Changing the copy number of transmitter transporters per vesicle - sorting versus expression under regime of day-night cycle

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Mahesh Darna

from Metpalli, India

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1. **Gutachter: Prof. Dr. Gudrun Ahnert-Hilger**

2. **Gutachter: Prof. Dr. Volker Haucke**

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To

My beloved parents, brothers and

My dearest wife

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1 INTRODUCTION

1.1 Synaptic neurotransmission

The central nervous system is composed of more than 100 billion neurons, each in turn forming from a few hundred to as many as 200,000 synaptic connections. Synapses are specialised junctions, through which neurons communicate with each other. Such a huge collection of inter-connected neurons make a neural network. Each neuron receives as many as 15,000 connections from other neurons. A neuron transports its information by way of a nerve impulse. When a nerve impulse arrives at the synapse, it releases neurotransmitters, which influence adjoining neurons, either in inhibitory or excitatory way. The basic principle of neural work is neural summation, i.e., the excitatory and inhibitory post synaptic responses that summate in the cell body. If the depolarisation of the neuron at the axon is above threshold, it will create a new action potential at its axon hillock, and the signal is transmitted to the next neuron, resulting in an experience or an action. This sort of neuronal transmission is also called synaptic transmission. Once nerve cells receive the neurotransmitters, exchanging them from the pre-synaptic neuron or terminal button to the post-synaptic neuron or dendrite of the second neuron and send it back out to several neurons. They do the same and create a wave of energy until the pulse has made its way across an organ or specific area of neurons.

The synapse plays a pivotal role in the processes of learning and memory formation. Information transfer should be translated into signals in accordance to the need of the organism. Synapses are perfect entities to control this signal transmission. Each synapse is individually regulated and shows selective action. A synapse can also modulate the strength of its transmission. This kind of ability to modify the strength of transmission is called "synaptic plasticity" which is thought to be the cellular basis of the brain's ability to compute, learn and remember. At the synapse, information transfer changes from an electrical to a chemical mechanism involving the release of small chemical messengers called neurotransmitters from the pre-synaptic terminal.

Classic neurotransmitters such as acetylcholine and amino acid transmitters are stored in uniform organelles known as small synaptic vesicles. Typically, when an action potential travelling along an axon arrives at the nerve terminal, voltage-gated calcium ion channels open and cause an influx of calcium ions. This influx triggers the fusion of synaptic vesicles (SVs)

with the plasma membrane, thus enabling the neurotransmitters to be released into the synaptic cleft, where they diffuse and subsequently bind to and activate specific receptors on the postsynaptic cell membrane. Subsequent to neurotransmitter release, SVs are endocytosed and reloaded with neurotransmitter for another round of exocytosis. These cycles are repeated many times and can function independently of the nerve cell body, thus making the synapse an autonomous unit containing all elements necessary for vesicle exocytosis and recycling. Yet, the long-term changes involved in memory formation are coupled to changes in protein synthesis. Thus, although the synapse can function autonomously, synaptic plasticity requires communication of the synapse with the cell body and vice versa.

1.2 The pre-synaptic terminal

A typical pre-synaptic button is a specialized portion of the axon. It is characterized by an active zone, a region where the pre-synaptic plasma membrane comes in close contact with the post-synaptic plasma membrane and an associated cluster of vesicles (De Camilli et al., 2001). All pre-synaptic functions either directly or indirectly involve SVs. In a neuron, SVs are uniformly small (~20 nm radius) abundant organelles whose only known function is to take up and release neurotransmitters. SVs are made of lipid bilayer from endocytic vesicles and from endosomes. It could be said that SVs have unique proteins in their membranes and this mode of selective sorting of proteins is not well understood. The vesicles are essential for the propagation of nerve impulses between neurons and are constantly recreated by the cell. Purified vesicles have a protein: phospholipid ratio of 1:3 with a remarkable lipid composition (40% phosphatidylcholine, 32% phosphatidylethanolamine, 12% phosphatidylserine, 5% phosphatidylinositol, 10% cholesterol, wt/wt; Takamori et al., 2006).

Regulated synaptic transmission depends on several factors that affect the release probability of SVs. Mobilisation and release probability of SVs in turn depend on several factors, like protein-protein interactions (reviewed by Van der Kloot, 1991). SVs are characterized by a large number of proteins that participate in several protein-protein interactions while the amount of neurotransmitter released depends on the expression levels of transporters (Wang et al., 1997). Biochemical studies on purified SVs have identified integral and peripheral associated major proteins. More than 1000 proteins function in the pre-synaptic nerve terminal, and hundreds are thought to participate in exocytosis. Many proteins that have been associated with SVs are

probably present only on a subset of vesicles or bind transiently to the vesicles, and the number of proteins that are constitutive parts of all SVs may be comparatively small (less than 50). Therefore, to understand the physiological properties of synaptic vesicle cycle and pre-synaptic plasticity, it is important to understand the functions of various protein-protein interactions and neurotransmitters residing in the vesicles.

Various biochemical studies proved a direct relation of SVs number to the amount of neurotransmitter stored and released. The biogenesis, trafficking and regulation of the multiple types of vesicles containing molecules that function in neurotransmission are distinct (Bean et al., 1994; Calakos and Scheller, 1996) and are independent of the nerve cell body. Communication between cells reaches its highest degree of specialization at chemical synapses. Some synapses talk in a 'whisper'; others 'shout'. The 'louder' the synapse, the more SVs are needed to maintain effective transmission, ranging from a few hundred (whisperers) to nearly a million (shouters). However, it has been reported that SVs in the nerve terminal are distributed between a numbers of different pools called synaptic vesicle pools.

1.2.1   Synaptic vesicle pools

SVs participate in a cycle (release/recycling) that permits them to be repeatedly used during sustained activity and hence a need for different vesicle pools. Also, reports identified that vesicle populations play distinct roles in neurotransmission. The study of vesicle pools began with the work of Birks and Macintosh (1957); and Liley et al., 1953. They proposed that there were two distinct pre-synaptic stores of transmitter a 'readily releasable' fraction, which is rapidly depleted at high frequencies of stimulation, and a 'non readily releasable' fraction. Later, Elmqvist and Quastel (1965) proposed the concept of three vesicle pools: A reserve pool that makes up 80–90% of the total pool, the recycling pool which is significantly smaller and readily releasable pool (RRP) consists of a few vesicles that seem to be docked and primed for release.

The reserve pool is defined as a depot of SVs from which release is only triggered during intense stimulation. The release of these vesicles requires stimulation frequencies of at least 5–10 Hz in frog neuromuscular junction (NMJ) (Delgado et al., 2000) and 30 Hz in *Drosophila* larval NMJ (Kuromi and Kidokoro, 2000). After a lot of research on these pools, it was suggested that depletion of recycling pool vesicles triggers reserve pools mobilization and release, although the underlying molecular mechanisms remain unclear.

The recycling pool is the pool of vesicles that maintain release on moderate (physiological) stimulation. This pool is thought to contain about 5–20% of all vesicles. Physiological frequencies of stimulation cause it to recycle continuously (Richards et al., 2003; Hanse and Gustafsson, 2001), and it is refilled by newly recycled vesicles.

The readily releasable pool (RRP) consists of SVs that are immediately available on stimulation. These vesicles are generally thought to be docked to the pre-synaptic active zone and primed for release, although docked vesicles are not all necessarily immediately releasable. The RRP is depleted rapidly by 5–15 shocks of high frequency electrical stimulation (Elmqvist and Quastel, 1965), a few milliseconds of depolarisation (Mennerick and Matthews, 1996) or about 1 s of hypertonic shock.

SVs contain two classes of obligatory components: one, transport proteins involved in neurotransmitter uptake, and second, trafficking proteins that participate in SV exo- and endocytosis and recycling. Transport proteins include a vacuolar-type proton pump (V-ATPase) that generates the electrochemical gradient, which fuels neurotransmitter uptake and neurotransmitter transporters that mediate the actual uptake. The trafficking proteome of SVs is complex. It includes intrinsic membrane proteins; proteins associated via posttranslational lipid modifications, and peripherally bound proteins. These proteins do not share a characteristic that would make them identifiable as SV proteins, and little is known about how these proteins are specifically deposited into SVs. As summarized in Figure 1, many but not the entire known synaptic vesicle proteins interact with non-vesicular proteins and are linked to specific functions.
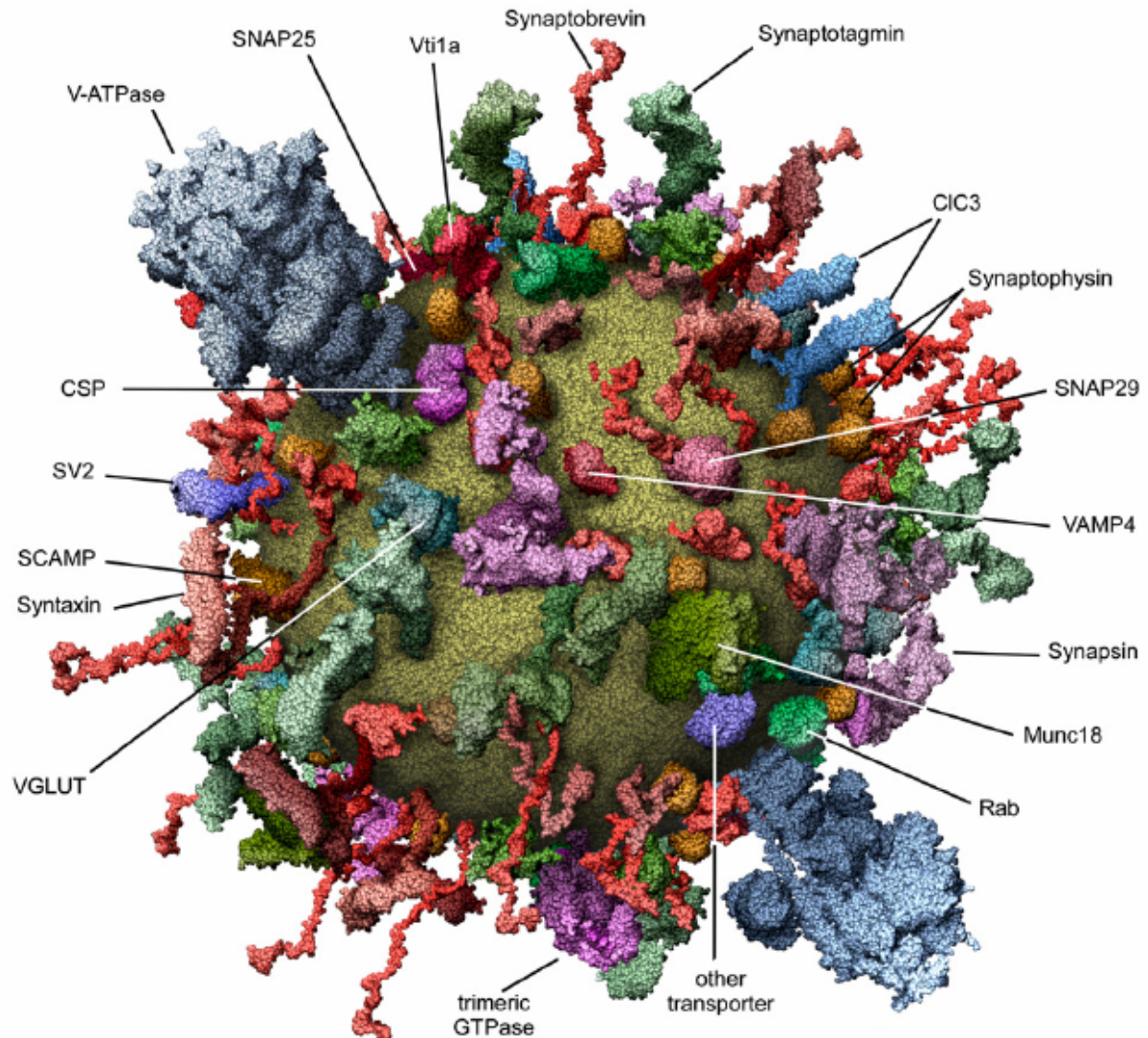
Figure 1: Out side view of SV and its key proteins (*Adapted from Takamori et al., 2006*)

To understand their interactive functions, an overview of different protein families identified so far, proteins involved in membrane fusion, key proteins of SVs and transporters proteins will be presented in the following:

1.2.2 Proteins involved in membrane fusion

The mechanism of exocytosis has been a subject of intense research. In very basic terms, the question has been whether bilayer fusion is mediated simply by lipids or whether intrinsic membrane proteins play a critical, causal role. It is now well established that efficient membrane fusion in vivo requires the interaction of small cytoplasmically exposed membrane proteins called SNAREs (Soluble N-ethlymaleimide sensitive factor Attachment protein Receptors) (Sollner et al., 1993). Fusion is driven by the progressive zippering of vesicle and plasma membrane SNAREs that form a four-helix bundle (Sutton et al., 1998). Although many other proteins appear to have critical roles in synaptic vesicle exocytosis, it seems likely that the SNAREs represent the minimal essential machinery for fusion (Weber et al., 1998).

