effects of phosphorylation of some SNARE related proteins are briefly described as below.

Synapsins (five isoforms: Ia,Ib,IIa,IIb and III), a family of highly conserved neuronal phospho- proteins that are specifically associated with synaptic vesicles, recruit synaptic vesicle from RP to refill the RRP. Both synapsin I and II are phosphorylated by CaMKI and PKA at their N termini. In addition, synapsin I is phosphorylated at both N and C termini by mitogen-activated protein kinase (MAPK) and at its C terminus by CaMKII (Greengard et al., 1993; Jovanovic et al., 1996). In living hippocampal terminals, synapsin Ia prevents transmitter release by anchoring synaptic vesicles to the cytoskeleton at rest; upon action potential (AP) firing, synapsin Ia can be phosphorylated by CaMKII as a result of rise in intracellular Ca^{2+} and dissociates from synaptic vesicles, disperses into axons and reclusters to synapses after the cessation of synaptic activity. The change in phosphorylation-state of synapsin Ia regulates synaptic vesicle mobilization and the kinetics of vesicle pool turnover, and hence, neurotransmitter release (Turner et al., 1999; Chi et al., 2003).

In addition, the Rab family of low-molecular-mass GTP-binding proteins are thought to guide membrane fusion between a transport vesicle and the target membrane, and to determine the specificity of docking. In Rab3A-deficient mice, Ca^{2+}-triggered fusion is slightly increased while the size of the RRP vesicles is normal, which is not due to an enhancement in release probability but maybe by rising the energy barrier for neighbouring vesicle fusion (Geppert et al., 1997).

Also in neurons with Syt12 expression, spontaneous neurotransmitter release can be increased by approximately 3 fold, but without effect on evoked release, which seems to be due to phosphorylation of Syt12 by cAMP-dependent PKA on serine97. Replacing serine97 by alanine eliminates synaptotagmin-12 phosphorylation and blocks its effect on spontaneous release (Maximov et al., 2007).

Moreover, as an important kinase related to SNARE, the effects of PKC on neurotransmitter release have also been the focus of study. Phorbol ester, a modulator which targets presynaptic PKC/munc-13 signaling cascades, change the properties of the dose-response curve, such as its Ca^{2+} sensitivity, steepness, maximum rate, etc (Betz et al., 1998; Hori, et al., 1999; Rhee et al., 2002; Silinsky and Searl, 2003; Wierda et al., 2007). At the calyx of Held, a large excitatory synapse of the CNS, phorbol esters potentiate evoked release and the spontaneous release

rate by increasing the apparent Ca^{2+} sensitivity of vesicle fusion (Lou et al., 2005). In endocrine secretion, phorbol esters were shown to shift the dose-response curve (Wan et al., 2004) or else to bring forward a component of release, which is very sensitive toward calcium concentration (Yang and Gillis, 2004).

Intriguingly, at frog NMJs, treatment of LY-294002, an inhibitor of phosphoinositide-3 kinase (PI3K) or casein kinase 2 (CK2) enhances the spontaneous release rate up to 100-fold of its basal level irrelevant of the extracellular calcium and inhibits vesicle cycling although the exact target of the kinases are not known yet (Rizzoli and Betz, 2002). CK2 is a ubiquitous, constitutively active, calcium- and cyclic nucleotide-independent protein kinase (for review, see Tuazon and Traugh, 1991), which is also present on synaptic vesicles and proved to be able to phosphorylate syt (Bennett et al., 1993; Davletov et al., 1993; Hirling and Scheller, 1996; Hilfiker et al., 1999). In all, vesicle release can be largely influenced by different kinases, which further broadens the means of neurotransmission in different physiological context. The function of S/T kinase AKT in controlling the functional status of release machinery will be the focus of the present study.

3.6.3 Other regulations

Except the above mentioned modulation of neurotransmitter release, other forms of release modulation also seem to exist. In the glycinergic synapses of two rat auditory brainstem nuclei, anteroventral cochlear nucleus and the mouse medial nucleus of the trapezoid body, ruthenium red, a Ca^{2+}-independent secretagogue that acts downstream of the Ca^{2+} receptor to induce transmitter release (Trudeau et al., 1996a) increased the frequency of mIPSCs independent of presynaptic calcium stores (Lim et al., 2003).

In all, neurotransmitter release seems to be simple, however, the properties of release can be modified with many possibilities. Therefore, neurological diseases could be caused by irregular field potentials in neural circuits or the dysfunction of certain synaptic kinases. This highlights the importance of neurotransmitter release regulation in the future study.

3.7 AKT structure and function

Among all the kinases, AKT (also named PKB) has a role in multiple cellular processes including glucose metabolism, apoptosis, cell survival and proliferation, transcription, protein synthesis and cell migration and is associated with several forms of cancer and psychiatric disorders (Dudek et al., 1997; Whiteman et al., 2002; Altomare and Testa, 2005; Stambolic and Woodgett, 2006). Mammalian genomes contain three genes encoding AKT (AKT1, 2, and 3) with multiple domains: an N-terminal pleckstrin homology (PH) domain, an interdomain linker, a kinase domain, and a C-terminal hydrophobic motif (Coffer et al., 1998; Alessi and Cohen, 1998). Before AKT activation, AKT and 3-phosphoinositide dependent protein kinase-1 (PDK1) are maintained in an inactive state through their PH domain and kinase domain interaction, also called ‘‘PH-in’’ conformer, which prevents PDK1 phosphorylation of PKB activation loop (Calleja et al., 2007). Activation of AKT requires its translocation to plasma membrane that is mediated by the interaction between the PH domain and phosphatidylinositol (3,4,5)-triphosphate (PIP3), which is a product of phosphatidylinositol 4,5-bisphosphate (PIP2) phosphorylation by phosphatidylinositol 3-kinase (PI3K) (Alessi and Cohen, 1998). This translocation to the membrane leads to a conformational change in AKT, producing a ‘‘PH-out’’ conformer and enabling PDK1 access the activation loop (Calleja et al., 2007). The phosphorylation of the two critical residues Thr308 and Ser473 of AKT can induce a greater than 1000-fold increase in its kinase activity (Alessi and Cohen, 1998). AKT can be activated in this membrane associated form in a context-dependent manner by a multitude of factors such as PDK, TORC, ILK, PKA or ATP (Lin et al., 2012). The lipid phosphatase PTEN acts as a counter-player of PI3K by dephosphorylating PIP3 back to PIP2 thereby removing the membrane localization signal for AKT and thus its membrane associated activities (Hyun et al., 2000).

At *Drosophila* NMJs, one copy of AKT related gene has been identified (Dakt1) (Franke et al., 1994). AKT has been shown to be expressed in presynaptic terminals where it has been proposed to be involved in a homeostatic negative feedback loop. The transmitter glutamate activates PI3K/AKT in a DmGluRA/CaMKII/DFak-dependent manner resulting in an inhibition of the transcription factor Foxo and subsequently in a decreased neuronal excitability (Howlett et al., 2008; Lin et al., 2011). In addition, a recent study suggested that a TORC2/AKT pathway regulates the size growth of synaptic boutons without effects on synaptic functions (Natarajan

et al., 2013).

Notably, mutations in PI3K/AKT pathway like TSC1/TSC2, NF1, or PTEN, lead to syndromic autism spectrum disorder (ASD) (Bourgeron, 2009; Ebert and Greenberg, 2013), and accumulating data indicate the interaction between activity-dependent neuronal signaling and ASD, schizophrenia or cancer. However, a direct synaptic role of AKT needs to be described.

3.8 Aims of this study

Neurotransmitter release is a fundamental process in the nervous system by which neurons are enabled to communicate with postsynaptic cells in a highly controlled manner. Although the general principles of neurotransmitter release have been clarified in the recent decades, there are still several open questions that have not been solved so far: is spontaneous vesicle release purely stochastic or is it subject to regulation? What is the relationship between vesicles released in a spontaneous or evoked manner or do both forms of vesicle release recruit vesicles with dedicated identities? We approached these and other questions at the larval NMJs of *Drosophila melanogaster*, a well accessible experimental system to study development and function of glutamatergic synapses. Specifically, we addressed the following issues:

• **Is spontaneous vesicle release subject to regulation?** In a screen for signaling pathways affecting the rate of spontaneous vesicle release we have identified the S/T-kinase AKT as a potent regulator. In this project we analyzed the physiological and molecular mechanisms by which AKT affects the rate of spontaneous vesicle release.

• **What is a potential regulator of AKT?** In this project we used an alternative ATP-depletion strategy that nerve stimulation can, in the absence of extracellular calcium, induce enhancement in spontaneous vesicle release. The data confirmed the functional existence of a Cacophony/AKT/release machinery complex and suggest that AKT is regulated by ATP. They suggest that AKT may serve at vesicle release sites as a local energy sensor that depending on the availability of ATP switches individual vesicle release machinery either into a tightly clamped mode for evoked release or at low ATP levels into a loosely clamped mode generating spontaneous release.

• **What limits synaptic transmission at *Drosophila* NMJs?** In a collaborative attempt to establish a mathematical model of synaptic bouton functions we assessed the depletion kinetics of preexisting vesicles during high-frequency stimulation.

4 Materials and methods

4.1 The *Drosophila* larval neuromuscular junction

The larval neuromuscular preparation is a widely used system for physiological and structural analyses of synaptic function and development, because it uses glutamate as neurotransmitter (Jan and Jan, 1976). The muscle cells are few in number (about

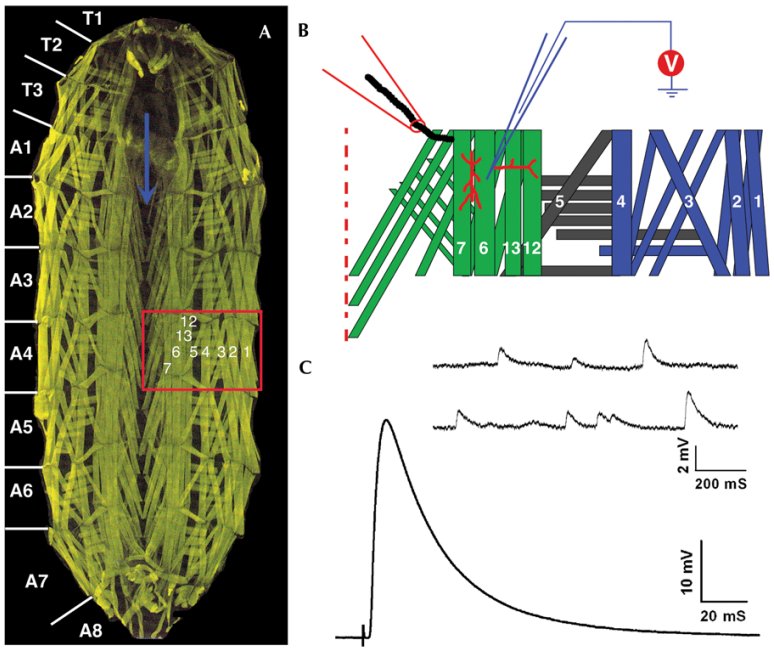

Figure 12. Intracellular recording of synaptic potentials from larval body-wall muscles.

A. An illustration of the thoracic (T) and abdominal (A) segments and the repeated pattern of musculature arrangements (the brain and the ventral nerve cord are removed in this image) in a dissected, flattened, and fixed third-instar larva. Major ventral, lateral, and dorsal muscles are labeled with numbers. The ventral midline is indicated by the blue arrow. **B.** One hemisegment of the body-wall muscles is illustrated here, with ventral (green), lateral (black), and dorsal (blue) muscles marked (Hoang and Chiba 2001). Major surface muscles are identified by their numbers. (Dashed red line) The ventral midline. A suction electrode is shown to pick up a segmental nerve and used to stimulate the motor axons within the nerve. A microelectrode is used to record miniature excitatory potential (mEJPs) and evoked synaptic potentials from muscle 6. Segments 4 and 5 are often used for synaptic transmission studies because the nerve leading to these muscles is long and easier to pick up by the suction electrode. Ventral longitudinal muscles such as muscles 6, 7, 12, and 13 are typically used for intracellular recordings, largely because of their large sizes and ventral positions. (C) Illustrative mEJPs and eEJP sample traces. (Adapted from Bellen and Budnik 2000; Zhang and Stewart, 2010.). For details on intracellular recordings see section 4.4.

30 in each abdominal hemisegment, Fig. 12), readily identifiable, and arranged in a regular pattern (Crossley, 1978). Their innervation consists of stereotyped terminal branches of typically two motor neurons (Johansen et al., 1989), many of which have been identified and named (Sink and Whitington, 1991; Atwood et al., 1993).

4.2 Larval and fly culture

All larvae were raised under tightly controlled standardized culture conditions (constant 25°C, 65% humidity, and high animal density, standard cornmeal fly food) and used as mid-third instar larvae shortly before the onset of the wandering stage. Fly stock were incubated at 18°C, 65% humidity.

Cornmeal fly food components:

Components	Weight	%
corn meal	800 g	6,4%
malt extract	800 g	6,4%
dry yeast	180 g	1.44%
soy bean meal	100 g	0.8%
molasses	220 g	1.76%
agar	80 g	0.64%
propionic acid	62.5 ml	0.5%
water	10-12 liter	

4.3 Fly Genetics: GAL4 - UAS

The GAL4-UAS system was developed by Andrea Brand and Norbert Perrimon in 1993 (Brand and Perrimon, 1993) as a powerful modular technique for the cell specific expression of genes. The system consists of two transgenic modules that are separated in two parental fly lines: one fly line is transgenic for the GAL4 gene, expressing the yeast transcriptional activator protein Gal4 according to a promoter or enhancer of choice (enhancer-trap in Fig. 13); another fly line carries a reporter transgene of choice that is preceded by the Upstream Activating Sequence (UAS), an enhancer element to which Gal4 can specifically bind to activate gene transcription (Fig. 13). Each of these driver- and reporter-fly lines is functionally neutral, only if a Gal4-driver line is crossed to a UAS-reporter line the F1-progeny will contain both modules and hence will express the reporter molecule in those cells in which the promoter/enhancer of the driver line is active.