SNARE Proteins

SNAREs form a novel super family of small and mostly membrane-anchored proteins that share a common motif of about 60 amino acids (SNARE motif). On the basis of their localisation and overall structure, SNAREs were initially classified into t-SNAREs (for SNAREs localised to the target membrane) and v-SNAREs (for SNAREs localised to the membrane of the trafficking vesicle) (Söllner et al., 1993). SNAREs are integral membrane proteins that associate into a tight complex to overcome the repulsive forces of two membranes for fusion (Chen and Scheller, 2001; Jahn et al., 2003). In neurons, the t-SNAREs were grouped into the syntaxin (Syn) and SNAP-25 families (plasma membrane proteins), whereas the v-SNAREs comprised synaptobrevin/VAMPs (Syb, the synaptic vesicle protein) and their relatives. The neuronal SNAREs Syb 2, Syn 1a, and synaptosome-associated protein (SNAP)-25 assemble into a stable ternary complex with a 1:1:1 stoichiometry that is referred to as the core complex. The complex could only be disassembled by means of ATP hydrolysis driven by a specialized chaperone, N-ethylmaleimide sensitive factor (NSF). NSF works in conjunction with adaptor proteins called SNAPs, for soluble NSF attachment proteins. NSF and SNAPs were identified before the identification of SNARE proteins as prerequisites for exocytosis.

Synaptobrevins/VAMPs (Vesicle Associated Membrane Protein) the synaptic vesicle protein Syb has recently been identified as one of the key proteins involved in exocytotic membrane

fusion. It interacts with the synaptic membrane proteins Syn 1 and SNAP-25 form a complex which precedes exocytosis (Söllner et al., 1993b). Syb was initially discovered in the *Torpedo* nervous system (Trimble et al., 1988). It is a small 18 kDa intergral membrane protein of SVs and is the most abundant protein with an average of 70 copies per vesicle (Takamori et al., 2006). It also forms another complex with synaptophysin (Syp) commonly known as the Synaptophysin/Synaptobrevin complex. Recent studies on Syb knock out mice models (Schoch et al., 2001) and *in vitro* assays (Hu et al., 2002) have shown that Syb also plays an important role in Ca $^{2+}$ triggered fusion.

Syntaxins (Syn) are integral plasma membrane proteins and function as target membrane receptors (t-SNAREs) for vesicle proteins (v-SNAREs) (Sollner et al., 1993; Südhof, 1999; Lin and Scheller, 2000; Fon and Edwards, 2001). There are different isoforms but Syn I is the main isoform found in the synaptic membrane of nerve terminal. Syn 1a is a 35 kDa protein and interacts with Syb and SNAP-25. It was also found to interact with the SV protein synaptotagmin (Syt) in Ca^{+2}-dependent manner (Bennett et al., 1992) and with N-type and P/Q type calcium channels (Yoshida et al., 1992; Leveque et al., 1998). In *Drosophila*, loss of function mutations in a Syn 1 homologue result in complete absence of spontaneous, and near absence of evoked, synaptic transmissions. Syn is regulated by other proteins to sustain its active participation in exocytosis.

SNAP-25 (synaptosome-associated protein) of 25 KDa is a membrane bound protein anchored to the cytosolic face of membranes via palmitoyl side chains in the middle of the molecule. SNAP-25 is a t-SNARE protein assembles with Syn 1 and Syb. SNAP-25 inhibits P/Q- and L-type voltage-gated calcium channels located presynaptically (Hodel, 1998) and interacts with the Syt. In glutamatergic synapses, SNAP-25 decreases the Ca^{2+} responsiveness, while it is naturally absent in GABAergic synapses (Pozzi et al., 2004). A mutant mouse that lacks the SNAP-25 gene is defective in neuronal dopamine signalling and exhibits similar behaviour as sufferers from hyperactivity disorders (Wilson et al., 1996).

1.2.3 Key proteins of synaptic vesicles

SV comprises of several other proteins, which are not directly involved in exocytosis but are required either prior to or subsequent to the fusion event. The most studied, Syp, Syt and associated protein Rab3 are briefly discussed in this section:

1.2.3.1 Synaptophysin - The first and still favourite synaptic vesicle marker

Syp was the first integral membrane protein of SVs to be isolated (Wiedenmann and Franke, 1985; Jahn et al., 1985) and cloned (Leube et al., 1987; Buckley et al., 1987; Südhof et al., 1987). The identification of Syp as a molecular component selectively and permanently associated with the membrane of SVs opened the possibility for tracing the movements of SVs in neurons, shedding light on the mechanisms of membrane trafficking. It is an abundant protein in the brain, corresponding to approximately 7% of synaptic vesicle protein (Jahn et al., 1987). Syp is a N-glycosylated membrane protein of 38 kDa. Syp has been found to interact *in vitro* with various nerve terminal proteins, which include the v-SNARE protein Syb (Edelmann et al., 1995), the vesicular proton pump V-ATPase (Galli et al., 1996), myosin V (Prekeris et al., 1997), dynamin I (Daly et al., 2000, 2002), and adaptor protein 1 (AP1) (Horikawa et al., 2002). Syp probably required for the exocytotic release of neurotransmitter by delivering monomeric Syb to the other SNARES in the right place and at the right time.

Mouse knockout of Syp alone produced no detectable changes in the phenotype. However, a defect in synaptic transmission was observed in a Syp/synaptogyrin double mutant, suggesting functional redundancy for these proteins (Fernandez-Chacon and Südhof, 1999).

1.2.3.2 Synaptotagmin

In 1981, Matthew et al., reported a 65 kDa protein, Syt present on the surface of SVs and LDCVs. Syt I is the best characterized isoform, and is the most abundant Ca^{2+} binding protein on secretory organelles. Syt I binds multiple Ca^{+2} ions involving its C2A and C2B domains (for review see Chapman, 2002). The first C2A domain binds Syn in a Ca^{+2} dependent manners. Binding of Syt to the SNARE complex via Syn probably induces a structural rearrangement in the fusion machinery, thereby triggering the opening of the fusion pore and neurotransmitter

release. Thus, Syt probably functions both in the last step of exocytosis and the first step of endocytosis (by initiating the assembly of clathrin coats).

1.2.3.3 Rab3A

Rab3A is an associated protein, which is not directly involved in exocytosis but is required either prior to or subsequent to the fusion event. Rab proteins constitute a large family (over 40 distinct members) of GTPases that participate with SNAREs in vesicle targeting and membrane fusion (Novick and Zerial, 1997). They are localised to the cytoplasmic face of organelles and vesicles involved in the secretory and endocytotic pathways. The major Rab protein in brain, Rab3A, associates with SVs. Rab3A isoforms associate with pre-synaptic vesicles to regulate Ca^{+2} dependent exocytosis and neurotransmitter release. Recently it was shown that Rab3A has a function upstream of vesicle fusion in the activity-dependent transport of synaptic vesicles to and their docking at the active zone (Miriam et al., 2001).

1.2.4 Vesicular transporter proteins

Changes in the post- and pre-synaptic structures mediate synaptic plasticity and are reflected as variations in the input and output at synaptic junctions. At the pre-synapse, the availability and fusion competence of SVs as well as the amount of neurotransmitter stored in individual vesicles determine the strength of the post-synaptic answer. Neurotransmitters are key molecules of neurotransmission. They are concentrated first in the cytosol and then in SVs of pre-synaptic terminals by the activity of specific neurotransmitter transporters of the plasma and the vesicular membrane, respectively. Irrespective of the type of vesicle, vesicular neurotransmitter transporters concentrate low molecular weight neurotransmitters such as monoamines (by vesicular monoamine transporters, VMATs), acetylcholine (by vesicular acetylcholine transpoters, VAChT), glutamate (by vesicular glutamate transporters, VGLUTs), γ-aminobutyric acid (GABA) and glycine (by VGAT). In this project, vesicular glutamate transporters, vesicular GABA transporters and vesicular monoamine transporter VMAT2 will be discussed (for review see Ahnert-Hilger et al., 2003).

1.2.4.1   Vesicular Glutamate Transporters (VGLUTs)

L-glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS) that contributes not only to fast synaptic neurotransmission, but also to complex physiological processes like memory, learning, plasticity, and neuronal cell death (Shigeri et al., 2004). Glutamate is synthesized in the cytoplasm and is transported into vesicles by VGLUTs. After glutamate has been released to the synaptic cleft, it can be either inactivated by enzymatic degradation or transported back to the neuron or glial cells by active transport. According to the structure and site of action, glutamate transporters can be divided in two super families: the plasma membrane transporters (EAATs) and the vesicular transporters (VGLUTs).

VGLUTs are dependent on a proton gradient which is created by hydrolysing adenosine triphosphate (ATP) by the V-type H^+-ATPase (Fig. 2). This enables the flow of protons (H^+) into the interior of the synaptic vesicle making it more acidic and generating a pH gradient across the vesicle membrane. The second consequence of the proton influx is that vesicle interior becomes more positive creating a corresponding membrane potential, thus forming electrochemical proton gradient. But glutamate uptake depends predominately on the existence of a vesicular membrane potential gradient, rather than a pH gradient, unlike other vesicular neurotransmitter transporters that depend more on the existence of a pH gradient as the driving force. Moreover, low concentration of chloride 1-5 mM is needed for optimal vesicular glutamate transport (Naito and Ueda, 1985).
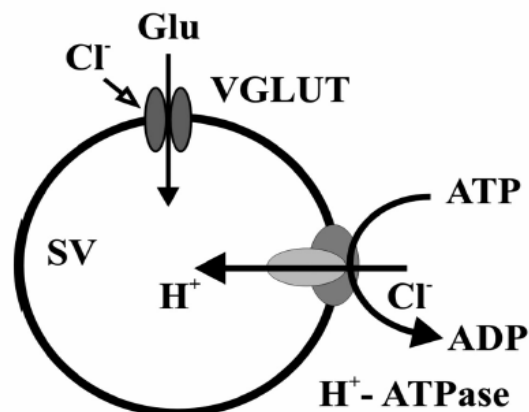
Figure 2: Mechanism of glutamate transport into SVs *(Adapted from Liguz-Lecznar and Skangiel-Kramska, 2007)*

Three vesicular glutamate transporters, VGLUT1 (Bellocchio et al., 2000; Takamori et al., 2000 a), VGLUT2 (Fremeau et al., 2001; Bai et al., 2001; Takamori et al., 2001; Hayashi et al., 2001), and VGLUT3 (Gras et al., 2002; Takamori et al., 2002; Fremeau et al., 2002; Schefer et al., 2002) have been cloned. VGLUT1 and 2 have been originally described as brain-specific or differentiation-associated, Na^+-dependent inorganic phosphate transporters, BNPI (Ni et al., 1994) and DNPI (Hisano et al., 2000), respectively, but later have been shown to transport glutamate with high avidity and specificity. In addition to their function as vesicular glutamate transporters, VGLUT1 and VGLUT2 appear to transport phosphate into the cytoplasm of nerve terminals (Ni et al., 1994) probably when integrated in the plasma membrane as BNPI/DNPI. So far, it is unclear how the decision between either function is regulated.

All three isoforms are highly homologous. The membrane spanning domains of VGLUTs have almost 90% homology, whereas the N- and C- terminal regions have little homology and contribute to functional differences (Fig. 3). It seems that the genes for these proteins are identified in many organisms from different branches of philogenetic tree. It also seems that transport properties of the VGLUTs are similar (Bellocchio et al., 2000; Fremeau et al., 2001; Takamori et al., 2000). Why then have three isoforms emerged in mammals? Difference in cellular and regional distribution of these transporters may, in part, answer that question.
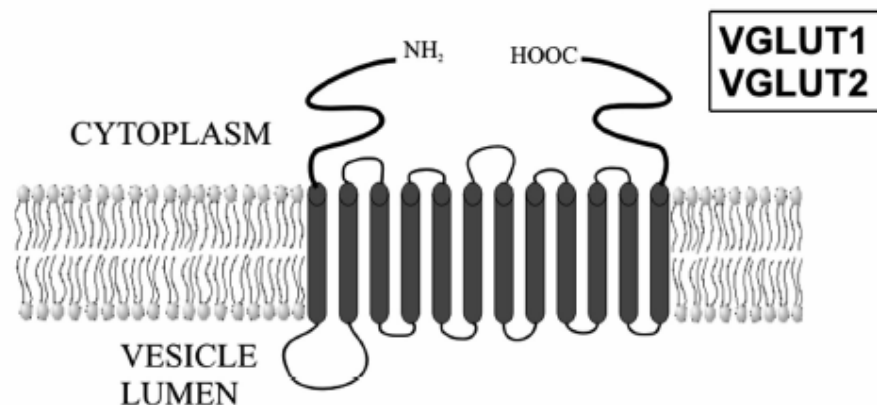
Figure 3: **Predicted secondary structure of VGLUTs: Putative models propose 12 transmembrane segments for VGLUT1 and VGLUT2** *(Adapted from Liguz-Lecznar and Skangiel-Kramska, 2007)*

VGLUT1 and VGLUT2 have a distinct and mutually exclusive distribution in brain, with VGLUT1 being the dominant transporter in cortex, hippocampus, and cerebellum (Fremeau et al., 2001; Fujiyama et al., 2001; Kaneko and Fujiyama, 2002) and VGLUT2 in thalamic and hypothalamic regions (Hisano et al., 2000; Sakata-Haga et al., 2001; Fujiyama et al., 2001; Fremeau et al., 2002; Kaneko and Fujiyama, 2002). In addition, VGLUT2 is expressed in the pineal gland and in cells of Langerhans islets, suggesting a role in endocrine function (Hayashi et al., 2001). VGLUT1 and VGLUT2 are targeted to functionally distinct synaptic release sites (Fremeau et al., 2002). A strict separation between VGLUT1 and VGLUT2 containing terminals has been shown for cerebellar cortex where parallel fiber terminals contain VGLUT1, whereas climbing fiber terminals have VGLUT2 (Fremeau et al., 2002). In contrast, VGLUT3 is found in serotonergic, cholinergic (Fremeau et al., 2002; Gras et al., 2002; Schefer et al., 2002) and GABAergic terminals (Fremeau et al., 2002), suggesting a role for glutamate as cotransmitter in these nerve terminals. The expression of VGLUTs is age-dependent and the developmental regulation of particular VGLUTs is different in distinct brain regions. Axon terminals containing VGLUT2 mature earlier than VGLUT1 and VGLUT3 loaded axons and VGLUT2 expression at birth is relatively high (Boulland et al., 2004). Expression of VGLUT1 increases during postnatal development in most brain regions (Minelli et al., 2003; Nakamura et al., 2005).

Identification of VGLUTs has raised many questions about excitatory neurotransmission. Because of a small pool of recycling vesicles at glutamatergic synapses, rapid refilling is needed (Harata et al., 2001; Schikorski et al., 1997). Wojcik et al (2004) have shown that the expression level of VGLUTs determines the amount of glutamate that is loaded into vesicles and released and thereby regulates the efficacy of neurotransmission. Therefore a need for the regulatory mechanisms underlying the rapid recycling of VGLUTs in vesicle pools is vital.

1.2.4.2   Vesicular GABA transporters (VGAT)

VGAT, also termed vesicular inhibitory amino acid transporter (VIAAT), was cloned in parallel from *Caenorhabditis elegans* (McIntire et al., 1997) and from mouse (Sagn et al., 1997). VGAT exhibits a very low affinity for its substrate GABA (in the millimolar range). VGAT also transports glycine and exhibits even lower affinity for glycine than GABA (Sagne et al., 1997; McIntire et al., 1997; Bedet et al., 2000). Its presence on a subset of SVs was confirmed by immunoisolation of GABA-specific vesicles from rat brain using a VGAT-antibody (Takamori et

al., 2000). VGAT has been found in terminals of GABAergic and glycinergic neurons, suggesting that it is the main vesicular transporter for inhibitory transmitters in brain (Chaudhry et al., 1998; Dumoulin et al., 1999).

1.2.4.3   Vesicular Monoamine Transporters (VMATs)

Two structurally related but pharmacologically distinct VMATs (VMAT1 and VMAT2) have been cloned. VMAT1 has been cloned from PC12 cells (Liu et al., 1992) and VMAT2 from rat brain (Liu et al; 1992., Erickson et al., 1992 and Liu et al., 1994). VMAT2 is the dominant transporter in the brain, but also occurs in a variety of peripheral cells like sympathetic neurons, enterochromaffin cells (Peter et al., 1995; Erickson et al., 1996), and also in blood platelets (Lesch et al., 1993). By contrast, VMAT1 appears to occur only in the periphery, at least in adult individuals. Both transporters accept monoamines such as serotonin, dopamine, noradrenalin and adrenaline at comparable concentrations with micro-molar K_m values for VMAT1 and sub micro-molar K_m values for VMAT2 in rat (Peter et al., 1994) and humans (Erickson et al., 1996). VMAT2 also transports histamine, barely recognized by VMAT1. VMAT2 has higher affinity for monoamines than VMAT1, which may be required for rapidly recycling SVs in brain in contrast to more slowly filling secretary granules in the adrenal medulla (Peter et al., 1994).

1.2.4.4   Vesicular proton-pump (V-ATPase or H$^+$-ATPase)

V-ATPases translocate protons across the membrane of various organelles (lysosomes, endosomes, trans-Golgi cisternae, secretory granules etc.); creating an electrochemical proton gradient that is acidic and positive within these intracellular compartments (Stevens and Forgac, 1997; Nelson and Harvey, 1999; Nishi and Forgac, 2002; Schoonderwoert and Martens, 2001). In neurons, V-ATPase is present in the membrane of SVs, the neurotransmitter-storing organelles (Stadler and Tsukita, 1984; Hicks and Parsons, 1992). The large electrochemical H$^+$-gradient generated by this enzyme (pH 5.2-5.5 inside the SVs) (Michaelson and Angel, 1980; Füldner and Stadler, 1982) is used by specific vesicular transporters to accumulate the neurotransmitter. In addition to this well established role in neurotransmitter storage, V-ATPase could also participate in the constitution of a fusion pore involved in neurotransmitter release (Morel et al., 2001) and

membrane fusion (Peters et al., 2001). This V-ATPase interacts with Syb and with the SNARE complexes involved in synaptic vesicle docking and exocytosis.

1.2.5 Endocytosis – Synaptic vesicle protein recycling

When an action potential arrives in the nerve terminal, the pre-synaptic membrane depolarizes, voltage gated calcium channels open and the influx of calcium ions in turn triggers exocytosis of SVs. Vesicles are then retrieved by endocytosis, and after being reloaded with transmitter by means of specific carriers in the vesicle membrane, they re-enter the pool of fusion-competent vesicles. Multiple mechanisms have been proposed to underlie the efficient recycling of synaptic vesicles that is required to sustain the high rates of neurotransmitter release observed at many synapses.

Kiss-and-run: in which the vesicle opens and closes transiently, presumably through a small fusion pore, thus does not require sorting of vesicular from plasma membrane proteins (Ceccarelli et al., 1973; Gandhi and Stevens, 2003; Staal et al., 2004; Harata et al., 2006). Newly reformed vesicles may then stay in place, be reloaded, and undergo a new round of exocytosis or may de-dock and allow other vesicles to take their place. Studies of Drosophila endophilin mutants have been invoked to support the kiss-and-run model of neurotransmitter release (Verstreken et al., 2002). Disruption of the *Drosophila* endophilin gene, which codes for an important endocytic protein, resulted in synapses that have very few vesicles but nevertheless sustain synaptic transmission at low frequencies (Guichet et al., 2002, Rikhy et al., 2002, Verstreken et al., 2002). Thus, the occurrences of kiss-and-run, and the mechanisms underlying it, remain open questions. If kiss-and-run exists, it will be important to determine the factors that prevent collapse of the vesicle into the plasma membrane.

Clathrin-mediated endocytosis: In contrast to kiss-and-run, full fusion, in which the vesicle collapses into the plasma membrane and is retrieved by clathrin-coat-dependent processes. Clathrin coats and their accessory factors function as molecular sorters that select proteins to be internalized and provide the driving force for membrane invagination (Schmid, 1997). Clathrin coats have two protein layers: an outer clathrin layer and an inner layer composed of clathrin adaptors. These adaptors are a set of proteins that function as molecular linkers between the membrane and the clathrin layer and that also have clathrin assembly properties. At synapses, the main adaptors are the heterotetrameric complex AP2 and AP3 or AP-180.

Alternatively, SVs have also been shown to bud from an endosomal intermediate, a process that involves the brefeldin A (BFA)-sensitive adaptor protein AP3 (Faundez et al., 1998). However, the relationship between these different mechanisms and indeed the role of the AP3 pathway has remained unclear.

Although many components of the endocytic machinery have been identified, the recycling of SV proteins is still not completely understood. The Ca^{+2} sensor Syt interacts with clathrin adaptor AP2 (Zhang et al., 1994; Jorgensen et al., 1995; Li et al., 1995), but the role of the interaction and the sequences involved remain unknown (Blagoveshchenskaya et al., 1999; Haucke and De Camilli, 1999; Grass et al., 2004; Poskanzer et al., 2006; Diril et al., 2006). The v-SNARE, Syb that mediates vesicle fusion also contributes to synaptic vesicle recycling (Desnos et al., 1995; Grote et al., 1995; Salem et al., 1998; Deak et al., 2004), but the sorting signals remain unclear. Among the transporters that fill synaptic vesicles with neurotransmitter, the vesicular monoamine and acetylcholine transporters depend on a dileucine-like motif for endocytosis (Tan et al., 1998; Krantz et al., 2000). More recently, the vesicular glutamate transporters (VGLUTs) have shown differences in trafficking that suggest sorting to distinct synaptic vesicle recycling pathways.

1.3   Pre- and post-synaptic plasma membrane associated proteins

So far, many pre-and post-synaptic plasma membrane proteins have been identified in neurons. In this study, post-synaptic plasma membrane protein NMDA-receptor 1 and pre-synaptic plasma membrane protein Na^+/K^+-ATPase will be discussed and used as plasma membrane markers.