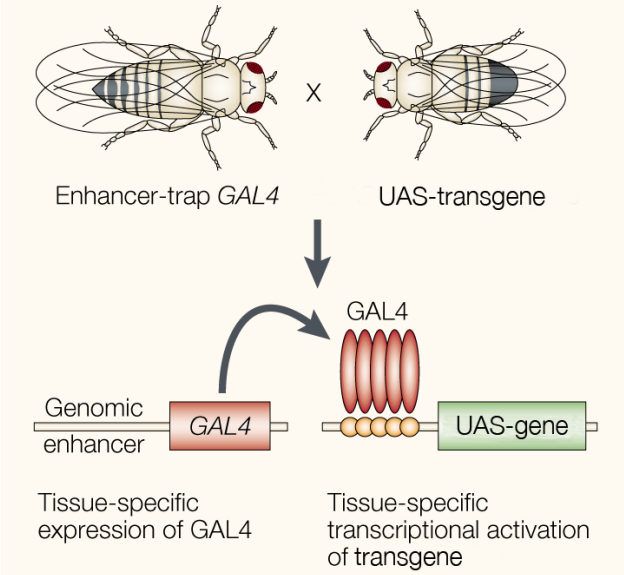

Figure 13. Model of GAL4/UAS system.

GAL4 is a yeast transcriptional activator; in the absence of ectopically expressed GAL4, the transgene is inactive in these transgenic flies. The transgene is activated by crossing these flies to transgenic flies that express GAL4 (enhancer-trap GAL4 fly), also known as the 'drivers'. (Adapted from Muqit and Feany, 2002.)

Most of the flies were obtained from Bloomington Stock Center or Vienna *Drosophila* RNAi Center if not specified. In all the experiment, Oregon R was used as wild type control. Male UAS-RNAi flies were mass mated to Dicer ok6-GAL4 or Dicer elav-GAL4 driver lines, and other UAS-transgene flies were mass mated to ok6-GAL4 or elav-GAL4 driver lines.

Lines	Nr.	Function	Donor
UAS-Akt-myr		Constitutive active Akt	Prof. Stephan Schneuwly, University of Regensburg, Germany
yw;UAS-Cpx	1436	Wild type Dcpx	Prof. Hugo Bellen, Baylor College of Medicine, US
yw;UAS-CpxI; CpxSH1/SM5-TM6	1418	Rat Cpx	Prof. Hugo Bellen, Baylor College of Medicine, US
yw;UAS-CpxIII; CpxSH1/SM5-TM6	1419	Mouse Cpx	Prof. Hugo Bellen, Baylor College of Medicine, US
$Para^{ts}$		Temperature sensitive VGSC paralytic	Prof. Chun-Fang Wu, University of Iowa, US
Cac^{ts2}		Temperature sensitive Cac	Prof. Richard W. Ordway, Pennsylvania State University, US
UAS-Ork1. Δ-NC	6587	Open rectifier K^+ channel mutant	Prof. Stephan Sigrist, Freie Universität Berlin
UAS-Ork1. ΔC	6586	Open rectifier K^+ channel mutant	Prof. Stephan Sigrist, Freie Universität Berlin
W^-;;pUASTi RNAi Brp C12; RNAi Brp C8	1025	Bruchpilot RNAi	Prof. Stephan Sigrist, Freie Universität Berlin

4.4 Electrophysiology

Experiments were performed on size-matched mid third instar male larvae at 25°C. All recordings of larval ventricular muscle 6 (segments A2/A3) were performed in bridge mode as described (Sigrist et al., 2003). In general, larvae were dissected and incubated in normal saline solution (NS) containing 2 mM Ca^{2+} or 1 mM EGTA without extracellular Ca^{2+} for 30 minutes (Jan and Jan, 1976). In the BAPTA-AM assay, hemolymph-like ringer's 3.1 (HL3 solution, [Stewart et al., 1994]) was used to efficiently load BAPTA-AM into presynaptic bouton. Muscle cells were impaled with a 15 - 20 MΩ microelectrode filled with 3 M KCl. Signals were acquired and amplified with AxoClamp 2B (Axon CNS, Molecular Devices, Sunnyvale, CA, USA), digitized using DigiData1322A and recorded on a computer with the Clampex software (Molecular Devices, Sunnyvale, CA, USA). Cells with a resting potential more negative than -50 mV were selected for further analysis. For nerve stimulation, the cut end of the intersegmental nerve was placed into a suction electrode, and suprathreshold voltage pulses were applied at indicated frequencies. In the depletion-recovery assay, a '100 stimuli plus a 5 s interval' recording paradigm was used continuously in 15 - 80 Hz. Data analysis was performed off-line. To load BAPTA-AM, 100 μM BAPTA-AM was first dissolved in 20 mM Mg^{2+} containing HL3 buffer for 10 minutes. Then the extracellular buffer was replaced with 2 mM Mg^{2+} containing HL3 buffer. Before testing in 1 mM EGTA containing solution, 10 pulses in 1 Hz were used to assure the fully chelating of the newly entered Ca^{2+}.

4.5 Drug and Chemicals

Most chemicals were first dissolved in dimethyl sulfoxide (DMSO) as stock solution, and the final concentration of DMSO in NS was minimized, showing no effects on the observed phenomenon. Other drugs were dissolved in NS. Reagents were bath applied by peristalic pump in a speed of 1 ml/min (IDEX Corporation, US).

4.5.1 Chemicals

All chemicals were purchased from Sigma (St. Louis, MO, USA), unless otherwise specified.

Chemicals	Company
740 Y-P	Tocris Bioscience, UK
Bafilomycin A1	LC labs, US
BAPTA-AM	Tocris Bioscience, UK
LY-294002	LC labs, US
PLTX - II	Alomone labs, Israel
Ryanodine	Enzo Life Sciences GmbH, Germany
Thapsigargin	Enzo Life Sciences GmbH, Germany
Tetrodotoxin	Tocris Bioscience, UK
Wortmanin	LC labs, US
Mk-2206	Selleck Chemicals, US
Oligomycin	Santa Cruz Biotechnology, US

4.5.2 Solutions

Normal saline solution

	concentration (in mM)
KCl	5
NaCl	130
$MgCl_2$	2
Sucrose	36
HEPES	5
Buffered at pH 7.3.	

Hemolymph-like ringer's 3.1 (HL3)

	concentration (in mM)
KCl	5
NaCl	70
$MgCl_2$	20
Sucrose	115
HEPES	5
$NaHCO_3$	10
Buffered at pH 7.2.	

4.6 Statistics

Data analysis was performed off-line (Clampfit 10, Axon Instruments) and Minianalysis 6.0(Synaptosoft Inc., US). Data are expressed as mean ±SEM (n), where n indicates the number of larvae examined. Statistical analyses were carried out using two-tailed Student's t-test for unpaired or paired data if not otherwise stated. In the figures, the level of significance is marked with asterisks: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

4.7 Resources for Drosophila study

Drosophila Databases

Flybase: http://flybase.org/

Stock Center

Vienna Drosophila RNAi Center: http://stockcenter.vdrc.at/control/main

Bloomington Stock Center: http://flystocks.bio.indiana.edu/

Kyoto Drosophila Genetic Resource Center:

http://kyotofly.kit.jp/cgi-bin/stocks/index.cgi

Berkely Drosophila Genome Project: http://www.fruitfly.org/

Antibodies & Cell Lines

The Developmental Studies Hybridoma Bank: http://dshb.biology.uiowa.edu/

5 Results

5.1 AKT activity controls functional status of vesicle release machinery

5.1.1 AKT activity modulates basal spontaneous release and evoked synchronous release

To shed light on the potential regulatory mechanisms underlying vesicle release machinery we started to re-examine basic vesicle release processes at NMJs of *Drosophila* melanogaster larvae. Intracellular recordings of the membrane potential of postsynaptic muscle fibers typically detected spontaneous mEJPs corresponding to individual vesicle release events from these NMJs at a frequency between 1 - 6 Hz (arrows in upper trace of Fig. 14A). Consistent with similar observations in this system (Buhl et al., 2013), we found that this rate of spontaneous quantal release shows no significant dependency on extracellular Ca^{2+} concentrations. At unphysiologically high extracellular Ca^{2+}-concentrations (8 mM), we observed a significant reduction in mEJP-amplitudes compared to all other tested conditions (Fig. 17J) likely resulting in an underestimation the mEJP-frequency. Thus, spontaneous vesicle release also persisted at the almost same rate in the absence of intra- and extracellular Ca^{2+}.

What can alter spontaneous vesicle release? To address that, we screened many signaling molecules including kinases and phosphatases. When we blocked the kinase activity of *Drosophila* AKT with a selective AKT blocker MK-2206 (50 μM) or knocking down AKT by RNAi-expression in larval motor neurons strongly increased the rate of spontaneous vesicle release from 4.81 ± 0.73 Hz to 41.00 ± 2.26 Hz and 5.87 ± 0.35 Hz in the presence of 2 mM extracellular calcium, 3.00 ± 0.23 Hz to 41.60 ± 5.54 Hz and 10.54 ± 4.37 Hz in nominally calcium-free buffer respectively (Fig. 14C and F). Conversely, overexpression of a membrane associated constitutive-active form of AKT (myr-Akt) resulted in a significant drop of spontaneous vesicle release to a level of ~ 1.6 Hz in the presence of 2 mM extracellular calcium (Fig. 14C and F) and ~ 0.5 Hz in nominally calcium-free buffer (Fig. 14F). Both gain of function (GOF) and loss of function (LOF) effects of AKT on spontaneous vesicle release were observable in the presence or absence of

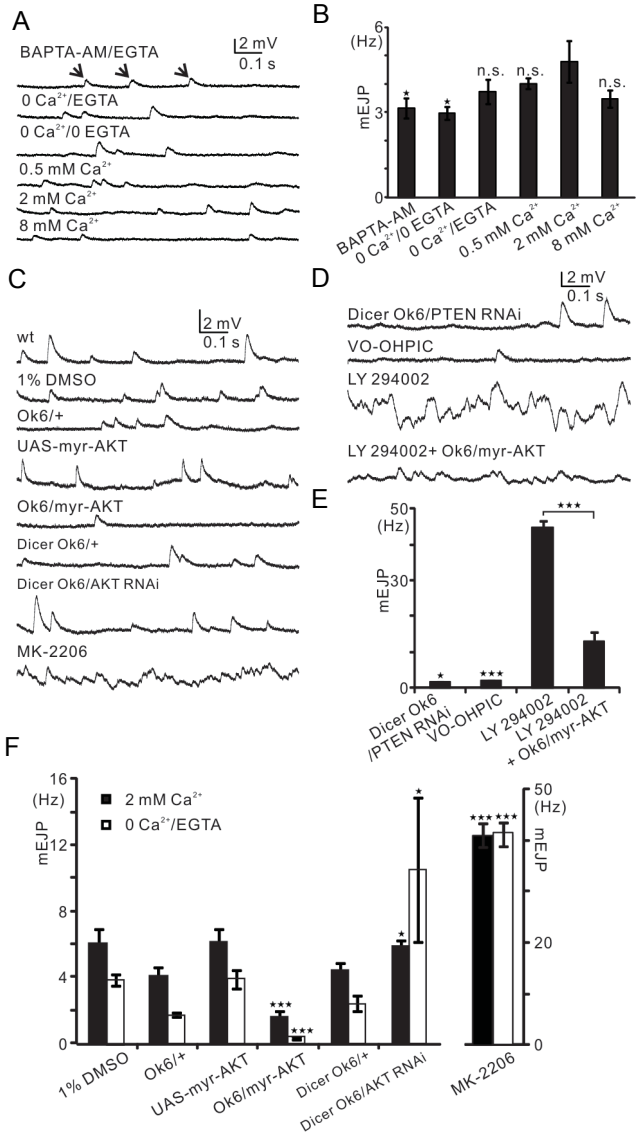

Figure 14. AKT activity regulates spontaneous and evoked neurotransmitter release.

A. Representative traces of mEJP recorded in the indicated conditions. Note the spontaneous vesicle release (arrows). **B.** Histogram of the mEJP-frequency data in A showing that spontaneous release is unaltered in the presence of different $[Ca^{2+}]_o$ (compared with 2 Ca^{2+}). In mM: 0.1 BAPTA-AM/1 EGTA (n=5), 0 Ca^{2+}/1 EGTA (n=7), 0 Ca^{2+} (n=6), 0.5 Ca^{2+} (n=5), 2 Ca^{2+} (n=6), 8 Ca^{2+} (n=6). **C.** Representative traces of mEJP recorded in 2 mM Ca^{2+} containing buffer. **D.** Representative traces of eEJP recordings in 0 mM Ca^{2+}/1 mM EGTA. **E.** Histogram of the mEJP-frequency data in D showing that spontaneous release is suppressed by motor neuron overexpression of PTEN RNAi (n=8) and PTEN inhibitor VO-OHPIC (n=7), but greatly enhanced by PI3K inhibitor LY-294002 (n=5) and LY-294002 effect can be furthered suppressed by overexpression of myr-AKT(n=5). Dicer Ok6/+ (n=5). **F.** Histogram of the mEJP-frequency data in C and also the mEJP-frequency data recorded in 0 mM Ca^{2+}/1 mM EGTA containing buffer showing that spontaneous release is suppressed by motor neuron overexpression of myr-AKT (n=5 and 5), but greatly enhanced by AKT inhibitor MK-2206 (n=5 and 5). wt (n=6 and 7), Ok6/+ (n=5 and 5), 1% DMSO (n=5 and 6), UAS-myr-AKT (n=5 and 8), Dicer Ok6/AKT RNAi (n=7 and 8). *p < 0.05; *** p < 0.001. Data represent means ± S.E.M. of the indicated number of preparations.

extracellular calcium indicating that AKT controls the rate of non-synchronous vesicle release independent of extracellular calcium and under otherwise physiological

conditions.