NMDA-receptors localised at the post-synaptic plasma membrane. NMDA-receptors represent a class of glutamate receptors that are of central importance in synaptic plasticity. Multiple NMDA receptor subtypes exist but R1 is the most important as it is required for activity. NMDA-receptors allow Ca^{2+} influx and are thought to trigger Ca^{2+} dependent postsynaptic processes involved in long term potentiation and depression.

Na^+/K^+-ATPase is a particle-bound enzyme that concentrates in the nerve ending or synaptosomal membranes. The arrival of the nerve impulse to the nerve endings leads to a series of events involving the entry of sodium and the exit of potassium. Restoration of ionic equilibria of sodium and potassium through the membrane is carried out by the sodium/potassium pump, which is the enzyme Na^+/K^+-ATPase.

1.4 Circadian clock and its effect on regulation of vesicular neurotransmitter transporters

Life on earth is under the influence of the daily changes of night and day. No other environmental factor has influenced evolution in such a steady rhythm. Life that depends on energy source light "internalizes" its periodic availability in the form of the circadian clock. The circadian clock prepares the body for tasks that typically occur in the course of a day. Its main function is to organize the time course of biochemical, physiological and behavioural processes thereby optimizing an organism's performance in anticipating changing environmental conditions. Circadian rhythms are defined as oscillations that display a cycle with a period length of about 24 hrs, hence the term circadian from the Latin *circa*, meaning "about" and *dies*, "day", meaning literally, "about a day". Circadian clocks regulate a diversity of activities in nature, such as the sleep-wake cycle, migration behaviour in birds, and seasonal reproduction (Dunlap et al., 1999).

1.4.1 Circadian clock: Suprachiasmatic nucleus (SCN)

A "master clock" or circadian pacemaker controls circadian rhythms in mammals and resides in the suprachiasmatic neucleus (SCN) that is located lateral to the third ventricle and atop the optic chiasm in the anteroventral hypothalamus. Each SCN consist of about 8000 cells packed into an approximately 0.5 mm by 1 mm football shape (Reppert et al., 2001; Klein et al., 1991) and is necessary for expression of most, perhaps all, sustained circadian rhythms. Destruction of these cells eliminates circadian rhythms including drinking, locomotor activity, body temperature, estrus cycling and secretion of prolactin, melatonin, growth hormone, and cortisol (Meyer-Berstein et al., 1999). These lesion studies indicated that a single master pacemaker either in the SCN or in upstream structures controlled all circadian outputs. Genes involved in the intracellular clock mechanism of the SCN were also found to be expressed rhythmically in other brain areas, in peripheral organs (Zylka et al., 1998) and even in cultured cell lines (Balsalobre et al., 1998; Yamazaki et al., 2000).

The effects of light on the SCN are mediated by unknown photoreceptors located in the retina. Light is the major photic entrainer (input) and this light mediated resetting is accomplished by the release of glutamate via the retinohypothalamic tract (RHT). The primary pathway for transmission of photic information from retina to pacemaker for entrainment is the RHT. RHT comprises a distinct subset of retinal ganglion cell axons that separate from the other

optic axons at the optic chiasm to innervate the SCN. The photic information transmitted plays a critical role in mediating photic regulation of the circadian system. There is evidence that the amino acid glutamate is a transmitter at the RHT/SCN synaptic connection and plays critical role in mediating photic regulation of the circadian system (Colwell and Manekar, 1996). Anatomical studies report that identified RHT terminals innervating the SCN show glutamate immunoreactivity associated with synaptic vesicles (Castel et al., 1993; DeVeries et al., 1993). A variety of glutamate receptors have been localized to the SCN by both *in situ* hybridization and immunocytochemistry (Gannon et al., 1994). Application of GluR agonists causes phase shifts of a rhythm of neural activity recorded from the SCN *in vitro* (Ding et al., 1994; Shibata et al., 1992). Finally, GluR antagonists block light-induced phase shifts and Fos-induction in the SCN *in vivo* (Abe et al., 1992; Colwell and Manekar, 1992). Despite this strong evidence that glutamate is a transmitter released by the RHT, there are many unanswered questions as to how the circadian oscillators in the SCN respond to this glutamatergic stimulation. In the simplest scenario, light causes the release of glutamate, which initiates a signal-transduction cascade in SCN neurons, ultimately resulting in a phase shift of the circadian system. More work is clearly needed to delineate the signal-transduction cascades by which glutamate acts in the SCN and to understand how these cascades influence the phase of the circadian system.

1.4.2   Mammalian circadian genes

Many of the clock genes are expressed in an oscillating manner on the transcriptional and on the translational level. Interestingly, clock gene expression is not restricted to the SCN and can be observed in most tissues (Albrecht et al; 1997; Shigeyoshi et al; 1997). Oscillating gene expression is maintained in peripheral organs (Yamazaki et al., 2000) and even in cell culture (Balsalobre et al., 1998). This indicates that circadian rhythmicity is not cell type specific and is a property of individual cells.

In mammals, the intracellular clock machinery comprises two loops; a positive and a negative feedback loops drive the expression of clock genes. The two transcription factors BMAL1 (Hogenesch et al., 1998) and CLOCK (Gekakis et al., 1998) members of the basic-helix-loop-helix (bHLH)-PAS (Period-Arnt-Single minded) family of proteins, constitute the positive loop. They heterodimerize and activate transcription of three *Period (Per1/2/3)* (Sun et al., 1997; Tei et al., 1997; Albrecht et al., 1997; Shearman et al., 1997; Takumi et al., 1998, Zylka et al.,

1998), two *Cryptochrome (Cry1/2)* (Miyamoto and Sancar, 1998; Okamura et al., 1999), two *Differentiated embryo chondrocyte expressed (Dec1/2)* genes (Honma et al., 2002), *Rev-erb α* (Preitner et al., 2002), probably *Rorα* (Sato et al., 2004) and several clock-controlled genes by binding to E-box enhancer elements present in the promoter of those genes.

The functioning of the clock at the molecular level has been elucidated by targeted mutations and deletions of candidate circadian clock genes in the mouse. Loss or mutation of specific clock genes can lead to altered period length (*Cry1, Cry2, Per1, Per3, CKI*) and slow (*Clock, Per2*) or immediate loss (*Bmal1*) of circadian rhythmicity under constant conditions. *Cry1/Cry2* double mutant animals (Van der Horst et al., 1999, Vitaterna et al., 1999) and *Per1/Per2* double mutant animals (Zheng et al., 2001; Bae et al., 2001) lose circadian rhythmicity immediately under constant conditions, confirming the importance of these genes in the clock mechanism. However, *Per3* seems to be dispensable for the clock because deletion of *Per3* has only a mild effect on circadian wheel running activity (Shearman et al., 1997), and *Per1/ Per3* and *Per2/Per3* double mutants display the phenotype of *Per1* or *Per2* mutants, respectively (Bae et al., 2001). The array of phenotypes observed after the disruption of the main molecular clock components suggests that not all clock gene products are equally important for the maintenance of circadian rhythmicity.

1.4.3   Regulation of neurotransmitter transporters in wild type and *Per2^{Brdm1}* mutant mice

Period (Per) genes are involved in regulation of the circadian clock and modulate several brain functions. Three putative mammalian homologues (mPer1, mPer2 and mPer3) of the Drosophila circadian clock gene *Period* (*Per*) have been identified. The mPer genes share a conserved PAS domain (a dimerization domain found in Per, Arnt and Sim) and show a circadian expression pattern in the SCN. To assess the in vivo function of mPer2, deletion mutation in the PAS domain of the mouse mPer2 gene was generated and characterized (Zheng et al., 1999). Mice carrying a deletion in the *Period2* gene (*Per2^{Brdm1}*) are defective in light mediated resetting (Albrecht et al., 2001) and are arrhythmic in constant darkness (Zheng et al., 1999). *Per2^{Brdm1}* mutant mice, which have a deletion in the PAS domain of the Per2 protein, show alterations in the glutamatergic system. Lowered expression of the glutamate transporter EAAT1 is observed in these animals, leading to reduced uptake of glutamate by astrocytes (Spanagel et al., 2005). As a

consequence, glutamate levels increase in the extracellular space of $Per2^{Brdm1}$ mutant mouse brains. This is accompanied by increased alcohol intake in these animals. Collectively, these data establish a link between glutamate and dysfunction of the circadian clock gene *Per2*. Another recent study showed (Johnston et al., 2004) that the isoform of *Period* gene, *Per1,* is co-expressed with VGLUT3 in amacrine cells of the retina suggesting that VGLUT3 amacrine cells might play an important role in the light entrainable circadian clock pathways. These studies strongly underscore the role of clock genes in maintaining the key pathways of glutamatergic neurotransmission.

Changes in the rate of neurotransmitter biosynthesis, storage, reuptake, and degradation each have the potential to influence extracellular neurotransmitter concentrations, and hence signalling. Individual steps in neurotransmitter release may therefore have an important influence, not only on the site and mode of transmitter release, but also on the amount of neurotransmitter released (Fon and Edwards, 2001). Recently it was shown that monoamine oxidase (Maoa) acitivity is reduced and dopamine levels, concentrated in vesicles by VMAT2, are elevated in $Per2^{Brdm1}$ mutant mice (Hampp et al., 2008). Further, Ehlen et al., 2006 had shown that GABA, concentrated in vesicles by VMAT2, suppresses Period1 and Period2 mRNA in the SCN during the mid-subjective day. A recent study reported that the amount of VGLUT1 undergoes strong diurnal cycling (Yelamanchili et al., 2006). VGLUT1 protein levels are high before the start of the light period, decline at noon, increase again before start of the dark period, and decline again at midnight. In contrast, mice lacking the period gene *Period2* did not show any light-cycle-dependent changes of VGLUT1 levels. Based on these arguments, it can be also speculated that synaptic vesicles containing VGLUT1 could be sorted transiently into a special compartment or pool that still needs to be characterized.

1.5   Aim of the project

Regulating neurotransmission is one of the foremost tasks of a synapse. At the pre-synapse, SVs release by exocytosis upon activation and the vesicle membrane is then retrieved by endocytosis, and SVs are then regenerated and re-filled with neurotransmitter. Although many aspects of vesicle recycling are understood, the fate of some of the vesicular proteins including VGLUTs after fusion is still unclear. Do they translocate or sort to the plasma membrane, or do they recycle immediately? Recently, it was shown that synaptotagmin I, a protein resident in the

vesicle membrane, remains clustered in isolated patches on the pre-synaptic membrane regardless of whether the nerve terminals are mildly active or intensely stimulated. Vesicular neurotransmitter transporters like VGLUTs recycling after exocytosis play a relevant role in synaptic plasticity because the number of VGLUTs per vesicle is crucial for the amount of stored neurotransmitter thereby influencing postsynaptic response. Besides the occurrence on synaptic vesicles VGLUTs may also function as sodium phosphate transporter at the plasma membrane, however physiological relevance for a switch between these membrane compartments is lacking. As the vesicular proteins play such incisive roles in synaptic neurotransmission, an understanding of vesicular proteins including VGLUTs transport mechanisms and trafficking of VGLUTs through the synaptic vesicle life cycle at pre-synaptic nerve terminals is needed. Therefore, trafficking of vesicular proteins including VGLUTs to different compartments in synaptosomes from adult neurons and the method to detect this trafficking still need to be characterised.

Circadian rhythm: So far, the role of clock genes has been only implicated in the post-response stages of neurotransmission, for example, the effects of altered glutamatergic signalling and post-synaptic receptors. It remains to be established whether clock genes are also involved in other synaptic plasticity related functions like pre-synaptic vesicle trafficking, sorting and regulation of neurotransmitter transporters. Circadian clock might be involved in the regulation of vesicular packaging and transport of neurotransmitters like glutamate by VGLUTs, GABA by VGAT and monoamines by VMAT2. The previous results from the circadian experiments indicate that synaptic vesicles containing VGLUT1 could be sorted into a special compartment or pool that still needs to be characterized.