5.1.2 AKT regulation of neurotransmitter release requires its binding to PIP3

As mentioned before, AKT activation requires its translocation to plasma membrane mediated by PIP3, which is a product of PIP2 phosphorylation by PI3K (Alessi and Cohen, 1998). However, PIP3 can also be dephosphorylated to PIP2 by lipid phosphatase PTEN thereby removing the membrane localization signal for AKT and thus its membrane associated activities (Hyun et al., 2000). Therefore, we challenged the membrane association of AKT by interfering either with the PI3K-mediated synthesis of PIP3 or the PTEN-mediated de-phosphorylation of PIP3. We found that the blockade of PI3K with the specific inhibitor LY-294002 (50 μM) had a similar effect on spontaneous vesicle release (Fig. 14D and E) as blocking AKT itself, in that it strongly increased the basal spontaneous release to 44.66 ± 2.04 Hz (Fig. 1E). This phenotype could be rescued by overexpressing the membrane-bound myr-Akt transgene (13.18 ± 2.57 Hz) suggesting that PI3K is the upstream kinase of AKT in such regulation. Furthermore, blocking the lipid-phosphatase PTEN pharmacologically with the inhibitor VO-OHPIC (100 nM) or genetically by expressing a PTEN-RNAi transgene in motor neurons resulted in a significantly reduced rate of spontaneous vesicle release (1.95 ± 0.16 and 1.54 ± 0.06 Hz, respectively; Fig. 14D and E), phenotypes that are consistent with an increased presence and function of membrane-bound AKT. Thus, the AKT-mediated regulation of neurotransmitter release requires a PIP3 dependent membrane anchoring of AKT.

5.1.3 Functional interaction of Cacophony with the release machinery is necessary for the AKT-mediated regulation of neurotransmitter release

How is spontaneous release regulated by AKT? As introduced above, VGCCs are multifunctional signaling complexes that are located at synapses in direct contact with the vesicle release machinery (Bajjalieh and Schenell, 1995; Südhof, 1995). We therefore assessed whether the N-type-VGCC Cacophony that is the primary mediator of action potential triggered Ca^{2+}-influx at *Drosophila* NMJs (Littleton and Ganetzky, 2000; Kawasaki et al., 2000; Macleod et al., 2006) is potentially involved in the regulation of spontaneous release by AKT. Cacophony can be pharmacologically blocked by a component of the Plectreurys spider venom Pltx and its synthetic analog PltxII (Bodi et al., 1995). As shown in Figure 15A and B, eEJPs were reduced

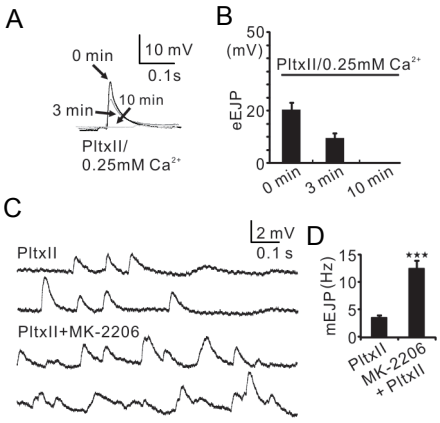

Figure 15. MK increases mini rate even in presence of PLTX.

A. Representative traces of eEJP recorded in 0.25 mM Ca^{2+}. **B.** Histogram of the eEJP amplitude data in A showing that evoked release is totally blocked after 10 minute PltxII (n=5). **C.** Representative traces of the mEJP-frequency data recorded with and without MK-2206 in 0.25 mM Ca^{2+}. **D.** Histogram of the mEJP-frequency data in C showing that spontaneous release induced by AKT inhibition also exists in the presence of PltxII (n=5). *** $p < 0.001$. Data represent means ± S.E.M. of the indicated number of preparations.

to zero after about 10 mintues incubation of PltxII (10 nM) in 0.25 mM Ca^{2+}, but the spontaneous release rate was not affected (3.45 ± 0.46 Hz). After the blockade of the evoked responses, MK-2206 (50 μM) was co-incubated with PltxII for 30 minutes. We found that spontaneous release was increased (12.50 ± 1.40 Hz, Fig. 15C and D), but was strongly suppressed comparing to treatment with only MK-2206 (Fig. 14B), which indicated the crucial role of Cacophony in AKT controlling of spontaneous release.

5.1.4 Akt regulates vesicle release via Syt-Cpx

In principle, the release machinery has been shown to be clamped by Syt and Cpx at the *Drosophila* NMJs (Huntwork and Littleton, 2007; Cho et al., 2010). In order to find out the downstream molecules of the release machinery interacting with AKT, we assessed the role of LOF and GOF genotypes of both genes and their potential relationship to AKT-functions. Consistent with previous observations we found that

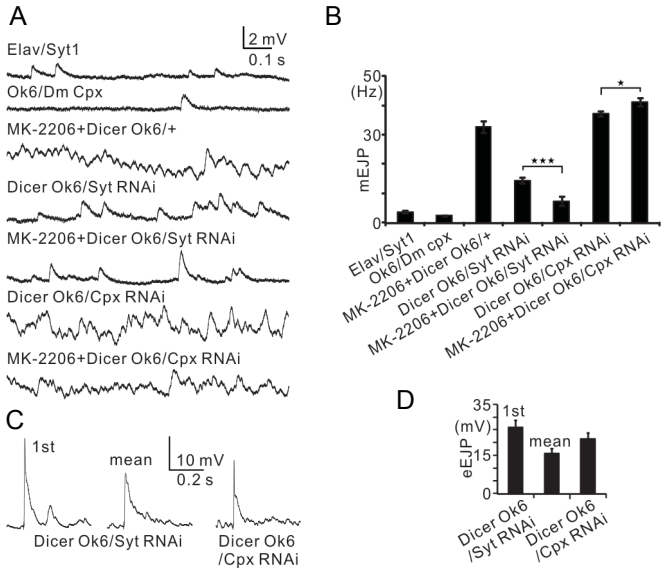

Figure 16. Syt is a downstream signaling molecule of AKT.

A. Representative traces of mEJP recorded in 2 mM Ca^{2+} containing buffer. **B.** Histogram of the mEJP-frequency data in A showing that spontaneous release is increased in motor neurons overexpressing Syt RNAi (n=5) or Cpx RNAi (n=5) and suppressed by overexpression of *drosophila* Cpx (n=5). Additional treatment with MK-2206 reduced spontaneous release when overexpressing of Syt RNAi (n=5) but slightly increased spontaneous release in Cpx RNAi (n=5). Elav/Syt1 (n=5), MK-2206/Dicer OK6/+ (n=5). **C.** Representative traces of eEJP recordings in 2 mM Ca^{2+} containing buffer. **D.** Histogram of the eEJP amplitude data in C showing that motor neuron overexpression of Syt (n=5) and Cpx RNAi (n=5) reduces eEJP amplitude. Note that the eEJP responses become smaller within a minute. * $p < 0.05$; *** $p < 0.001$. Data represent means ± S.E.M. of the indicated number of preparations.

RNAi mediated knock down of Cpx resulted in a strongly increased rate of spontaneous vesicle which shows similar phenotype to blockade of AKT and decrease in the amplitude of evoked release, application of MK-2206 slightly enhance the mEJP frequency (37.20 ± 0.97 Hz, 21.33 ± 2.34 mV and 41.20 ± 1.53 Hz, Fig. 16A to D). Conversely, overexpression of a wild type *Drosophila* Cpx transgene in motor neurons significantly reduced spontaneous release frequency (0.90 ± 0.08 Hz, Fig. 16A and B). Both observations support the view that Cpx acts as a fusion clamp at glutamatergic synapses of *Drosophila* (Huntwork and Littleton, 2007). In addition, overexpressing wild type Syt in motor neurons resulted in similar

spontaneous vesicle release rate as wild type (3.69 ± 0.48 Hz, Fig. 16A and B), and knockdown of Syt by RNAi expression resulted as expected in an increased basal mEJP frequency (14.37 ± 1.00 Hz, Fig. 16A and B) and gradual reduction in the evoked release accompanied by an increase in the asynchronous release (25.87 ± 2.83 mV and 15.72 ± 1.91 mV Fig. 16C and D). However, spontaneous release was reduced upon treatment with MK-2206 (7.36 ± 1.51 Hz, Fig. 16A and B). The above phenotypes indicated that the Syt/Cpx-based fusion clamp is a target of AKT.

5.1.5 The same pool of vesicles are involved in AKT regulated spontaneous release and evoked release

AKT can control spontaneous vesicle release via synaptotagmin. What is the identity of the vesicles released by Akt signaling blockers? In order to assess the origin of the spontaneously released vesicles in the above experiments, we examined the effect of the AKT-blocker MK-2206 in the absence of vesicles of the RRP. Therefore, we treated larval filet preparations with 2 μM Baf-A1 which makes the release of newly recycled vesicles invisible to postsynaptic membrane potential recordings and hence can be used to "deplete" preexisting vesicles of the RRP with stimulation. As expected, Baf-A1-treated preparations showed a rapid decay of eEJP-amplitudes during continuous 30 Hz motor nerve stimulation (Fig. 17B) resulting in an almost complete "depletion" of preexisting mature vesicles within 5 minutes of stimulation (0.65 ± 0.08 mV, Fig. 17A and D). The same stimulation in vehicle without Baf-A1 elicited typical eEJPs (15.97 ± 1.41 mV, Fig. 17 A and D), indicating that the observed eEJP-depletion in Baf-A1-treated preparations is not due to general synaptic limitations induced by continuous high-frequency simulation. It is rather due to the "depletion" of preexisting mature vesicles of the RRP. At this point of eEJP depletion, mEJPs were still detectable (0.20 ± 0.05 Hz, upper traces in Fig. 17C), however, at a significantly lower rate compared to that of BafA1-treated cells without prior stimulation (3.92 ± 0.21 Hz, lower traces in Fig. 17C and Fig. 17D) or untreated cells (Fig. 14B). Importantly, in cells depleted of their RRP, MK-2206 treatment had no effect on the rate of spontaneous vesicle release (0.27 ± 0.05 Hz, middle traces in Fig. 17C and Fig. 17E). This finding demonstrates that MK-2206 and hence AKT does not directly affect spontaneous vesicle release. It rather suggests that the bidirectional effects of AKT on the rate of spontaneous vesicle release require the presence of RRP vesicles that apparently are the primary source of spontaneously

released vesicles. RRP vesicles are prevented from spontaneous release by AKT hyperactivity (Fig. 14F) whereas AKT hypoactivity results in enhanced spontaneous

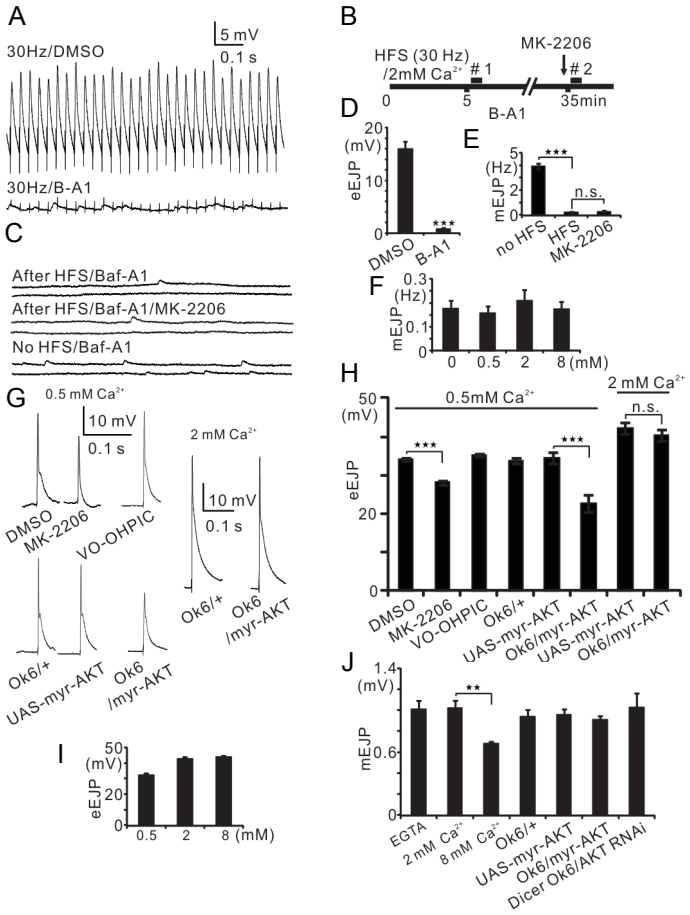

Figure 17. Same pool of vesicles drives synchronous release and AKT regulated spontaneous release.

A. Representative traces of eEJP trains recorded during HFS in the presence or absence of blocker of the vesicular proton pump B-A1 in 2 mM Ca^{2+} containing buffer. **B.** Schematic drawing of the experimental protocol used. Filet preparations were incubated for 5 minutes in the presence or absence of Baf-A1 in 2 mM Ca^{2+} containing buffer. Intracellular mEJP recordings (small bars #1) were then performed and followed with 30 minute incubation in the presence of B-A1/MK-2206, then continued with mEJP recordings (small bars #2). **C.** Representative traces of mEJP recorded after or without HFS followed by with or without MK-2206. **D.** Histogram of the eEJP amplitude data in A showing that evoked release is totally depleted in the presence of B-A1 (n=5), but largely remained in the presence of vehicle DMSO (n=5). **E.** Histogram of the mEJP-frequency data showing that spontaneous release is strongly depleted after HFS (n=5) comparing to no HFS (n=5), and a further MK-2206 has less impact (n=5). **F.** Histogram of the mEJP-frequency data showing that spontaneous release maintains at a similar level in different $[Ca^{2+}]_o$ after depletion. n=5 in all the concentrations. **G.** Representative traces of eEJP recorded in the indicated condition. **H and I.** Histogram of the eEJP amplitude data in G and that of wild type in different Ca^{2+} concentration. wt/8 mM Ca^{2+} and MK-2206 (n=7), wt/2 mM Ca^{2+} and VO-OHPIC (n=6), the other conditions (n=5). **J.** Histogram of the mEJP amplitude data in the indicated conditions. wt/EGTA (n=7) /2 mM Ca^{2+} (n=6)/8 mM Ca^{2+} (n=7), Ok6/+ (n=5), UAS-myr-AKT (n=5), Ok6/myr-AKT (n=5), Dicer Ok6/AKT RNAi (n=7). n.s. $p > 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data represent means ± S.E.M. of the indicated number of preparations.

release of RRP vesicles (Fig. 14F). At rest, the AKT activity seems to be balanced such that a typical spontaneous release rate of about 3 Hz can be observed.