Therefore, in this project, the following points were addressed:

1. Establishment of a pronase digestion assay to study the translocation of vesicular proteins to the plasma membrane
2. Time-dependent trafficking or sorting of VGLUTs in wild type mice entrained in LD cycle
3. Effect of circadian rhythm on regulation of VGAT and VMAT2 in wild type and *Per2^{Brdm1}* mutant mice.

2   MATERIALS

2.1   Animals

1. Adult wister rats (150-200gm) and mice were purchased from Harlan Winkelmann, Germany.

2. Wild type and *Per2^{Brdm1}* mutant mice entrained in LD and DD conditions were provided from Department of Medicine, Division of Biochemistry, University of Fribourg, Fribourg, Switzerland.

2.2   Antibodies

2.2.1   Primary antibodies

The following antibodies are purchased from Synaptic systems (Göttingen, Germany) and used in the given final dilution.

Anti-VGLUT1 (vesicular glutamate transporter 1), Monoclonal, mouse, 1:10,000

Anti-VGLUT1 (vesicular glutamate transporter 1), Polyclonal, rabbit, 1:10,000

Anti-VGLUT 2 (vesicular glutamate transporter 2), Polyclonal, rabbit, 1:10,000

Anti-VGLUT2 (vesicular glutamate transporter 2), Polyclonal, rabbit, 1:10,000

Anti-VGAT (vesicular GABA transporter), Monoclonal, mouse, 1:2000

Anti-VGAT (vesicular GABA transporter), Polyclonal, rabbit, 1:2000

Anti-VMAT2 (Vesicular mono amine transporter 2), Polyclonal, rabbit, 1:2000

Anti-Synaptobrevin I (Clone 69.1), Monoclonal, mouse, 1:10,000

Anti-Synaptophysin (Clone 7.2), Monoclonal, mouse, 1:10,000

Anti-Syntaxin 1A/B (Clone HPC), Monoclonal, mouse, 1:10,000

Anti-Synaptotagmin 1 (clone 41.1), Monoclonal, mouse, 1:5000

Anti-Synaptotagmin 1 (clone 604.4), Monoclonal, mouse, 1:5000

Anti-Proton Pump, Polyclonal, rabbit, 1:1000

Anti-Rab3A, Monoclonal, mouse, 1:10,000

Anti-NMDA-receptor, Monoclonal, mouse, 1:5000

Anti-AP-180 (AP3), Polyclonal, rabbit, 1:5000

Anti-Endophilin 1, Polyclonal, rabbit, 1:5000

Anti-Syntaxin 16, 13 and 6, Polyclonal, rabbit, 1:2000

Anti-VGLUT1 (vesicular glutamate transporter 1), Polyclonal, Guine pig, 1:10,000 purchased from Millipore, Chemicon International, USA.

Anti-SNAP 25 (synaptosomal attachment protein), Monoclonal, mouse, 1:10,000, purchased from Sternberger Monoclonals (Baltimore, USA)

Anti-Na^+/k^+-ATPase α-3, Monoclonal, mouse, 1:5000, purchased from Upstate Cell Signalling, NY, USA

Anti-Adaptin (AP2), Monoclonal, mouse, 1:2000, purchased from Affinity Bioreagents, USA.

2.2.2   Secondary antibodies

Horse anti-mouse, IgG peroxidase-labelled, Monoclonal, purchased from Vector Laboratories (Burlingame, CA, USA)

Goat anti-rabbit, IgG peroxidase labelled, Polyclonal purchased from Vector Laboratories (Burlingame, CA, USA)

Goat anti-guinea pig, IgG Peroxidase labelled, Polyclonal purchased from Jackson Immuno Research Lab (PA, USA).

2.3   Chemicals and Reagents

40% Acrylamide / Bisacrylamide-mix (40:1)   BioRad (Hercules, CA, USA)

APS (Ammonium persulfate)   Sigma (Munich, Germany)

BCA (Sodium Bicinchoninic acid-4,4-dicarboxy-2,2-Bichinolin)	Sigma (Munich, Germany)
BSA, Fraktion V (Bovine Serum Albumin)	Roth (Karlsruhe, Germany)
Brefeldin A	Sigma (Munich, Germany)
Bromphenolblue	Sigma (Munich, Germany)
β-MCD (β-methyl cyclo dextrin)	Sigma (St Louis, MO, USA)
DMSO (Dimethylsulfoxide)	Sigma (Munich, Germany)
DTT (Dithiothreitol; threo-1,4-Dimercapto-2,3-butandiol)	Sigma (Munich, Germany)
Dynasore	Sigma (Munich, Germany)
ECL TM (Enhanced Chemiluminescence)	Amersham (Buckinghamshire, UK)
EDTA (Ethylene Diamine Tetra Acetic acid)	Roth (Karlsruhe, Germany)
Ficoll	Pharmacia Biotech (Uppsala, Sweden)
L-[G-3H] Glutamate	Amersham (Dreieich,Germany)
Glycerol	Roth (Darmstadt, Germany)
Hybond C	Amersham (UK)

HEPES (N-2-Hydroxyethylpiperazin-
N´-2-ethansulfonicacid)   Biochrom (Berlin, Germany)

LMW (Low Molecular Weight Marker-Marker)   Amersham
(Buckinghamshire, UK)

Lubrol   Serva (Heidelberg, Germany)

Lovastatin (Mevostatin)   Sigma (St Louis, MO, USA)

Milk powder (for western blotting)   Molkerei Heideblume
(Elsdorf, Germany)

Methanol   Merck (Darmstadt, Germany)

MES (2-[N-Morpholino ethane sulfonic acid) Sigma

Pronase   Calbiochem
(Schwalbach, Germany)

PMSF (Phenylmethylsulfonylfluoride)   Sigma (Munich, Germany)

Ponceau S   Sigma (Munich, Germany)

Porablot (Nitrocellulose membrane)   Machery & Nagel
(Düren, Germany)

Protease inhibitor cocktail   Sigma (Munich, Germany)

AEBSF   104 mM

Aprotinin   0,08 mM

Leupeptin   2 mM

Bestatin   4 mM

Pepstatin A   1,5 mM

E-64   1,4 mM

p-Nitrophenol   Sigma (Munich, Germany)

p-Nitrophenyl phosphate liquid substrate   Sigma (Munich, Germany)

SDS (Sodiumdodecylsulphate)   Sigma (Munich, Germany)

Sucrose   Roth (Karlsruhe, Germany)

TEMED (N, N, N´, N´-tetramethylethylendiamin)   Sigma (Munich, Germany)

Triton X-100   Roth (Karlsruhe, Germany)

Tween-20   Merck (Darmstadt, Germany)

Western blot stripping buffer   Pierce (Rockford, USA)

2.4   Buffers and Solutions

(Composition)

Antibody solution

1.5% bovine serum albumin in Tris-buffered Saline (TS) buffer

Blocking solution

5.0%   low fat milk powder

0.1%   Tween-20 in TS buffer

Electrophoresis buffer (10 X)

30 g	Tris
144 g	Glycine
10 g	SDS

Extraction buffer for immunoprecipitations

144 mM	KCl	
20 mM	HEPES-KOH	pH 7.4
2 mM	EDTA	
1%	Triton X-100	

Krebs Ringer Hepes buffer (KR-Hepes)

140 mM	NaCl	
5 mM	NaHCO3	
1 mM	MgCl2	
1.2 mM	Na2HPO4	
10.0 mM	glucose	
20.0 mM	HEPES	pH 7.4

MES Buffered saline (MBS)

25 mM	MES	
150 mM	NaCl	pH 6.5

MBS Extraction buffer

25 mM	MES	
150 mM	NaCl	pH 6.5

with 1% Triton or 2% Lubrol

Phosphate buffered saline (PBS) buffer

140 mM	NaCl
2.7 mM	KCl

| 10 mM | Na_2HPO_4 |
| 1.8 mM | KH_2PO_4, pH 7.4 |

Ponceau solution

| 0.5 % w/v | Ponceau S |
| 3.0 % v/v | Trichloroacetic acid |

Sample buffer (3 X)

12.48 ml	stacking gel buffer (4 X)	
1.50 ml	0.1 M EDTA (Na^+-salt)	pH 6.8-7.0
15 g	Sucrose	
5.00 ml	1.5 M DTT	make up to 50 ml using dH_2O

Separating gel buffer (4 X)

| 181.7 g | Tris, | pH 8.8 |
| 4.0 g | SDS | make up to 1 L using dH_2O |

Sodium buffer

10.0 mM	Glucose	
5.0 mM	KCl	
140.0 mM	NaCl	
5.0 mM	$NaHCO_3$	
1.0 mM	$MgCl_2$	
1.2 mM	Na_2HPO_4	
20.0 mM	HEPES,	pH 7.4

Solution A (for BCA assay)

1.00 g	BCA-disodium
1.70 g	Na_2CO_3
0.16 g	Na_2-tartrate
0.40 g	NaOH

0.95 g   NaHCO$_3$

100 ml   dH$_2$O,   pH 11.25 (NaOH).

Solution B (for BCA assay)

4 g   CuSO$_4$ x 5H$_2$O

in 100 ml of dH$_2$O

Sucrose/ Homogenisation solution

0.32 M   sucrose in dH$_2$O

Stacking gel buffer (4 X)

60.5 g   Tris pH 6.8

 4.0 g   SDS   make up to 1 L using dH$_2$O

TBST buffer

25 mM   Tris

150 mM   NaCl

3 mM   KCl

0,05 %   Tween 20   pH 7,4

TS buffer (Tris-buffered saline) (10 X)

20 mM   Tris

150 mM   NaCl   pH 7.5 (HCl)

Western blotting buffer (for semidry transfer)

48 mM   Tris

386 mM   Glycine

3.7 g / l   10 % SDS

800 ml   H$_2$O

200 ml   Methanol

3 METHODS

3.1 Preparation of synaptosomes and SVs

3.1.1 Preparation of crude synaptosomes from rat and mouse whole brain

Synaptosomes were prepared from rat and mouse whole brain as described by Edelmann et al., 1995. The rat was anaesthetized with ether prior to decapitation. The whole brain was removed quickly and placed in ice-cold homogenisation/sucrose buffer containing protease inhibitors Pi 10 µl (1:1000) and PMSF 45 µl (1mM). Each rat brain was homogenised in 10 ml of homogenisation buffer and each mouse brain was homogenised in 5 ml of homogenisation buffer using a dounce homogenizer (900 rpm, 9 strokes). The homogenate (H) was subsequently centrifuged at 1,200 x g for 10 minutes in a Beckman Ti 70 rotor. The resultant pellet P1 containing nuclei and tissue debris was stored for analysis and the supernatant (post nuclear supernatant, PNS) was centrifuged for 20 minutes at 12,000 x g to obtain the crude synaptosomal pellet (P2). All steps were performed at 4°C.

3.1.2 Preparation of pure synaptosomes from rat whole brain by ficoll gradient

The crude synaptosomal pellet was reconstituted in 2 ml homogenisation buffer and loaded onto a ficoll gradient made up of three layers containing 6% (4ml), 9% (1 ml) and 13% (4 ml) Ficoll® 400 prepared in homogenisation buffer. The gradient was then centrifuged in a Beckman SW 40 Ti rotor at 90,040 x g for 35 minutes. After centrifugation, synaptosomal fraction was collected from interface between the 9% and 13% ficoll layer. This fraction was collected carefully with a glass pasteur pipette which was bent thermomechanically at the tip. The fraction was pelleted again at 12000 x g for 20 minutes to obtain a pure synaptosomal pellet.

3.1.3 Isolation of crude synaptic vesicles from rat whole brain

Crude SVs were prepared from adult rat brain synaptosomes based on the procedure described by Huttner et al., 1983. Adult rat brain synaptosomal fraction was isolated as described above (section 3.1). Crude or pure synaptosomal pellets were resuspended in 1ml of homogenisation buffer and were given a water shock (homogenisation at 2000 rpm, 3 strokes) by the addition of 9

volumes of sterile water, 10 mM HEPES (pH 7.4) and protease inhibitors. The lysate was centrifuged at 25,000 x g for 20 minutes resulting in the first lysed pellet (Lysed pellet 1, LP1), which was stored for analysis and the corresponding first lysed supernatant (LS1). The LS1 was carefully removed and centrifuged at 350,000 x g for 30 minutes in a Beckman TLA 101.4 rotor. This last centrifugation step yields a pellet (LP2) constituting of synaptic vesicles and its corresponding diluted synaptosomal supernatant LS2. For resuspension, the SV fraction was drawn 6 times through a 23 gauge needle and subsequently 9 times through a 27 gauge needle in buffer chosen for the preferred experimental conditions. All steps were performed on ice.

3.1.4 Preparation of crude SVs from wild type and *Per2*^{Brdm1} mutant mouse strains

All animal work was performed according to the guidelines of the Schweizer Tierschutzgesetz (TSchG, SR455, Abschnitt 2 (Art. 5 und 7), 5 (Art. 11) and 6 (Art. 12-19) of Fribourg. The *Per2*^{Brdm1} mutants lack a major part of their PAS domain responsible for protein-protein interaction resulting in a truncated non-functioning protein (Oster et al., 2002; Zheng et al., 1999). Wild type and *Per2*^{Brdm1} mutant mice were bred in BioZone ventilated caging systems (Cage Model CA20, VR Classic TM, BioZone, Margate, UK) in Hannover and in filter top open cages (Type2 Polycarbonate Cage with top wiring, Tecniplast, Italy) in custom made racks in Fribourg in a 12h light and 12h dark cycle. Matings were normally set up as pairs or triples with rotation of the females in a one to two week turn during the expansion of the colonies. Litters were weaned three to four weeks after birth and separated for their gender. Store cages held up to 6 animals. Genotype was determined by southern blot hybridization with genomic DNA extracted from tail tissue before weaning *(Personal communication: Yalamanchili S Thesis).* Wild type and *Per2*^{Brdm1} mutant mice were bred as described before and maintained under 12h light-12h dark (LD) conditions or under constant dark conditions (DD). The mice were sacrificed periodically at the required "zeitgeber time" (ZT), timing described as ZT24 (onset of lights period); ZT6 (middle of light period); ZT12 (onset of dark period) and ZT18 (middle of dark period) or at circadian time (CT), time points were taken as described above. SVs were prepared as described in section 3.1.3. All fractions H, P1, P2, LP1 and LP2 were stored in Laemmli for Western blotting or frozen at –80°C.
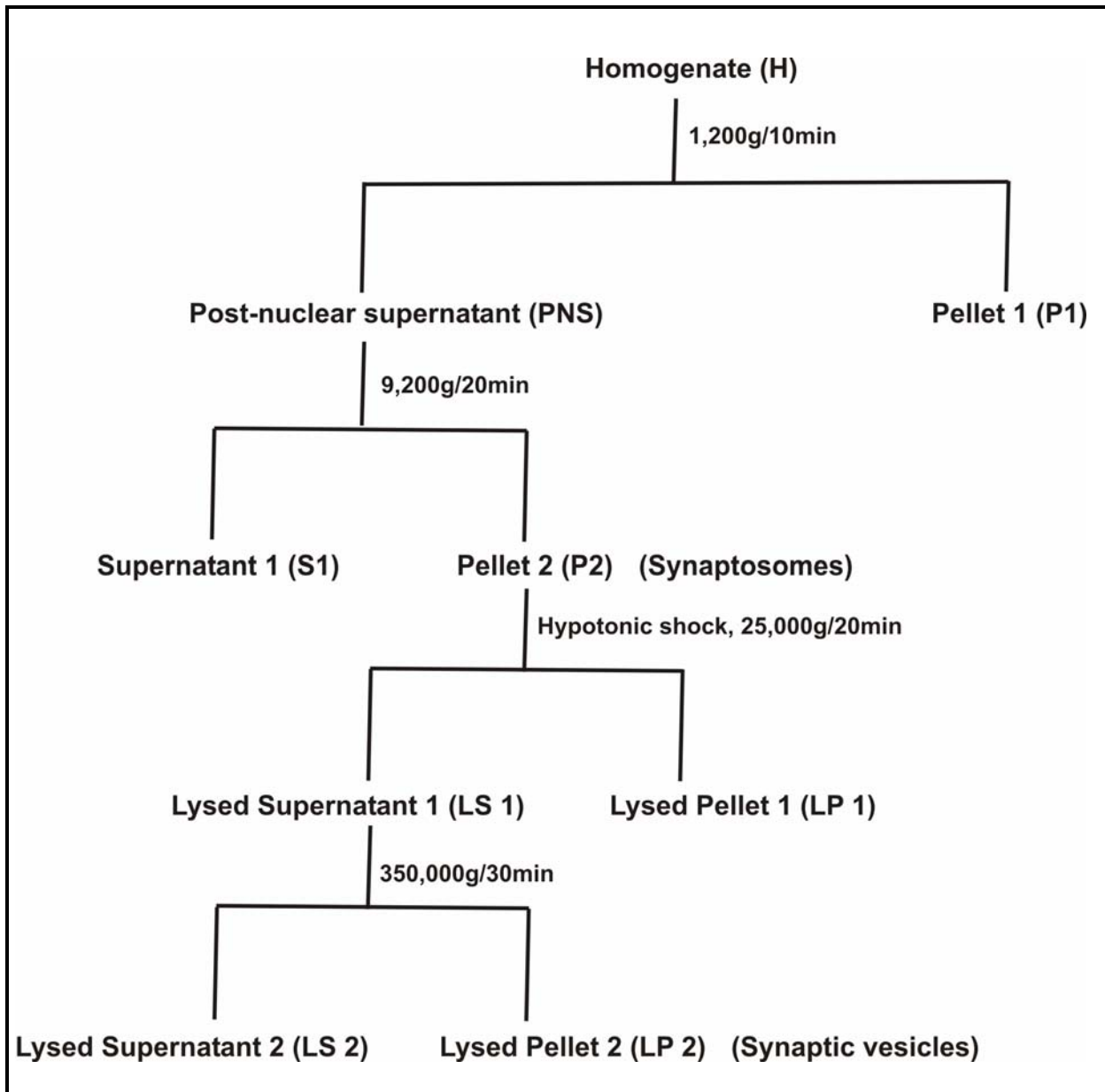
Scheme of sub-cellular fractionations of the rat brain; synaptic vesicle isolation

3.2 Pronase treatment on synaptosomes and SVs

Pronase experiments were done on crude and purified synaptosomes, and SVs prepared from rat and mice whole brain as described in sections 3.1.2, 3.1.3; and on crude synaptosomes prepared from wild type mice which entrained in LD cycle as described in section 3.1.4. Pronase was made as 1 mg/ml in distilled water. Synaptosomes prepared in sodium buffer (see section 2.4) were incubated with 50 µg/ml pronase for 30 minutes at 37°C. To see temperature dependant

digestion, some experiments were also performed at 4°C. Later, pronase was removed by centrifugation at 9000 rpm for 5 min at 4°C in an eppendorf centrifuge. The pellets were resuspended in sodium buffer and stored in Laemmli for analysis on SDS PAGE and immunoblotting.

3.3   SLO and Triton X-100 treatment on intact synaptosomes

SLO permeabilisation: Synaptosomes were prepared from whole rat brain by ficoll gradient as described in 3.1.2. Synaptosomes were resuspended in KR-Hepes buffer and incubated with 100 µl SLO and 1 mM DTT at 4°C for 10 min. Then, SLO was removed by centrifugation at 9000 rpm for 5 min at 4°C in an eppendorf centrifuge. After resuspending all samples in sodium buffer, they were incubated at 37°C for 5 min. These permeabilized synaptosomes were further used for pronase treatment (see section 3.2).

Triton X-100 extraction: Synaptosomes were prepared from whole mouse brain as described in 3.1.1. 1 mg/ml synaptosomes were extracted with 100µl Triton X-100 (from 10% stock) in extraction buffer (see section 2.4) at room temperature, 300 rpm for 1 hr. Sample tubes were packed in ice during incubation. After 1 hr extraction, samples were centrifuged at 9000 rpm for 5 min at 4°C in an eppendorf centrifuge. Supernatant was taken for further pronase experiments.

3.4   Stimulation of synaptosomes with sucrose, ionomycin and α-latrotoxin

Crude and purified synaptosomes prepared from rat or mouse whole brain were used for stimulation. The stimulation was done using either sucrose, ionomycin or α-latrotoxin before subjecting to pronase treatment.

Sucrose stimulation: Rat and mouse synaptosomes (200 µg), prepared in KR-Hepes buffer, were incubated with 0.5 M sucrose for 15 min at 37°C. Later, stimulation was stopped by keeping samples on ice and isoosmolality reduced by diluting samples with excess amount of KR-Hepes buffer. Sucrose was removed by centrifugation at 9000 rpm for 5 min at 4°C in an eppendorf centrifuge.

Ionomycin stimulation: Rat and mouse synaptosomes (200 µg), prepared in KR-Hepes buffer, were incubated with 5 µM ionomycin for 15 min at 37°C. Later, stimulation was stopped by

keeping samples on ice and ionomycin was removed by centrifugation at 9000 rpm for 5 min at 4°C in an eppendorf centrifuge.

α-Latrotoxin stimulation: Rat and mouse synaptosomes (200 µg), prepared in KR-Hepes buffer, were incubated with 5 nM α-latrotoxin for 15 min at 37°C. Later, stimulation was stopped by keeping samples on ice and α-latrotoxin was removed by centrifugation at 9000 rpm for 5 min at 4°C in an eppendorf centrifuge.

3.5 Inhibition of dynamin and AP3 dependent pathways by using dynasore and brefeldin A (BFA) on purified synaptosomes

Purified rat synaptosomes, prepared in KR-Hepes buffer, were incubated with 10 µM BFA (AP3 inhibitor) and 80 µM dynasore (dynamin inhibitor) with or without 0.5 M sucrose for 30 min at 37°C. The reaction was stopped by keeping samples on ice and diluted by adding more volume of KR-Hepes buffer to reduce hyperosmolality. BFA, dynasore and sucrose were removed by centrifugation at 9000 rpm for 5 min at 4°C in an eppendorf centrifuge. These control and treated synaptosomes were resuspended in sodium buffer and further used for pronase treatment.

3.6 Isolation of lipid rafts from purified synaptosomes

Isolation of lipid rafts was carried out as described by Parkin et al., 1999 and Becher et al., 2001. Synaptosomal fractions were prepared by ficoll gradient as described in section 3.1.2. The synaptosomal pellet was resuspended in MBS buffer (see section 2.4). Synaptosomes were prepared in MBS extraction buffer containing either 1% Triton or 2% Lubrol as the final concentrations. The samples were extracted for 1 hr on ice followed by the addition of equal amount (1 ml) of 80% sucrose solution to the extracted samples. A 5-30 % sucrose gradient, containing 1ml each of 5%, 10%, 15%, 25% and 30% sucrose solutions with a supplementation of 0.1% Triton (for Triton extracted samples) or 0.2% Lubrol (for Lubrol extracted samples) in each fraction were prepared in MBS buffer and equilibrated overnight at 4°C. The extracted protein sample was introduced with a glass pipette to the bottom of the gradient carefully without disturbing the equilibrated layers. The gradient was spun at 140,000 x g for 18 hours in a

Beckman SW 40 swing out rotor. 1 ml fractions were harvested from the top of each gradient and were stored in 4 x Laemmli for further analysis on SDS PAGE and immunoblotting.