Consistent with the idea that RRP vesicles are the primary source of spontaneously released vesicles we found that acute blocking of AKT by MK-2206 resulted not only in a strongly enhanced rate of mEJPs (Fig. 14F) but in concurrently reduced eEJP-amplitudes (28.10 ± 0.60 mV, Fig. 17G and H). However, acute AKT hyperactivity by overexpressing myr-AKT strongly suppressed spontaneous release (Fig. 14E) and also resulted in smaller eEJP-amplitudes in lower Ca^{2+} (0.5 mM, 22.73 ± 2.34 mV) but similar to wild type eEJP-amplitudes in higher Ca^{2+} (2 mM, 40.50 ± 1.46 mV, Fig. 17G and H); blockade of PTEN was associated with fewer mEJPs (Fig. 14E) and unaltered eEJPs (35.28 ± 0.49 mV, Fig. 17G and H). The reduced eEJP amplitudes in hypo- and hyper- AKT activity were unlikely due to the change in quantal size because the amplitude of mEJPs were almost stable at around 1 mV range among these treatments (Fig. 17J).

These effects of acutely altered AKT activities on mEJP rates and eEJP amplitudes suggest that AKT can switch the modality of vesicle release within a common pool of vesicles such that either evoked vesicle release is favored over spontaneous release or vice versa. Thus, AKT activity seems to maintain the release machineries of RRP vesicles in a clamped and Ca^{2+}-sensitive mode to be used in evoked release whereas reduced or blocked AKT activity renders release machineries fusogenic and only loosely Ca^{2+}-dependent resulting in spontaneous vesicle release.

5.2 AKT regulates depolarization triggered vesicle release by sensing the local ATP level

5.2.1 Local ATP level regulates spontaneous vesicle release

In an attempt to identify potential synaptic regulators of AKT we assessed whether the recently identified adenosine 5′-triphosphate (ATP)-dependent on-off switch of AKT might play a role in the regulation of its synaptic function. We found that 2 hours treatment with oligomycin (2 μg/ml, 20.48 ± 2.69 Hz, Fig. 18A), a specific blocker of ATP-synthase, resulted in enhanced spontaneous vesicle release (20.48 ± 2.69 Hz, Fig. 18A) that was close to that elicited by AKT blockade. Shorter incubation (0.5 and 1.5 hours) with oligomycin can't facilitate spontaneous vesicle release (5.45 ± 0.64 Hz and 2.21 ± 0.06 Hz, Fig. 18A). As Ca^{2+} is an important ion during cellular ATP synthesis, we removed the extracellular Ca^{2+} and also incubated with 1 mM EGTA in order to speed up the depletion of intracellular ATP. Indeed, the spontaneous release was strongly enhanced during these treatments (7.21 ± 3.15 Hz and 26.83 ± 1.03 Hz, Fig. 18A).

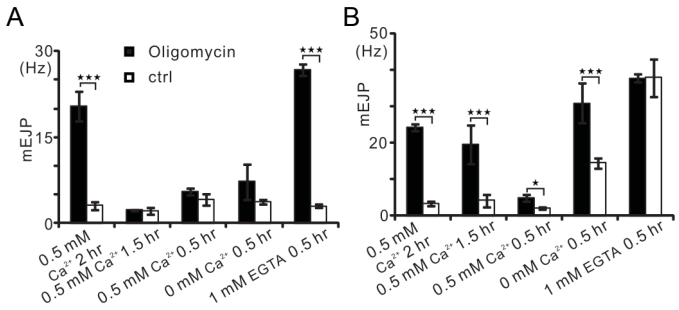

Figure 18. Depletion of ATP increases spontaneous vesicle release.

A. Histogram of the mEJP frequency in the presence of mitochondrial H^+-ATP synthase inhibitor oligomycin (filled bar) or 0.1% DMSO (empty bar). n=5 in the all indicated conditions. **B.** Histogram of the mEJP frequency altered by short trains of stimulation in the presence of mitochondrial H^+-ATP synthase inhibitor oligomycin (filled bar) or 0.1% DMSO (empty bar). n=5 in the all indicated conditions.* $p < 0.05$; *** $p < 0.001$. Data represent means ± S.E.M. of the indicated number of preparations.

In addition, we investigated whether short trains of nerve stimulation (Fig. 19B) can also affect the spontaneous release. We found that after half an hour incubation of oligomycin in the presence of 0.5 mM Ca^{2+}, spontaneous vesicle release was slightly increased (4.96 ± 0.92 Hz, Fig. 18B) by voltage stimulation, 1.5 hours incubation led to a stronger increase in vesicle release by voltage stimulation (19.70 ± 5.30 Hz, Fig. 18B) and longer incubation (2 hours) resulted in no more facilitation

by voltage stimulation (24.23 ± 0.92 Hz, Fig. 18B). However, the same treatments with vehicle showed no effects. Upon removal of extracellular Ca^{2+}, stimulation can trigger strong enhancement of vesicle release with or without oligomycin treatments (30.94 ± 5.50 Hz and 14.50 ± 1.41 Hz, Fig. 18B) in 0 Ca^{2+}/0 EGTA. In nominally Ca^{2+} free buffer (0 Ca^{2+}/1 mM EGTA), spontaneous release was enhanced to a similar level (37.84 ± 1.18 Hz and 37.94 ± 5.08 Hz in Fig. 18B and Fig. 19 A and C) in the presence or absence of oligomycin. Thus, acute depletion of local ATP enhances spontaneous vesicle release and short train of nerve stimulation in nominally Ca^{2+} free buffer also increases vesicle release to a similar level as ATP depletion and AKT blockade.

5.2.2 Depolarization enhances vesicle release in an inversely calcium dependent manner

Previous data showed that nerve stimulation strongly enhances vesicle release by increase ATP consumption in nerve terminals resulting in ATP-depletion and perhaps in AKT-inactivation. To test this hypothesis, we made use of an alternative ATP-depletion strategy as mentioned above that allowed us to make postsynaptic recordings during periods of intense nerve stimulation. When the motor nerve was briefly stimulated in the absence of extracellular calcium (0 mM Ca^{2+}/1 mM EGTA, 30 Hz for 5 sec, Fig. 19B), the vesicle release rate abruptly increased to 12.60 ± 1.07 fold (Fig. 19C), where it was maintained over the next few minutes before returning to baseline values (data not shown). The effect of nerve stimulation on vesicle release was significantly weaker or absent if EGTA was omitted or when physiological Ca^{2+}-concentrations (2 mM) added in the extracellular solution, respectively (4.02 ± 0.46 fold, triangle and 0.37 ± 0.09 fold, circle in Fig. 19C). Therefore, depolarization triggered vesicle release increase is inversely dependent on extracellular Ca^{2+}.

To find out the potential role of intracellular Ca^{2+} in depolarization induced vesicle release increase, we loaded cell-permeant fast Ca^{2+}-chelator BAPTA-AM (100 μM) into presynaptic terminals which eliminated nerve-stimulation evoked synchronous vesicle release (Fig. 19D, left panel). However it did not affect depolarization induced vesicle release increase (Fig. 19D, right panel). In addition, application of thapsigargin (TG, 20 μM), an inhibitor of the intracellular calcium pumps which increases intracellular Ca^{2+}, resulted in a mild but significant reduction of depolarization induced vesicle release increase. Whereas an inhibitor of

intracellular-store calcium channels - ryanodine (1 μM), which reduces intracellular Ca^{2+} level, showed no effect on depolarization induced vesicle release increase

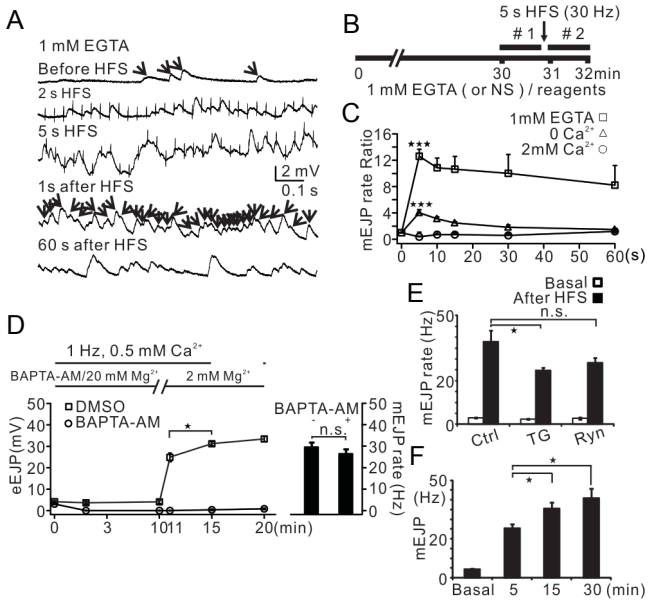

Figure 19. Inverse relationship between calcium concentration and depolarization triggered vesicle release. **A.** Representative traces of mEJP recordings before, during and after a brief train of high-frequency stimulation (HFS, 30 Hz, 5 sec.) in 0 mM Ca^{2+}/1 mM EGTA containing buffer. Note the strong enhancement of the rate of spontaneous vesicle release (arrows). **B.** Schematic drawing of the experimental protocol used. Filet preparations of *Drosophila* larvae were incubated in the indicated conditions for 30 minutes. Intracellular mEJP recordings (small bars #1 and #2) were then performed before and after HFS. **C.** The mEJP-frequency-ratios revealed a strong enhancement of the rate of spontaneous vesicle release in the absence of extracellular Ca^{2+} (squares; n=7). Without EGTA this enhancement is weaker (triangles; n=6) or as in the presence of Ca^{2+} absent (circles; n=6). **D.** Loading of NMJs with the intracellular Ca^{2+}-chelator BAPTA-AM (circles n=5) did not alter depolarization triggered vesicle release increase relative to the vehicle application (squares n=5). **E.** Depolarization triggered vesicle release was slightly reduced by depletion of intracellular Ca^{2+}-stores with the inhibitor of the intracellular Ca^{2+}-pump thapsigargin (20 μM; n=5), but unchanged with the antagonist of the intracellular store Ca^{2+}-channel ryanodine (1 μM; n=5). **F.** Histogram of the vesicle release frequency data showing that after 15 (n=6) and 30 (n=6) minutes incubation in 0 mM Ca^{2+}/1 mM EGTA containing buffer, depolarization triggered vesicle release becomes stronger than 5 (n=6) minute incubation. n.s. $p > 0.05$; *$p < 0.05$; *** $p < 0.001$. Data represent means ± S.E.M. of the indicated number of preparations.

(Fig. 19E). Moreover, depolarization-induced effects could be potentiated by increasing the incubation time of larval fillets from 5 to 30 minutes in the presence of EGTA (Fig. 19F). These observations indicated that in the near absence of extracellular and intracellular Ca^{2+} nerve stimulation can directly or indirectly elicit inversely Ca^{2+} dependent reversible increase in the rate of non-synchronous vesicle release.

5.2.3 Depolarization triggered vesicle release is independent of depolarization frequency

How is vesicle release dependent on depolarization in the absence of Ca^{2+}? To address that, firstly, we incubated the larval filet preparation in nominally Ca^{2+}-free extracellular solution for 30 minutes, then applied a total number of 150 stimuli at 2 and 10 Hz, which is the same number of stimuli as in 5s 30 Hz stimulation. Only after reaching a count of more than 20 APs, vesicle release enhancement became detectable and further stimulation led to a tonic plateauing release of about 40 vesicles at around 100 stimuli, which reduce to a basal level within the following 120 seconds (Fig. 20A-C). During the recovery, PAR showed significantly faster recovery kinetics after 150 stimuli in 2 Hz than in 10 Hz. This difference suggested that each

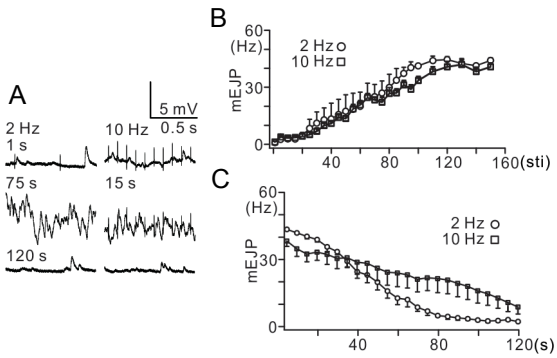

Figure 20. Depolarization triggered vesicle release is independent of stimulation frequency.
A. Representative traces of vesicle release induced by 2 (n=8) and 10 (n=8) Hz stimulation recorded during and after HFS in 0 mM Ca^{2+}/1 mM EGTA containing buffer. **B.** Onset of vesicle release induced by 2 (n=8) and 10 (n=8) Hz stimulation. **C.** Vesicle release decays close to basal spontaneous release level after cessation of 2 (n=8) and 10 (n=8) Hz stimulation. Data represent means ± S.E.M. of the indicated number of preparations.

AP-induced secondary signal has a defined half-life so that “old” secondary signals already start to decay earlier following the end of the long 2 Hz stimulation period compared to the decay following a short 10 Hz stimulation. Given that half-lives of protein phosphorylation range between seconds to hours (e.g. Hess et al., 1988; Igo et al., 1989; Zapf et al., 1998) and we have shown that the S/T-kinase AKT is a potent regulator of spontaneous vesicle release (see chapter 5.1.1), therefore we assessed whether AKT is involved in depolarization triggered vesicle release (see chapter 5.2.2).

5.2.4 AKT controls the clamp for depolarization triggered vesicle release

In previous part, we showed blockade and constitutive activation of AKT dramatically increases and reduces the basal spontaneous release frequency. In order to know the role of AKT in depolarization triggered vesicle release, we used the same paradigm as in Figure 19B. We found that AKT-hyperactivity (overexpression of myr-Akt, rather than wild type AKT) almost completely eliminated depolarization triggered vesicle release (4.40 ± 1.40 Hz and 34.12 ± 3.89 Hz, Fig. 21A and C) and AKT-blockade resulted in no additional enhancement of vesicle release compared to spontaneous release (45.23 ± 5.63 Hz, Fig. 14D, 21A). We also found that ATP depletion induced vesicle release in the presence of oligomycin can be suppressed by expressing of myr-AKT (16.24 ± 3.15 Hz, Fig. 21A), additional voltage stimulation resulted in less impact on the vesicle release rate (19.60 ± 3.09 Hz, Fig. 21A).

Consistently, alterations of the membrane association of AKT as above, we found that decreasing membrane binding of AKT by blocking PI3K with LY-294002 (50 μM) showed no further increase in depolarization triggered vesicle release (44.52 ± 2.83 Hz, Fig. 21B) compared to without stimulation (Fig. 14F), although expression of dominant negative form of PI3K did not change depolarization triggered vesicle release (1.70 ± 0.29 Hz before stimulation and 44.52 ± 2.83 Hz after stimulation, Fig. 21C). Increasing membrane binding of AKT by inhibiting PTEN with its inhibitor VO-OHPIC (100 nM) and expression of RNAi suppressed depolarization triggered vesicle release (1.95 ± 0.16 and 1.54 ± 0.06 Hz before stimulation, 24.77 ± 2.38 and 13.00 ± 3.36 Hz after stimulation, Fig. 21B and 1F), although increasing activation of PI3K by overexpressing catalytic subunit DP110 of PI3K, constitutively active form of PI3K-CAAX or with its activator 740 Y-P (5 μM) had no effect on the depolarization triggered vesicle release (3.69 ± 0.86, 1.73 ± 0.07 and 2.99 ± 0.48 Hz before stimulation, 35.84 ± 2.40, 30.05 ± 3.06 and 34.04 ± 3.56 Hz after stimulation, Fig. 21C). Importantly, overexpression of myr-Akt was sufficient to inhibit the depolarization triggered vesicle release even in the presence of 50 μM LY-294002 (11.80 ± 2.10 Hz, Fig. 21B). Therefore, a PIP3 dependent membrane anchoring of AKT is necessary for the regulation of depolarization triggered vesicle release.

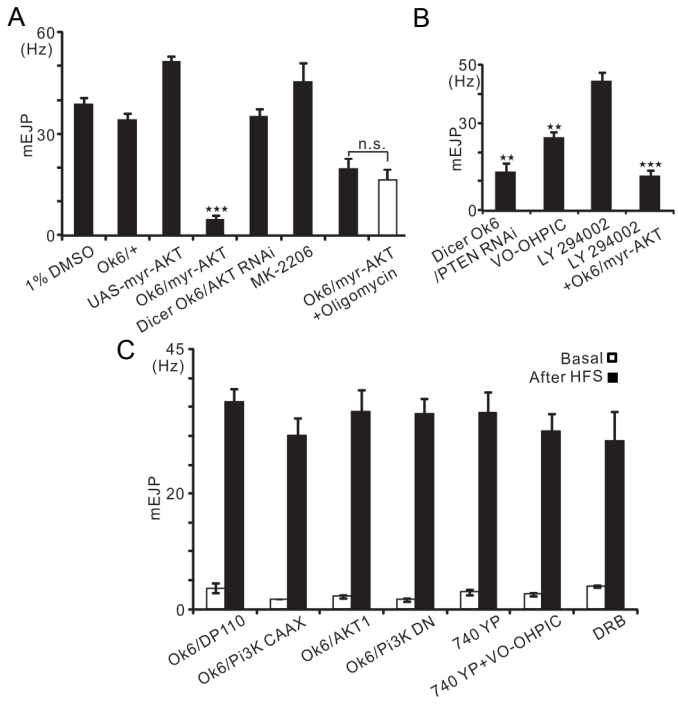

Figure 21. AKT activity senses local ATP level and controls depolarization triggered vesicle release.

A. Bar graph showing that oligomycin incubation induced vesicle release (empty bars, n=5) and depolarization triggered vesicle release (filled bars) were strongly suppressed by motor neuron overexpression of myr-Akt, but unaltered by AKT blockade. Mean value of 5-8 animals are included in each condition. **B.** Increasing AKT membrane binding by PTEN RNAi (n=8) and its inhibitor VO-OHPIC (n=7) reduces depolarization triggered vesicle release. Overexpression of myr-AKT suppresses depolarization triggered vesicle release in addition to inhibition of LY-294002 effect. **C.** Treatments have no effect on depolarization triggered vesicle release. Mean value of 5-8 animals are included in each condition. All show no significance compare to control. n.s. $p > 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data represent means ± S.E.M. of the indicated number of preparations.

A similar effect of LY 294002 on increasing spontaneous release was also observed in frog NMJ (Rizzoli and Betz, 2002). They indicated that casein kinase 2 (CK2), a ubiquitous, constitutively active, calcium- and cyclic nucleotide-independent protein kinase (for review, see Tuazon and Traugh, 1991) is responsible for the effect. To know whether CK2 is also involved in the regulation of depolarization triggered vesicle release, we applied 300 μM 5,6-dichlorobenzimidazole riboside (DRB), a specific CK2 inhibitor, which did not alter either basal spontaneous or depolarization triggered vesicle release (3.96 ± 0.17 Hz and 29.2 ± 5.03 Hz, Fig. 21C). Therefore, CK2 is not involved in both the regulation of spontaneous and depolarization triggered vesicle release.

5.2.5 Synaptotagmin is an effector of voltage stimulation in depolarization triggered vesicle release

What is the downstream signaling in depolarization triggered AKT control of vesicle release? In the above chapter, we showed that AKT regulates spontaneous release via the interaction with Syt-Cpx. Could this be a common mechanism that also underlies depolarization triggered vesicle release regulation? As shown above,

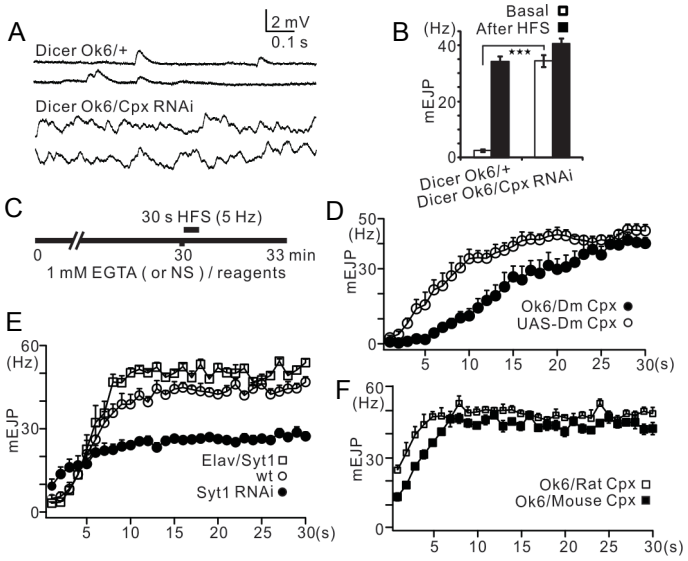

Figure 22. Syt KD reduces depolarization triggered vesicle release.

A. Representative traces of the mEJP-frequency data recorded in 0 mM Ca^{2+}/1 mM EGTA containing buffer. **B.** Histogram of the mEJP-frequency data in A showing that basal spontaneous release (empty bar) is strongly enhanced in motor neuron overexpressing Cpx RNAi (n=7) comparing to control (n=5). Vesicle release is further increased after 30 Hz stimulation in Cpx RNAi (n=7). **C.** Schematic drawing of the experimental protocol used. Filet preparations of *Drosophila* larvae were incubated in the indicated conditions for 30 minutes. Intracellular mEJP recordings were then performed during and after 30s 5 Hz stimulation. **D-F.** Onset kinetics of vesicle release induced by 5 Hz stimulation showing that motor neuron overexpressing *drosophila* Cpx (filled circle in D, n=5) has a slower kinetics than control (empty circle in D, n=5), and overexpression of rat (empty square in F, n=5) or mouse (filled square in F, n=5) Cpx indicate a faster kinetics but the same plateau. Surprisingly, overexpression (empty square in E, n=5) of Syt increases the plateau and Syt KD (filled circle in E, n=5) reduces the plateau vesicle release comparing to wild type (empty circle in E, n=5). *** $p < 0.001$. Data represent means ± S.E.M. of the indicated number of preparations.

knockdown of complexin has a similar phenotype to depolarization triggered vesicle release, which indicated that the Syt/Cpx-based fusion clamp could be one of the targets of AKT. We therefore assessed the role of LOF and GOF genotypes of both genes with the above paradigm (Fig. 19B). RNAi mediated knock down of Cpx resulted in a strongly increased rate of spontaneous vesicle release (34.40 ± 2.16 Hz Fig. 22A and B), which was slightly further increased by a brief train of 30 Hz

stimulation (40.65 ± 1.76 Hz Fig. 22A and B). In order to see the onset of depolarization triggered vesicle release better, we tested the genotypes with a total of 150 stimuli in 5 Hz for 30 seconds (Fig. 22C). Overexpression of *Drosophila* wild type Cpx transgene in motor neurons significantly slowed down the onset kinetics of depolarization triggered vesicle release without altering its peak frequency (40 – 45 Hz Fig. 22D). Thus, *Drosophila* Cpx worked more as a passive clamp in depolarization triggered vesicle release. What about the other complexins? As described before, mammalian complexins have different effects on releasing machinery to *Drosophila* complexin. Indeed, when we overexpressed mouse and rat complexins in larval motor neuron, the basal spontaneous releases were higher, but both of them arrived at similar plateau frequency of 40 - 50 Hz (Fig. 22F). These data suggest that all the known complexins worked as release machinery clamp rather than regulatable effector in depolarization triggered vesicle release, and depolarization triggered vesicle release is similarly dependent on the removal of the complexin fusion clamp as regular evoked vesicle release.

What could be the downstream regulatable effector of depolarization and AKT? LOF and GOF of Syt gave less expected results: overexpressing wild type Syt in motor neurons had similar spontaneous vesicle release rate as in wild type (3.20 ± 0.49 Hz Fig. 22E), however, it showed an increased sensitivity to depolarization as indicated by the faster onset kinetics and the higher plateau frequency of depolarization triggered vesicle release (50 Hz, squares in Fig. 22E). Syt-RNAi expression resulted as expected in an increased mEJPs frequency (9.33 ± 2.49 Hz, filled circles Fig. 22E), however, its sensitivity to depolarization was strongly reduced as indicated by the shallow slope and reduced plateau frequency of depolarization triggered vesicle release (~ 25 - 28 Hz, filled circles in Fig. 22E). Thus, at rest the Syt expression level showed phenotypes consistent with its role as fusion clamp. However, during nerve stimulation the sensitivity to depolarization of depolarization triggered vesicle release appeared to be proportional to the amount of Syt, and reduced Syt expression limited the maximal plateau of depolarization triggered vesicle release. These phenotypes suggested that Syt translated the depolarization signals that have been initiated by certain depolarization sensor towards loosening the complexin fusion clamp, which is also under the control of AKT.

5.2.6 Cacophony is the voltage sensor for depolarization triggered vesicle release

What senses the depolarization in depolarization triggered vesicle release? One of the most widely used voltage sensitive complex in nervous system is VGCC. We therefore investigated the potential role of Cacophony in depolarization triggered vesicle release. We first pharmacologically blocked Cacophony with PltxII in the absence of Ca^{2+}, and the basal spontaneous release was not altered (3.49 ± 0.92 Hz, Fig. 23D). Cacophony null mutants are embryonically lethal and could not be

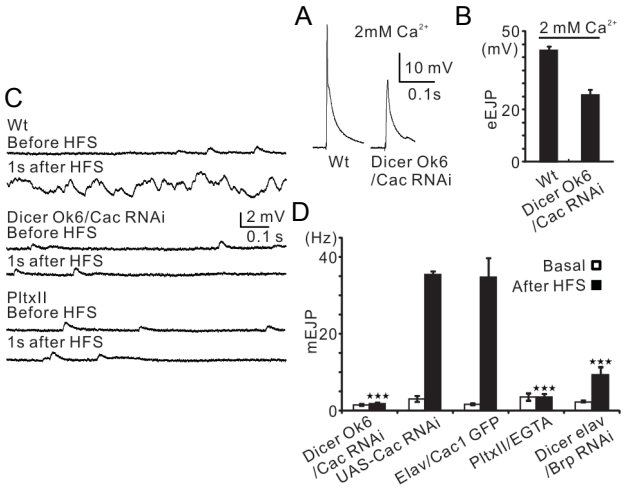

Figure 23. Down regulation of cacophony suppresses depolarization triggered vesicle release.