Depletion of cholesterol by MβCD in purified synaptosomes: Cholesterol depletion was carried out in purified synaptosomes before isolating lipid rafts in some experiments. Purified synaptosomes were resuspended in MBS buffer and incubated with 20 mM MβCD for 30 minutes at 37°C and 300 rpm. Later, MβCD was removed by centrifugation at 9000 rpm for 5 min at 4°C in an eppendorf centrifuge. Finally, synaptosomal pellet was resuspended in 1 ml of MBS buffer and further used for isolation of lipid rafts.

3.7   Protein determination

Protein concentrations were determined according to the Bicinchoninic acid (BCA) method of Smith et al., 1985. A standard curve was prepared using six duplicate serial dilutions of bovine serum albumin (BSA) ranging from 50 μg/ml to 500 μg/ml. BSA was prepared in PBS. Standards and appropriately diluted samples were pipetted in duplicates into the wells of a 96 well microtitre plate (20 μl/well). 200 μl of reaction solution (solution A and solution B (50:1 v/v) was added to each well, and the plate was incubated at 60°C for 20 minutes. The plate was then allowed to cool for 10 minutes before the absorbance of the samples was measured in an ELISA reader at 550 nm. Sample protein concentrations were estimated from the BSA standard curve.

3.8   Protein gel electrophoresis

Protein samples were electrophoresed on a denaturating sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) system under discontinuous conditions according to the method of Laemmli, 1970. The preferred percentage of the polyacrylamide gel depended on the molecular weight of the proteins to be analysed. In most cases, 8%, 10% and 12% gels were used in combination with a 4% stacking gel buffer in all cases to allow optimal separation. A standard low molecular weight (LMW) protein marker was run as a reference. Protein samples to be analysed were dissolved in Laemmli buffer and heated to 90°C for 5-10 minutes prior to loading. The gels were prepared as follows:

Separating gel percentage	12%	10%	8%
Separating gel buffer, pH 8.8 (4X stock)	2.5 ml	2.5 ml	2.5 ml
Acrylamide/bis-acrylamide Stock (40%)	3.0 ml	2.5 ml	2 ml
Water	4.5 ml	5 ml	5.5 ml
TEMED	5 µl	5 µl	5 µl
Ammonium persulfate (APS)	50 µl	50 µl	50 µl
Stacking gel percentage	4%		
Stacking gel buffer, pH 6.8 4X stock	1.25 ml		
Acrylamide/bis-acrylamide Stock (40 %)	0.5 ml		
Water	3.25 ml		
TEMED	2.5 µl		
Ammonium persulfate (APS)	25 µl		

Gels were electrophoresed at 80 V until the samples reached the stacking gel and then were run at a constant voltage of 140 V.

3.9   Western blotting and immunodetection

Proteins were transblotted from SDS gels onto Hybond C nitrocellulose membranes (see section 2.3) at a constant voltage of 25 V/2 gels for 75 minutes in a semi-dry blot transfer chamber using western blotting buffer (see section 2.4) for semi-dry transfer. The nitrocellulose membranes were then immersed in ponceau-s solution (see section 2.4) to visualize the molecular weight marker and to ensure proper protein transfer and integrity, and then destained with distilled water. Using the molecular weight marker as a reference, blots were carefully cut at appropriate heights into strips carrying the protein of interest. These strips were carefully labelled with a pencil, rinsed in TS buffer, and incubated in blocking buffer (see section 2.4) for 1 hr at room

temperature with intermittent changes of blocking buffer. Incubation with the primary antibody was performed at 4°C overnight or for 2 hrs at room temperature in the antibody solution (see section 2.4). On the next day, blots were blocked again for 1 hr in blocking buffer. Incubation with the secondary antibody was performed for 1 hr at room temperature in antibody solution. After secondary antibody incubation, blots were washed for 1-2 hrs in TS buffer with 0.1% Tween with intermittent change for every 20 minutes. All incubations and washes were performed on a shaker. All the immunoblots were invariably incubated with secondary antibodies tagged with HRP (horse radish peroxidase), so ECL detection was used to detect proteins. The blots were bathed in ECL solution for one minute, excess solution was drained off the blots and films were exposed at different time intervals depending on the intensity of the signals. After the development, the blots were eventually dried and stored at 4°C for subsequent use.

3.10 Quantification and calculation of percentage digested proteins after pronase treatment

ECL-developed films were scanned and protein bands were densitometrically quantified using the Labimage 1D 2006 programme purchased from Kapelan Bioimaging, Halle, Germany. Calculation was done by measuring the band intensity from the band of interest. Each experiment was repeated for at least three times. All band intensities were normalized with respect to the control level. Statistical calculations were performed using the two-tailed Student's T-test (Hinz et al., 2001). In pronase digestion experiments, % of protein digestion was estimated by calculating the ratio between ODs of control samples and ODs of pronase treated samples.

4 RESULTS

4.1 Analysis of proteins in various sub-cellular fractions of the brain

Crude synaptosomes (P2) and crude SVs (LP2) were prepared as described in section 3.1. Additionally, an aliquot of homogenate (H) was collected prior to centrifugation for gel analysis. Equal amounts of protein from H, P2 and LP2 fractions were loaded on SDS PAGE and analysed by immunoblotting for synaptic vesicle specific proteins like synaptobrevin (Syb), synaptophysin (Syp), vesicular neurotransmitter transporters like VGLUT1, VGLUT2 and VGAT, and plasma membrane marker like NMDA-Receptor (NMDA-R). It was observed that Syb, Syp, VGLUTs and VGAT were particularly abundant in the LP2 fraction, indicating an enrichment of SVs in this fraction (Fig. 4). On contrary, NMDA-R was less in the LP2 fraction indicating that isolated SVs are almost free from contamination with plasma membrane fractions.
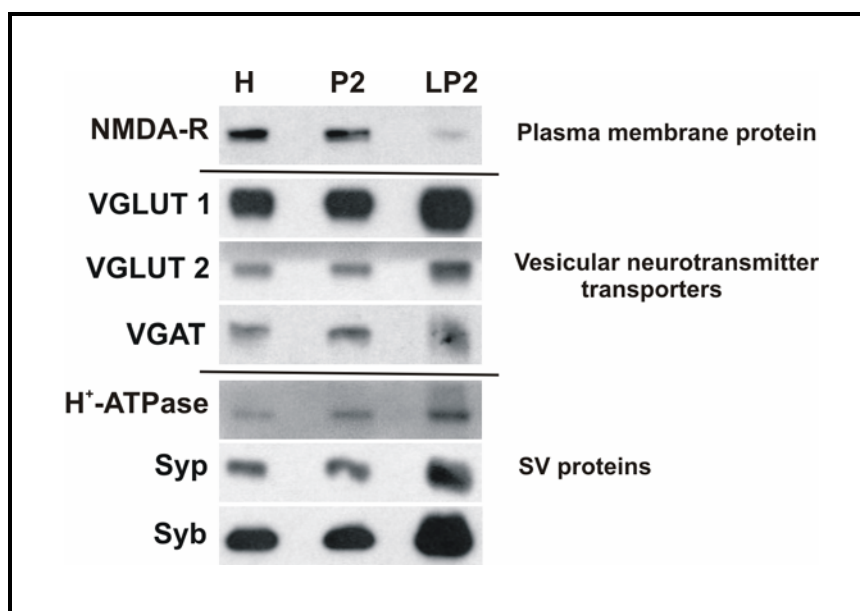
Figure 4: Distribution of proteins in sub-cellular compartments of the brain

Homogenate (H), crude synaptosomal pellet (P2) and synaptic vesicle fraction (LP2) were analysed via SDS PAGE and immunoblotting. Equal amounts of protein were loaded and looked for the distribution of vesicular proteins Syb, Syp and proton pump (H$^+$-ATPase); vesicular neurotransmitter transporters VGLUT1, VGLUT2 and VGAT, and plasma membrane protein NMDA-R.

4.2 Pronase digestion of synaptosomes to study the translocation of vesicular proteins to the plasma membrane

SVs locally recycle within the pre-synaptic terminal for multiple rounds of neurotransmitter release (Heuser and Reese, 1973 and Ceccarelli et al., 1973). This involves a fusion process, in which the luminal peptide chains of vesicular membrane proteins are transiently exposed to the extracellular space. It has recently been suggested that synaptotagmin Ia and Syb interchange with a large pool on the cell's axonal surface (Willig et al., 2006 and Alfonso et al., 2006). It could be said that besides occurrence on SVs, VGLUT may also function as sodium phosphate transporter at the plasma membrane, however physiological relevance for a switch between these membrane compartments is lacking. Taken together, it seems that some of the synaptic vesicle proteins localise at the plasma membrane at least transiently during periods of changed synaptic activity. Therefore, to analyse changes in the association of vesicular proteins with the plasma membrane, pronase treatment of synaptosomes prepared from adult rat or mice whole brains was established. Pronase is a mixture of proteolytic enzymes that cleaves specifically the peptide chains of membrane proteins facing the extracellular space. Initially, control experiments were performed to determine the optimum pronase concentration, time of incubation and its entry into the cytosolic compartment.

4.2.1 Characterisation of pronase treatment

Synaptosomes isolated from whole rat brains were resuspended in sodium buffer and treated with pronase as described in section 3.2. In pronase digestion experiments, Rab3, and NMDA-R and Na^+/K^+-ATPase proteins were taken as negative and positive controls, respectively, since Rab3 is not accessible to pronase whereas NMDA-R and Na^+/K^+- ATPase are more accessible for pronase digestion.

Different concentrations of pronase ranging between 75, 50, 25 and 12.5 µg/ml were applied to 200 µg synaptosomes for 30 min at 37°C, 300 rpm. It was observed that 50 µg/ml pronase led to a significant digestion of NMDA-R and VGLUTs (Fig. 5A). Similarly, pronase incubation time was optimised by treating 200 µg of synaptosomes with 50 µg/ml pronase for 15, 30, 45 and 60 min, respectively. Results from Fig. 5B indicate that 30 min incubation period

yielded a significant digestion of NMDA-R. Hence, in all further experiments 50 µg/ml pronase and 30 min incubation time was used.
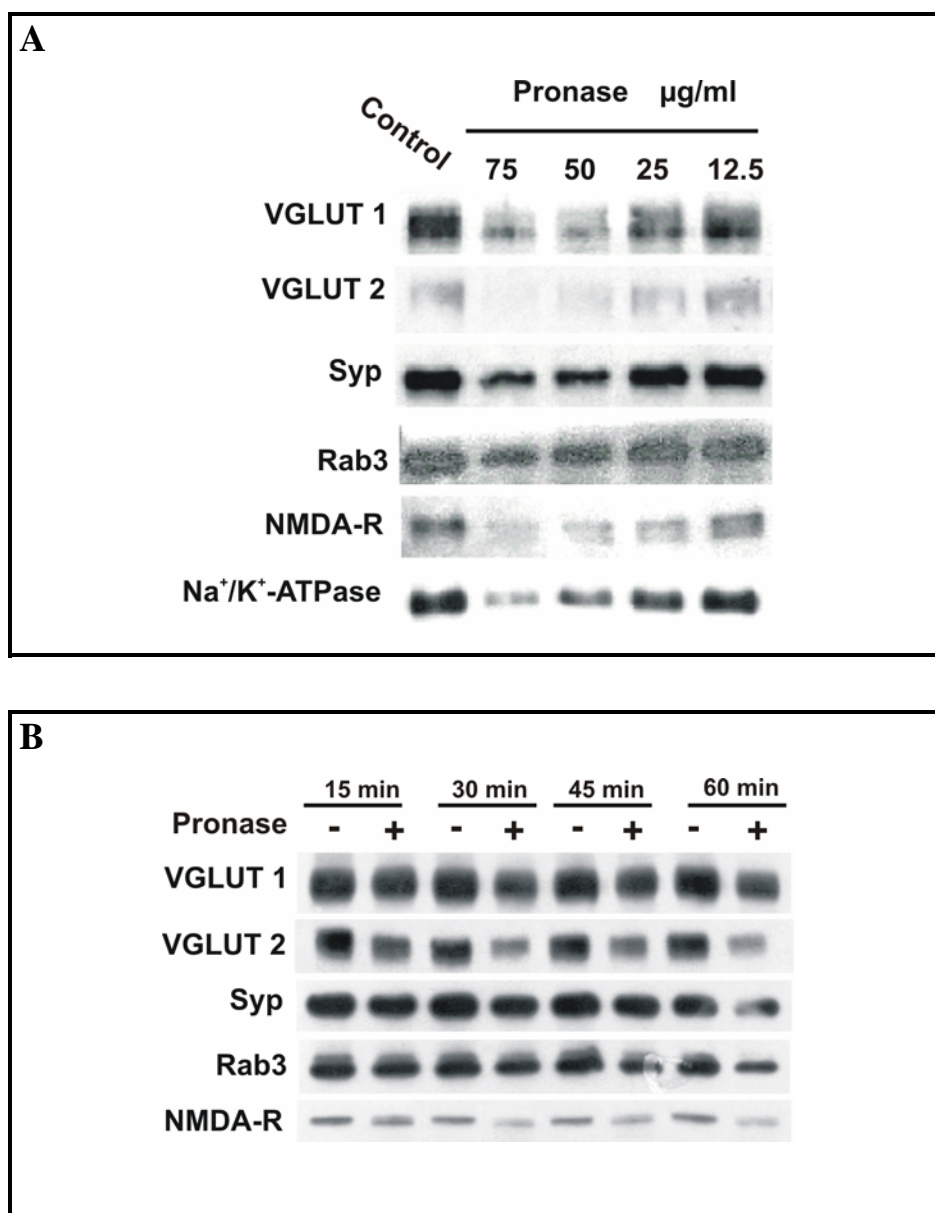
Figure 5: Pronase treatment: variation of concentration (A) and time of incubation (B)