A. Representative traces of eEJP recorded in 2 mM Ca^{2+}. **B.** Histogram of the eEJP amplitude data in A showing that evoked release is reduced in motor neuron expression of Cac RNAi (n=6). **C.** Representative traces of the mEJP-frequency data recorded before and after HFS in 0 mM Ca^{2+}/1 mM EGTA containing buffer. **D.** Histogram of the mEJP-frequency data in C showing that depolarization triggered vesicle release is strongly reduced when cacophony is down-regulated by Cac RNAi (n=6) or Brp RNAi (n=5), and blocked by PltxII (n=5), but unaltered when it is overexpressed (n=5) or in control UAS-Cac RNAi (n=5). *** $p < 0.001$. Data represent means ± S.E.M. of the indicated number of preparations.

used in larval stages. We therefore chose to knock down cacophony expression in larvae by RNA interference, and the eEJP was reduced to 26.4 mV in 2 mM Ca^{2+} (Fig. 23A and B). Intriguingly, both treatments strongly suppressed depolarization triggered vesicle release to 3.52 ± 0.92 and 1.83 ± 0.41 Hz respectively (Fig. 23C-D), suggesting that cacophony function other than the voltage-dependent gating of Ca^{2+}-channel and Ca^{2+}-influx is necessary for initiating depolarization triggered vesicle release. In addition, we found that RNAi-mediated knockdown of Bruchpilot, an essential protein of the active zone (Wagh et al., 2006) that has been shown to be necessary for clustering VGCCs at the release site (Kittel et al., 2006), results in a

similarly suppressed depolarization triggered vesicle release (1.47 ± 0.18 empty bar and 9.08 ± 1.84 Hz filled bar, Fig. 23D). It therefore emerges that cacophony is indeed involved in triggering depolarization triggered vesicle release presumably by sensing changes of the presynaptic membrane potential with its voltage sensor domain that depletes the synaptic ATP and triggers a signaling cascade which is active if Ca^{2+} is very low or absent. This signaling mechanism appears to act directly or indirectly on the vesicular release machinery to transiently elicit long lasting vesicle release.

5.2.7 Depolarization triggered vesicle release recruits the same RRP vesicles as synchronous vesicle release

What is the identity of the vesicles released in depolarization triggered vesicle release? To assess this, we treated larval filet preparations with a blocker of vesicular proton pump Bafilomycin A1 (2 μM). As described above, BafA1-treated preparations showed a rapid decay of eEJP-amplitudes during continuous 30 Hz motor nerve stimulation (Fig. 24A) resulting in an apparent complete depletion of preexisting mature vesicles within 5 minutes of stimulation (0.81 ± 0.09 mV, Fig. 24A

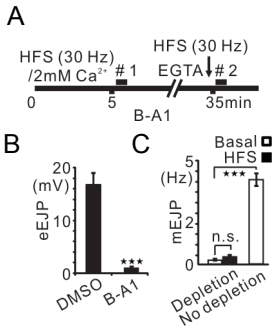

Figure 24. Same population of vesicles drive depolarization triggered vesicle release and synchronous release.

A. Schematic drawing of the experimental protocol used. Filet preparations were incubated for 5 minutes in the presence or absence of B-A1 in 2 mM Ca^{2+} containing buffer. Intracellular mEJP recordings (small bars #1) were then performed and followed with 30 minute incubation in the presence of B-A1/1 mM EGTA. Then HFS was applied for 5 s and continued with mEJP recordings (small bars #2). **B.** Histogram of the eEJP amplitude data in A showing that evoked release is totally depleted in the presence of B-A1 (n=5), but largely remained in the presence of vehicle DMSO (n=5). **C.** Histogram of the mEJP-frequency data showing that spontaneous release is strongly depleted after HFS (n=5) comparing to no HFS (n=5), and a further HFS can't induce vesicle release increase (n=5). n.s. $p > 0.05$; *** $p < 0.001$. Data represent means ± S.E.M. of the indicated number of preparations.

and B). The same stimulation in DMSO (1%) vehicle without BafA1-treatment

resulted in a small eEJP-depression (16.78 ± 1.12 mV Fig. 24A and B), indicating that the depletion of preexisting RRP vesicles. At this point of eEJP depletion mEJPs were still detectable (0.18 ± 0.04 Hz, lower trace in Fig. 24A), but at a significantly lower rate compared to that of Baf-A1 treated cells without stimulation (4.16 ± 0.32 Hz, Fig. 24D) or untreated cells (Fig. 14A). This basal mEJP rate of about 0.2 Hz did not show any change during or following 30 Hz nerve stimulation in NS (0 mM Ca^{2+}; 1 mM EGTA) (0.35 ± 0.05 Hz, Fig. 24D). Thus, depolarization triggered vesicle release seems to recruit vesicles from the same readily releasable vesicle pool as classical Ca^{2+}-dependent evoked vesicle release. This suggests that the release machinery that classically functions in an exquisitely Ca^{2+}-dependent manner can be switched by a voltage-dependent mechanism to an inversely Ca^{2+}-dependent mode of operation in which vesicles are transiently released in a spontaneous mode. We therefore reasoned that the triggering of depolarization triggered vesicle release requires at least two consecutive steps: a voltage sensor that is capable of sensing voltage changes thereby deplete synaptic ATP that in turn switches the release machinery of readily releasable vesicles from being Ca^{2+}-dependent and clamped to become unclamped and triggering vesicle fusion.

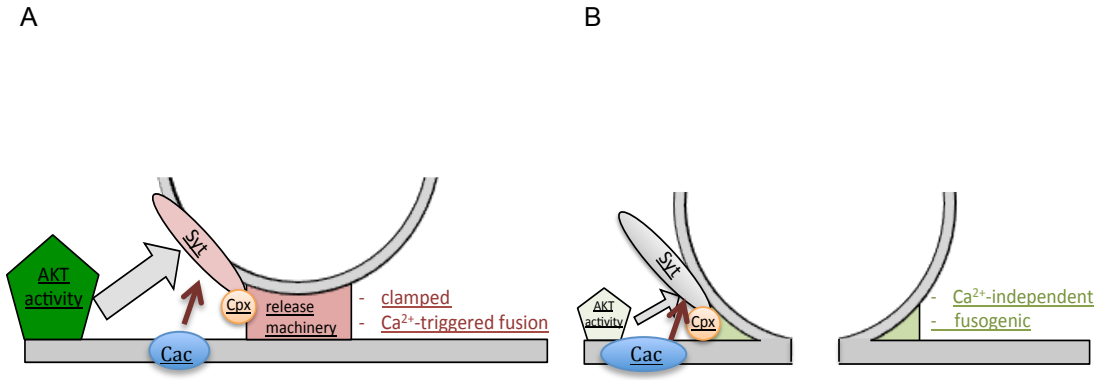

Figure 25. AKT switches between functional modes of the vesicle release machinery: a mechanism to dynamically regulate the function of individual vesicle release sites.

A. In the presence of Ca^{2+}, Syt phosphorylation by AKT signaling stabilizes the Syt1-Cpx dependent fusion clamp. In this "tight fusion clamp" configuration action potentials activates VGCC cacophony and leads to calcium influx at many release sites and hence a rapid synchronous and asynchronous release of many vesicles. In this transmission mode release sites participate in neuronal information processing. **B.** In the absence of calcium, down regulation of AKT most likely by reduced mitochondrial ATP-synthesis reduces the phosphorylation status of Syt via Cac and loosens the fusion clamp resulting in a fusogenic mode which vesicle tends to fuse spontaneously or be triggered by depolarization triggered vesicle release paradigm.

Taken together we have identified a novel Akt- and Syt-dependent switch in the

vesicle fusion machinery that regulates whether the release machinery is tightly clamped and calcium-sensitive and hence optimized for fast synchronous release (Fig. 25A) or whether it is in a calcium-insensitive fusogenic mode (Fig. 25B). The switch is controlled by the level of kinase activity of membrane-bound AKT at release sites and hence depends upon signaling pathways that affect the activity of AKT.

5.3 Disproportional depletion of vesicle release at high stimulation frequencies is due to limited vesicle diffusion

To examine whether the RRP vesicles can sustain the fast neurotransmitter release with the same efficiency under different firing intensities, we stimulated the larval nerves at different frequencies as in figure 26e. In the depletion essay, Baf-A1 was

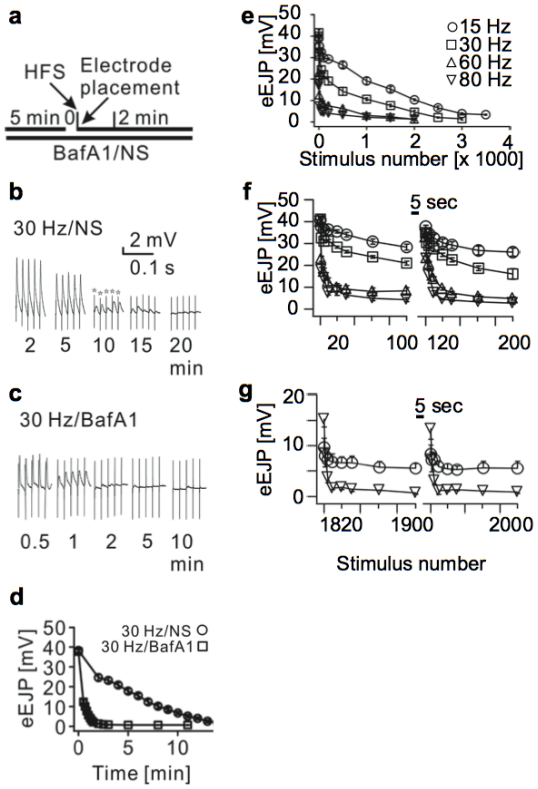

Figure 26. Disproportional depletion of eEJPs is due to limited availability of presynaptic vesicles.
a. Experimental design: larval filet preparations were pre-incubated in normal saline (NS) or NS containing 2 µM Baf-A1 to inhibit transmitter filling in recycling vesicles. Motor nerves were then continuously stimulated with a suction electrode at various frequencies (HFS) while we simultaneously recorded the membrane potential of the postsynaptic muscle fiber 6. **b-c.** Representative traces of these recordings taken at the indicated time-points of stimulation (stars: stimulation artifacts). **d.** Quantification of eEJP-depletion dynamics during continuous 30 Hz stimulation with and without Baf-A1 incubation. **e-g.** eEJP-depletion dynamics in Baf-A1 treated preparations caused by the indicated stimulation frequencies and expressed as a function of the stimulus number. (e) shows the eEJP-depletion dynamics during continuous long-term stimulation. In (f) and (g) stimulation trains of 100 stimuli were separated by pausing intervals of 5 seconds. Note, that the depletion of eEJP-amplitudes was disproportionally faster during 60 Hz and 80 Hz stimulations compared to 15 Hz and 30 Hz stimulations. Short pausing intervals resulted in an almost complete recovery of eEJP-amplitudes at the beginning of the experiment (f). In later phases of the experiment (g) the eEJP recovery was significantly higher in 80 Hz stimulated NMJs than in 15 Hz stimulated NMJs ($p<0.033$ at stimulus 1800 and $p<0.019$ at stimulus 1900). Data in d-g represent means ± SEM of 4 to 6 independent preparations.

applied shortly before the depletion begins. As expected, eEJP amplitude was reduced significantly faster during continuous 30 Hz nerve stimulation in BafA1-

treated larval preparations than in untreated preparations (Fig. 26 b-d). Therefore, BafA1 was efficient to block the refilling of the newly recycled vesicles, which is similar to temperature-sensitive mutants of dynamin (shibire^{ts}) (Siddiqi and Benzer, 1976) that all endocytic processes are inhibited at restrictive temperatures (Delgado et al., 2000) despite of the different mechanisms. In all the stimulation frequencies, eEJP amplitudes showed a fast initial decay and continuing with a slower decay after same number of stimuli (Fig. 26e). However, the evoked responses were significantly smaller in higher stimulation frequencies (60 and 80 Hz) comparing to lower stimulation frequencies (15 and 30 Hz) after the initial depletion (Fig. 26e), this unexpected phenomenon was unlikely due to desensitization of postsynaptic glutamate receptors, since the quantal amplitudes (mEJPs) were unaltered under all tested conditions (data not shown). Instead, a presynaptic limitation of vesicle supply most likely explains the disproportionally rapid decay of eEJP amplitudes at high stimulation frequencies. If so, one would expect that a short pausing interval allows vesicles from recycling pool or reserve pool to functionally reoccupy empty release sites after stimulation train resulting in recovered eEJP amplitudes. Indeed, when we used 'go-and-stop' experimental paradigm that the nerves were stimulated for 100 stimuli and then stopped for 5 seconds and repeated for 20 times, a fast and almost full recovery can be observed in all the stimulation frequencies (15-80 Hz) after 100 stimuli (Fig. 26f), however, eEJP amplitude recovery was significantly larger in boutons after 1800 stimuli at 80 Hz than at 15 Hz ($p<0.033$, $n=4$) or after 1900 stimuli ($p<0.019$, $n=4$). Both observations were consistent with the idea that vesicle motility was limiting the availability of mature vesicles at active zones. This leads to a rapid failure in reoccupying empty active zones with mature vesicles during higher frequency stimulation and thus to a rather small total number of released vesicles per train. Therefore, after prolonged stimulation periods, more vesicles were remained for the eEJP with higher stimulation frequency (triangle in Fig. 26g) and fewer vesicles were left during lower stimulation frequency (circle in Fig. 26g). Moreover, the *in silico* simulations of the vesicle depletion experiments approximated the experimental observations in that the simulated total amount of vesicular release decreased at various stimulation frequencies with similar time courses as in the *in vivo* experiments (detailed information is available in Fig. 4 of Knodel, 2013).

6 Discussion

Autism spectrum disorder is mostly a genetic disorder that occurs in about 1 out of 100 children typically with impairments in social interaction and communication, repetitive and stereotyped behavior (Baird et al., 2006). Mutations screened out a large amount of genes related to ASD, among which activity dependent genes and AKT/TSC signaling are crucial risk factors (review by Ebert and Greenberg, 2013). As well, Schizophrenia, showing symptoms of delusions including paranoia and auditory hallucinations, disorganized thinking reflected in speech, and a lack of emotional intelligence, occurs in 0.3-0.66% lifetime prevalence percent of the general population (McGrath et al., 2008). It has also been proved that AKT signaling plays a role in schizophrenia (Emamian et al., 2004). In the present study, we demonstrated AKT controlled the functional state of vesicle release machinery by the interfering Ca^{2+} channel Cacophony and synaptotagmin. In addition, we revisited the neurotransmitter release process and revealed an AKT switch in controlling cacophony mediated depolarization triggered vesicle release by sensing the local synaptic ATP levels. Finally, we showed that high frequency nerve stimulation led to a disproportional decay of evoked EJP amplitudes due to limited vesicle supply at the *Drosophila* larval NMJs, which provides experimental basis for three-dimensional *in silico* simulation.

6.1 AKT controls vesicle release machinery: a new mechanism for activity dependent induction of ASD

6.1.1 AKT regulating synaptic activity could be a potential mechanism for ASD

AKT is the hot spot of many biological subjects because of its central role in many diseases like ASD, cancer, diabetes and stroke. Our data indicated a direct AKT function in synaptic vesicle release regulation that hyperactivity of AKT clamps the releasing machinery tightly, leading to less willingness of spontaneous release, a suppression in depolarization triggered vesicle release, and reduced quantal content of synchronous release in low extracellular Ca^{2+} (0.5 mM, Fig. 17H) due to the tight clamp, but not due to smaller quantal size since the average mEJP amplitudes were comparable (~ 1mV) among different treatments (Fig. 17J). However, the eEJP

amplitude was the same as wild type in 2 mM Ca^{2+}, which implies the same amount of total RRP vesicles comparing to wild type. On contrary, AKT blockade resulting in more spontaneous release due to the loose clamping of releasing machinery and also reduction in evoked release, which is very likely due to the vacancy of RRP vesicles after prolonged intensive spontaneous vesicle release. Therefore, clamping of eEJP by AKT can be overcome by high Ca^{2+} concentration. Moreover, AKT regulating spontaneous vesicle release is evolutionarily conservative, as we observed AKT blockade also strongly enhance both excitatory and inhibitory spontaneous release, as well as eliminating the spontaneous neural network bursting in hippocampal neuron culture (Data not shown). Interestingly, a similar application of LY-294002 on frog NMJs induces a Ca^{2+}-independent increase in spontaneous neurotransmitter release which is induced by CK2 inhibition and blocks membrane cycling in a PI3K dependent way (Rizzoli and Betz, 2002). We ruled out the function of CK2 in *Drosophila* by treatment with 300 μM DRB and convinced the effect of PI3K in such regulation. This discrepancy might be the differences between species that the neurotransmitter is ACh in frog NMJs but glutamate in *Drosophila* NMJs.

By comparison, diverse synaptic phenotypes have been observed by the modulation of AKT and its relevant signaling. Consistent with our observations at *Drosophila* NMJs, AKT activation through DmGluRA results in a decreased neuronal excitability (Howlett et al., 2008). In heterozygous loss-of-function mutation $Tsc2^{-/-}$ mouse, late LTP is enhanced in CA1 region as Tsc is negative regulator of protein synthesis. However, spatial learning is impaired accompanying with abnormal brain enlargement in the same mice (Ehninger et al., 2008). On contrary, mGluR-LTD is abolished in the same mutant, together with less protein synthesis but enhanced AMPA and NMDA receptor mediated synaptic function (Bateup et al., 2011; Benjamin et al., 2011). Similarly, synapses of dentate granule cells of PTEN KO mouse, theta burst-induced-LTP is facilitated only in young mice but impaired in old mice and deficient mGluR-LTD occurs during all the ages (Takeuchi et al., 2012). Despite the intriguing data on synaptic function of AKT related signaling, how AKT regulation of synaptic activity influencing ASD needs to be clarified in the future study.

6.1.2 Synaptotagmin phosphorylation by AKT signaling switches functional state of release machinery

The function of synaptotagmin and complexin in neurotransmitter release has been investigated in depth in recent years, e.g. vesicle docking and priming, SNAREs oligomerization, with clamping the SNAREpins as the primary function (Huntwork and Littleton, 2007; Xue et al., 2008; Malsam et al., 2012; Südhof, 2012). In our study, KD of *Drosophila* complexin by RNAi results in massive increase in spontaneous release and a reduction in eEJP amplitude comparable to complexin KO (Huntwork and Littleton, 2007) and overexpression of *Drosophila* complexin suppresses spontaneous release. In contrary, overexpressing mouse and rat complexin strongly enhances basal spontaneous release as the identified facilitatory effect of mammalian complexin (Xue et al., 2008). However, despite of the respective inhibiting or facilitating effect on basal spontaneous release of *Drosophila* and mammalian complexin, after 30 s 5 Hz voltage stimulation, depolarization triggered vesicle release of both animals arrives at the same plateau level ~ 40 Hz. This strongly indicates the in total clamping effects of both complexins and the same amount of total vesicles can be zippered by complexins in depolarization triggered vesicle release.

As expected, KD of Dsyt by RNA interference reduces evoked synchronous release, accompanying by increased asynchronous release and spontaneous release. However, the depolarization triggered vesicle release is inhibited as the arrival of significantly lower plateau which indicates fewer synaptic vesicles than wild type can be released by depolarization when the amount of Syt is reduced. Surprisingly but consistently, AKT blockade reduces rather than enhances spontaneous vesicle release in Dsyt KD mutants but not in Dcpx KD mutants, which indicated Dsyt is a downstream signaling molecule in AKT regulated spontaneous release. How can syt trigger vesicle release in depolarization triggered vesicle release? In *Drosophila*, point mutations of Ca^{2+}-binding site of C2A and C2B (D282/284N in C2A and D416/418N in C2B) dramatically increases spontaneous release in a Ca^{2+}-independent manner, which might due to the conformational changes in the Syt1 structure (Lee et al., 2013; Fuson et al., 2007). Thus, it's intriguing to propose AKT signaling phosphorylation of Syt changes Syt conformation which kicks complexin out and releases the synaptic vesicles. Ca^{2+} binding to Syt seems to dominantly maintain the AKT phosphorylation site and synchronous release, because in the presence of Ca^{2+} no depolarization triggered vesicle release can be observed but with unaltered evoked response upon the arrival of depolarization, and the evoked release was only partially reduced even with AKT

blockade. The phosphorylation site of Syt is yet unclear and needs to be clarified in further study. In addition, during BAPTA-AM loading, the preparation was first incubated in 20 mM Mg^{2+} containing HL3 buffer, which resulted in a total inhibition of depolarization triggered vesicle release in addition to eEJP blockade (data no shown). To exclude the inhibition of depolarization triggered vesicle release is due to high Mg^{2+} or the difference between HL3 and normal saline buffer, we investigated depolarization triggered vesicle release in 2 mM Mg^{2+} containing HL3 buffer and observe the same depolarization triggered vesicle release as in normal saline (data not shown). Thus, high Mg^{2+} can inhibit depolarization triggered vesicle release, but the underlying mechanism is unknown yet. One explanation might be the binding of Mg^{2+} with Syt1 stabilizes the conformational structure of Syt1 and prevents depolarization triggered signaling (Sutton et al., 1999). Another explanation is high intracellular Mg^{2+} alters the electrostatic equilibrium of Syt1 and leads to the conformational change which is unaccessible to depolarization triggered signaling, as the Syt triggered fusion process is largely dependent on the intracellular ionic strength (Van den Bogaart et al., 2011). Therefore, AKT signaling phosphorylation of Syt determines the state of release machinery, which can be switched between synchronous release, depolarization triggered vesicle release and spontaneous release.

6.2 Depolarization triggered vesicle release is mediated by AKT via sensing local ATP level

6.2.1 AKT regulating synaptic activity depends on local ATP concentration

AKT clamping of releasing machinery can be regulated by presynaptic ATP level, as ATP binding determines the phosphorylation state of AKT (Lin et al., 2012). Indeed, the depolarization triggered vesicle release is further increased after 15-30 minutes incubation of larval fillet preparation in nominally Ca^{2+} free buffer compared to 5 minutes incubation (Fig. 19F), and longer incubation (90 minutes) resulted in enhanced spontaneous release without voltage stimulation (~ 40 Hz). An outcome of longer incubation in the absence of Ca^{2+} might be the reduction in the presynaptic mitochondria Ca^{2+}, which leads to a less ATP production and weakened AKT clamp of the releasing machinery. In the cellular system, AKT binding to ATP stabilizes its conformation in which both phosphorylated sites (threonine 308 and serine 473) were

inaccessible to phosphatases; otherwise adenosine 5′-diphosphate (ADP) binding increases the accessibility to phosphatases (Lin et al., 2012). In addition, long-term treatment (2 hours) in the presence of 0.5 mM Ca^{2+} or 30 minutes incubation in nominally Ca^{2+} free buffer with oligomycin, which depleted cellular ATP, also resulted in enhanced vesicle release (Fig. 18A). Therefore, the local ATP level can be sensed by AKT which in turn regulating synaptic activity.

6.2.2 Inversely calcium dependency of depolarization triggered vesicle release

Our data show that 30 Hz electronic shock rapidly increases vesicle release to 12.60 ± 1.07 fold within 5 seconds in the absence of Ca^{2+}, and can be suppressed when increasing Ca^{2+} concentration by either incubation in higher extracellular Ca^{2+} or thapsigargin (as in Fig. 19E). In the meantime, the postsynaptic membrane potential is hyperpolarized to around -40 mV (compared to -50 mV in resting), and the baseline also becomes shaky and not easily identifiable. Till now, Ca^{2+} is convinced to be essential component in most of the transmitter release. In synchronized release, extracellular calcium influxes into the AZs (Kittel et al., 2006) and sensed by Syts is crucial. Asynchronous release very often occurs and prospers during and after stimulus trains, which elevate the presynaptic residual calcium in a relatively longer time window than in synchronous release. In spontaneous release, it is thought to be random or accidental events that the predocked presynaptic vesicles fuse to the presynaptic membrane. Most studies in both excitatory and inhibitory synapses also show this random events varied dramatically corresponding to the extracellular or presynaptic calcium manipulations (Llano et al., 2000; Lou et al., 2005; Xu et al., 2009). The calcium sensors for synchronous release (Syt1, 2 and 9) are very well identified and characterized by their different Ca^{2+} binding kinetics, however the calcium sensor for spontaneous release still remains to be clarified because a clear reduction in spontaneous release resulting from the Ca^{2+} sensor KO is lack although some studies indicate Syt1 and Doc2 are the right molecules for such function (Xu et al., 2009; Groffen et al., 2010). For a long time, calcium sensor for asynchronous release has been hard to be identified because the dependency of asynchronous release on synchronous release albeit many studies show its existence, until recently Doc2 is confirmed as a Ca^{2+} sensor for asynchronous release (Yao et al., 2011). Our results from *Drosophila* NMJs show spontaneous

release rate is not dependent on Ca^{2+} (Fig. 14A) although long term chelating of intracellular Ca^{2+} has the tendency to reduce the spontaneous release rate, which is consistent with the previous observation (Lee et al., 2013). In unphysiological high Ca^{2+} concentration (8 mM), mEJP amplitude was smaller than in lower Ca^{2+} concentration, which might due to the desensitization of postsynaptic glutamate receptors.

By comparison to calcium dependent release, only a few treatments have been demonstrated to enhance transmitter release without calcium participating. For example, hypertonic potentiation of transmitter release (Hubbard et al., 1968; Kashani et al; 2001), can release "readily releasable" pool of vesicles, which is only a small proportion of the whole vesicle pools, in a Ca^{2+} independent manner (Stevens and Tsujimoto, 1995; Rosenmund and Stevens; 1996). In addition, ruthenium red mediated exocytosis of predocked vesicles is directly triggered by ruthenium red binding to the presynaptic membrane, a process that is inhibited by heparin, which also only affects "readily releasable" pool of vesicles (Trudeau et al; 1996; Sciancalepore et al., 1998; Congar and Trudeau, 2002). Thus, inversely calcium dependent depolarization triggered vesicle release is a new spontaneous release regulating mechanism which functions through ATP/AKT clamp.

6.2.3 Depolarization triggered vesicle release is independent of stimulation frequency

Voltage stimulation starts to facilitate vesicle release with an accumulation of more than 20 stimuli (1ms for 1 stimulus) and depolarization triggered vesicle release maximize at 100-120 stimuli in nominally Ca^{2+} free buffer (Fig. 20C). Consistently, when we depolarized the presynaptic motor neurons by applying extracellular high KCl (40 mM) buffer made by replacing the equivalent amount of NaCl to keep all the solutions isotonic, a fast increase of vesicle which maximizes within several seconds and remains for more than 30 minutes can be observed (data not shown). Different from synchronous and asynchronous release, which are immediate intensive vesicle release (within ~1-3 and ~100 ms) triggered by Ca^{2+} binding to Syt after AP invading the presynaptic terminals, depolarization triggered vesicle release activation requires a minimum summation of depolarization duration for about 20 ms.

In addition, 2 and 10 Hz stimulation showed no difference in the onset of depolarization triggered vesicle release, which indicated its dependency on total

stimuli number rather than stimulation frequency. By comparison, in evoked synchronous release depletion of RRP vesicles requires the refilling of the empty sites by recruiting RP vesicles, where a minimum stimulation frequency of 30 Hz is needed (Kuromi and Kidokoro, 2000) and accumulation of stimulus seems to be invalid. Moreover, the vesicle release decays relatively faster in 2 Hz than 10 Hz stimulation, although both of them arrive at basal level within ~ 120 s after the cessation of stimulation in both stimulation frequencies. This suggests each AP-induced secondary signal has a defined half-life so that "old" secondary signals already start to decay earlier following the end of the long 2 Hz stimulation period compared to the decay following a short 10 Hz stimulation. It also implies the existing of a homeostatic mechanism underlying depolarization triggered vesicle release related signaling which is most likely the tilting between phosphorylation and de-phosphorylation in AKT/Syt signaling.

Depolarization induced vesicle release potentiation is also reported in other species. In superior cervical ganglion neurons, AP trains can enhance hypertonicity induced vesicle release independent of Ca^{2+}, through the direct interaction between synprint site of N-type calcium channel and the SNARE proteins (Mochida et al., 1998). Whereas, the explanation why hyperosmotic sucrose is necessary for the phenomenon and a direct effect of depolarization on vesicle release are missing. Moreover, the synprint-SNAREs interaction doesn't apply for *Drosophila*, because such a synprint site is lack. Furthermore, membrane capacitance can be increased by depolarization in DRG neurons, but with limited characterization and mechanism analysis (Zhang and Zhou, 2002). Hence, voltage stimulation can trigger depolarization triggered vesicle release by accumulating single voltage signal irrelevant of stimulation frequency.

6.2.4 Cacophony is the voltage sensor for depolarization triggered vesicle release

The findings that both pharmacological blockade and genetically KD of cacophony channel almost totally suppress depolarization triggered vesicle release, as well as Brp KD potently reduces depolarization triggered vesicle release, indicate N-type calcium channel cacophony is the voltage sensor for depolarization triggered vesicle release signaling. Why depolarization triggered vesicle release was not partially suppressed since the eEJP amplitude of Cac RNAi mutant is slightly higher than half

of wild type in the presence of 2 mM Ca^{2+}? One should be aware that the percentage of Cac KD is not linearly corresponding to the eEJP reduction, as we can see the mean eEJP amplitude is around 20 mV in 0.25 mM Ca^{2+}. Therefore, the estimated leftover Cac is close to 12.5-25% which is consistent with the observed suppression of depolarization triggered vesicle release. Voltage regulation of neurotransmitter release has also been continuously studied by Parnas, who proposed a Ca^{2+}-voltage hypothesis that GPCR activity modifies Ca^{2+} triggered synchronous release and exampled five voltage sensitive GPCRs like M2-muscarinic receptor and mGluR (Parnas and Parnas, 2007). We also knocked out *Drosophila* mGluR, and didn't see the blockade of depolarization triggered vesicle release (data not shown). Therefore mGluR does not serve as voltage sensor for depolarization triggered vesicle release. How can voltage be sensed by cacophony? Structural analysis of calcium channel indicates the existence of voltage sensor in the fourth transmembrane domain (Yu et al., 2005), which most likely senses voltage change and leads to conformational change of calcium channel. In addition, a direct link between Cacophony and Syt might exist, which is important in AKT regulated vesicle release as PltxII reduced the spontaneous vesicle release induced by AKT blockade (Fig. 15C and D). Nevertheless, the intermediate interaction or signaling between cacophony and AKT/Syt remains to be clarified.

6.2.5 Depolarization triggered vesicle release uses the same vesicles as synchronous release

Which vesicles are employed by synchronous and spontaneous releases is long time debating topic with controversial opinions: some researchers support the same vesicles drive both releases (Prange and Murphy, 1999; Groemer and Klingauf, 2007; Hua et al., 2010; Wilhelm et al., 2010); the others are against that (Sara et al., 2005; Fredj et al., 2009). In *Drosophila* NMJ, studies show the same vesicles drive both releases (Wilhelm et al., 2010). In our study, after the depletion of vesicles for evoked release in B-A1, a release rate of 0.2 Hz is still observable and depolarization triggered vesicle release is eliminated. This suggests the existing of small pool of vesicles which only fuse and recycle spontaneously in addition to the vesicles shared by synchronous and spontaneous releases which lost most of spontaneous vesicle release after depletion. Moreover, depolarization triggered vesicle release and AKT blockade induced vesicle release were also totally eliminated by eEJP depletion

indicates a same pool of vesicles supply for all the synchronous, spontaneous and depolarization triggered vesicle release, as well as AKT blockade induced vesicle release. Thus, we hypothesize that presynaptic vesicles can be separated into a primary pool of vesicles involved in evoked release, spontaneous and depolarization triggered vesicle release, as well as AKT blockade induced vesicle release, and a small pool of vesicles only involved in spontaneous release.

6.3 Vesicle diffusion limits the availability of preexisting vesicles at active zones

Our experimental data revealed that vesicle motility is a limiting factor for reduced eEJP amplitude during HFS, and mathematical simulations fit very well to the biological results (see Knodel et al., 2013). Nevertheless, it was quite debating whether active transport system like Myosin/Actin is involved in synaptic transmission (Tokuoka and Goda, 2006; Seabrooke et al., 2010; Seabrooke and Stewart, 2011; Kisiel et al., 2011). In the present study, we simplified the vesicle release process by specifically blocking vesicle recycling with Baf-A1, therefore only the mobility of preexisting vesicles was investigated during high frequency neurotransmission (15 - 80 Hz). With these treatments, the eEJP depletion during HFS can be explained by the vesicle diffusion limitation induced shortage of synaptic vesicles in release site, but with no necessity of an active transport system. By comparison, the same HFS triggered longer lasting eEJP response in the absence of Baf-A1 indicating vesicle recycling contributes mostly in prolonged nerve stimulation. Hence it is very likely that active transport mechanisms are needed in vesicle recycling and refilling to AZs during HFS. Obviously, both passive and active vesicle transport mechanisms are present in boutons of *Drosophila* NMJs, how they interact and contribute to vesicle transportation in natural activity needs to be further clarified. According to our experimental results that vesicle diffusion is a major limiting factor in refilling AZs, we further studied how it affects synaptic output under different stimulation paradigms. During HFS, Local vesicle concentration reduced quickly resulting in smaller or failures of eEJPs. In a given release site, synaptic activity largely relates to its release probability, stimulation frequency and RRP vesicle size. How can bouton size shape these synaptic parameters? With the help of *in silico* simulations, we presume that a large type Ib bouton is more suitable for long-lasting high frequency stimulation with its larger number of low P_0-release sites, whereas a small type Is bouton with

fewer high P_0-release sites is improper for such transmission but maybe important in initiating a firing train. Indeed, in many synaptic depression assays, a fast reduction of evoked response can be observed before the arrival of plateau. It seems that Is and Ib are not fired in the meantime *in vivo*, while Is bouton is most likely first fired with a low stimulus frequency or with only a few stimuli. In contrary, Ib bouton is responsible for neurotransmission in longer lasting high frequency stimulation patterns. These predictions matched the natural firing patterns of both motor neurons, indicating a well-adapted synaptic properties in larval NMJ.

6.4 Conclusions

The present work clarifies a so far unknown key function of AKT in switching the functional state among synchronous release, spontaneous release and depolarization triggered vesicle release and implies a potential role of AKT in ASD etiology. In addition, it also unravels a voltage stimulation induced inversely Ca^{2+} dependent depolarization triggered vesicle release and its underlying mechanism that depolarization signal is sensed by N-type VGCC cacophony, then transduced to AKT/Syt-Cpx controlled releasing machinery.

Moreover, three-dimensional functional model allows us to dissect the physiological meaning of experimentally inaccessible parameters like the bouton size, vesicle diffusion and the release probability. In all, our data enriches the present knowledge about vesicle release and synaptic role of AKT, and also provides new avenues for therapy design for autism spectrum disorder and schizophrenia as well sets the framework for a theoretical reconstruction and functional dissection of further realistic parameters such as vesicle recycling, the membrane potential and its dynamics during stimulation, etc.

7 Abbreviations

ACh	Acetylcholine
ADP	Adenosine 5′-diphosphate
AKT	Protein kinase B
AP	Action potential
ASD	Autism spectrum disorder
ATP	Adenosine triphosphate
AZ	Active zone
Baf-A1	Bafilomycin A1
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)
CaM	Calmodulin
CaMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CaSR	Calcium-sensing receptor
CCK	Cholecystokinin
CK2	Casein kinase II
CNS	Central nervous system
Cpx	Complexin
DFak	Drosophila focal adhesion kinase
DMSO	Dimethyl sulfoxide
Doc2	double C2 domain
DRB	5,6-dichlorobenzimidazole riboside
DRG	Dorsal root ganglion neurons
eEJP	excitatory junctional potential
EGTA-AM	ethylene glycol tetraacetic acid (acetoxymethyl ester)
EM	Electron microscopy
GABA	Gamma-aminobutyric acid
GOF	Gain of function
GFP	Green fluorescent protein
GPCR	G-protein-coupled receptor
HFS	High-frequency stimulation
HL3	Hemolymph-like ringer's 3.1
IP3	Inositol triphosphate

KD	Knock down
KO	Knock out
LOF	Loss of function
LTD	Long-term depression
LTP	Long-term potentiation
M2R	M2-muscarinic receptor
MAPK	mitogen-activated protein kinase
mEPP	miniature end plate potential
mGluR	Metabotropic glutamate receptor
mIPSC	miniature inhibitory postsynaptic current
myr-Akt	myristoylated-Akt
NCX	Na^{+}-Ca^{2+} exchanger
NMDA	N-methyl-D-aspartic acid
NMJ	Neuromuscular junction
NS	Normal saline solution
NSF	N-ethylmaleimide–sensitive factor
PAR	Prolonged asynchronous release
PBS	Phosphate buffered saline
PDK1	3-phosphoinositide dependent protein kinase-1
PH	Pleckstrin homology
PI3K	Phosphoinositide-3 kinase
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
Pltx II	Plectreurys toxin II
PKA	Protein kinase A
PKC	Protein kinase C
PPF	Paired-pulse facilitation
PTEN	Phosphatase and tensin homolog
PV	Parvalbumin
PVDF	Polyvinylidene fluoride
RNAi	RNA interference
RP	Reserve pool
RRP	Readily releasable pool
RyR	ryanodine receptor
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SNAP	Soluble NSF attachment protein

SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor protein
SpH	Synapto-pHluorin
STED	Stimulated emission depletion microscopy
Syt	Synaptotagmin
TEV	Tobacco etch virus
TORC	Target of rapamycin complex
TSC	Tuberous sclerosis complex
UAS	Upstream Activation Sequence
VAMP7	Vesicle-associated membrane protein
VGCC	Voltage-activated calcium channel
VGLUT1	Vesicular glutamate transporter 1
Vti1a	Vps10p-tail-interactor-1a

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Publications

Phd

L. Ge, Y. Leung, D. Parisotto, T. H. Söllner, C. M. Schuster. AKT activity controls functional status of vesicle release machinery. (in preparation)

L. Ge, Y. Leung, C. M. Schuster. AKT regulates depolarization triggered vesicle release by sensing the local ATP level. (in preparation)

M. M. Knodel, R. Geiger, **L. Ge**, D. Bucher, A. Grillo, G. Wittum, C. M. Schuster & G. Queisser. Synaptic Bouton Properties Are Tuned to Best Fit the Prevailing Firing Pattern. (in submission)

Master

Zhang XJ, **Ge LH**, Liu J, Yang XL. Functional expression of the glycine transporter 1 on bullfrog retinal cones. Neuroreport. 2008 Nov 19;19(17):1667-71

Ge LH, Lee SC, Liu J, Yang XL. Glycine receptors are functionally expressed on bullfrog retinal cone photoreceptors. Neuroscience. 2007 Apr 25;146(1):427-34

Acknowledgement

Here is the end of my Phd journey, which started at the time I touched the ground of Frankfurt with exciting and uncertainty in the spring of 2008. This is not a lonely journey. It's an adventure where I knew and got accompanied by numerous people, who supported me, helped me and encouraged me. Hereby it is a pleasant time to say my gratitude to all those who made this thesis possible and an unforgettable experience for me.

First and foremost, my deepest homage goes to my guide, Prof. Dr. Christoph M. Schuster, for his kindness, patience and guidance. Under his supervision, I overcame different hard times and made crazy ideas feasible with great confidence. With his help, I acquired a broader sight in research, a more cautious attitude towards observation and a more relaxing way to do science. With his company, scientific discussion is no more profound, but a funny chat during lunch. With his guidance, research is no more tedious work. It's an inspiring game where you will get yes or no.

I also thank my second supervisor, Prof. Dr. Hilmar Bading, for the helpful discussions and support during my study.

My special thanks to YeungYeung Leung and Daniel Parisotto for their contribution to some of the crucial investigation, and also to the people I spent every working day with, my colleagues, Yon Leefmann, Lucas Vicuña, Daniel Bucher, Emilia Sancho Vargas, Franziska Bender, Ming Zou, Matthias Heindorf, Salskia Vernaldi, Jean Louis Thomas, Jan-Niklas Hörtzsch, Mirjam Senges, Eckehard Freitag, Tairi Aljand, Max Ross, Maria Müller, Eliana Nachman, Carmen Schäfer, Sofie, Innes Schmierer and Sara Khouaja. It's you who helped me to get used to the life in Germany and make my stay in Germany pleasant. Also thank Peter Bengtson for his help with in depth electrophysiology knowledge and Desiree Ditzel, Romina Geigar, Christian Schütz and for their help with German translation.

It's essential to thank Prof. Thomas Söllner for his insightful discussion and help, which bring the project forward. Additional thanks to Prof. Stephan Schneuwly, Prof. Chun-Fang Wu, Richard W. Ordway, Prof. Dr. Stephan Sigrist, Prof. Bruce Edgar and Prof. K. VijayRaghavan for their generous offering of the flies, and also Prof. Hugo J. Bellen for the precious antibody.

I take this opportunity to sincerely acknowledge my landlord and also good friend, Barbara Engelmann von Carnap, who kindly accommodated me before I slept

on the street and helped me in many facets. And Ekkehard Werner, thanks a lot for your help at the beginning of my Deutschland trip, life could have been much boring and hard without knowing you. As this is in Germany, language is always an obstacle for non-German speaking students like me. Therefore I want to thank all my German teachers and those who motivated and practiced German with me. You brought me from oriental culture into western culture, broke the cold glass in front of me and made my life more enjoyable in Germany.

I will never forget to say to the friends, with whom we cooked, ate, drank, traveled and played, you are the memory of fun, relaxing, help whenever needed and good times. It won't be only a short-term memory but a memory with abundant protein synthesis that will never be extinct.

Last but not least, I want to thank my beloved family. I know my stay in Germany is also hard for you too. The long distance made my visits fewer, however the distance doesn't make your love less. You always worried about my life here, supported and courage me. May all the healthiness, fortune and joy come along with you!