A. Synaptosomes prepared from whole rat brain were incubated with different amounts of pronase (75, 50, 25 and 12.5 µg/ml) for 30 min at 37°C. After pronase treatment, control and pronase treated samples were loaded on SDS PAGE and transferred to nitrocellulose membranes. The blots were analysed using antibodies against the indicated proteins. Rab3, and NMDA-R and Na^+/K^+-ATPase were used as negative and positive controls, respectively. Note that the optimum digestion of VGLUTs was obtained with pronase 50 µg/ml

B. Synaptosomes prepared from whole rat brain were incubated with 50 µg/ml pronase at different incubation times 15, 30, 45 and 60 min at 37°C. After pronase treatment, control and pronase treated samples were loaded on SDS PAGE and transferred to nitrocellulose. The blots were analysed using antibodies against the indicated proteins. Rab3, and NMDA-R and Na$^+$/K$^+$-ATPase were used as negative and positive controls, respectively. Note that the considerable digestion of VGLUTs was obtained after 30 min of incubation with pronase.

In the next control experiments, to analyse the effects of pronase leakage into the cytosolic compartment of synaptosomes, pronase digestion was performed on intact, SLO permeabilised, cholesterol depleted or triton extracted synaptosomes from adult rat and mice whole brain.

Pronase digestion on intact and differentially treated synaptosomes

To determine whether pronase respects the plasma membrane, synaptosomes were permeabilised with SLO. It is known that SLO forms monomers by binding to cholesterol domains at the plasma membrane at 4°C and after incubation at 37°C these monomers generate pores at the plasma membrane which lead to pronase entry into the plasma membrane. Accordingly, 200 µg synaptosomes were extracted with or without Tritin X-100 and digested with pronase. Triton X-100 disrupts the plasma membrane and extracts all proteins including SV proteins.

Purified synaptosomes were prepared from rat whole brain as described in section 3.1.2 and permeabilised by incubating them with or without SLO as described in section 3.3. These permeabilised and non-permeabilised synaptosomes were further incubated with pronase. Equal amounts of protein from all fractions were separated on SDS PAGE and analysed via immunoblotting. As shown in Fig. 6A, all synaptic vesicular proteins including SV associated protein Rab3 were completely digested in permeabilised samples compared to non-permeabilised samples. Similar results were obtained when pronase was applied on triton extracted synaptosomes (Fig. 6B). These results indicate that pronase does not cross the plasma membrane so that it can not enter into the cytoplasm.
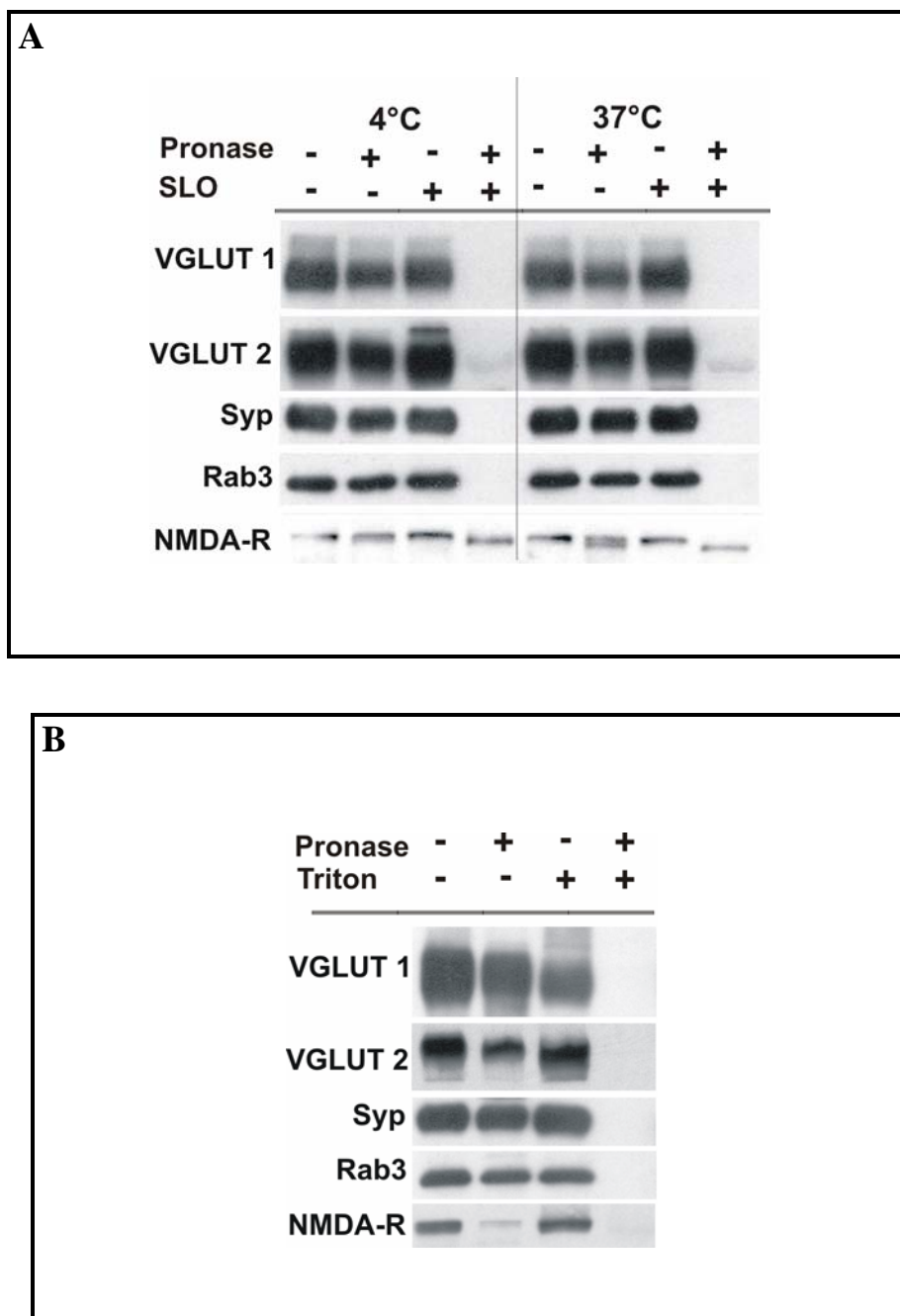
Figure 6: Pronase treatment on intact, permeabilised or triton extracted synaptosomes

A. Synaptosomes were subjected to permeabilisation with SLO before incubation with or without pronase at 4°C and 37°C. After SDS PAGE and transfer to nitrocellulose, blots were analysed using antibodies against the indicated proteins. Note that complete digestion of all vesicular proteins was obtained in permeabilised synaptosomes compared to intact synaptosomes at both temperatures.

B. Synaptosomes prepared from mouse whole brain were extracted with or without 1% Triton X-100 before they were incubated with or without pronase for 30 min at 37°C. After SDS PAGE and transfer to nitrocellulose the blots were analysed using antibodies against the indicated proteins. Note that the complete digestion of all vesicular proteins was obtained in triton extracted synaptosomes compared to intact synaptosomes.

In another approach, pronase assay was performed on cholesterol depleted synaptosomes to validate pronase leakage into the plasma membrane. Cholesterol is a basic component of the plasma membrane, and depletion of cholesterol with methyl-β-cyclodextran (MβCD) dissociates plasma membrane structure (Haynes et al., 1997). Purified synaptosomes prepared from adult rat brain were pretreated with or without 40 mM MβCD at 4°C and 37°C followed by pronase digestion. All fractions were loaded on SDS PAGE and analysed by immunoblotting. As shown in Fig. 7, all synaptic vesicular proteins including SV associated protein Rab3 were completely digested in cholesterol depleted fractions compared to non depleted fractions incubated at 37°C. On contrary, there was no digestion of vesicular proteins in cholesterol depleted synaptosomes which were incubated at 4°C.
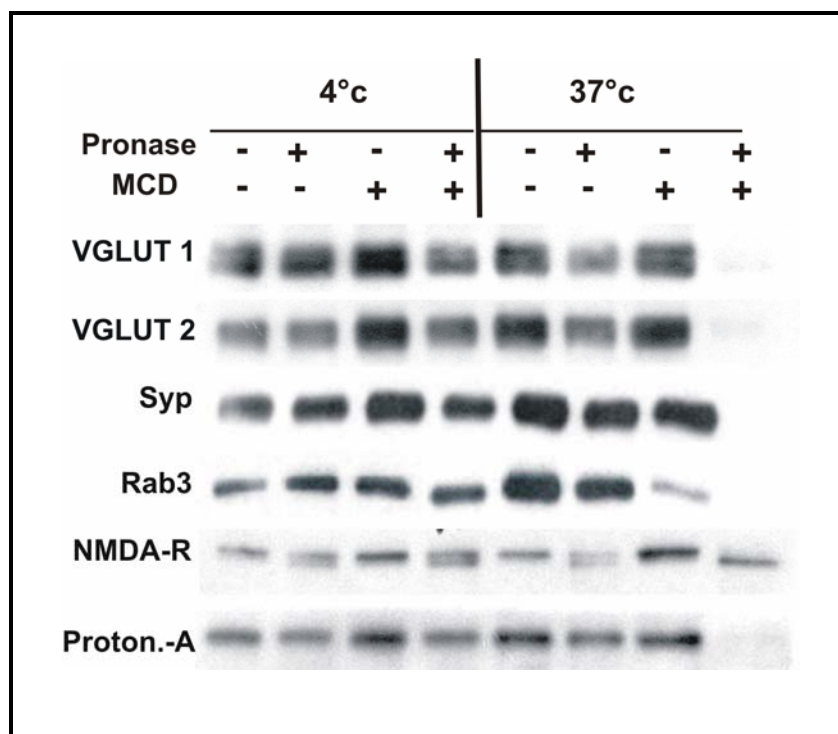
Figure 7: Effect of pronase on cholesterol depleted synaptosomes

Synaptosomes from rat brain were treated with or without MβCD to deplete cholesterol prior to pronase treatment for 30 min at 4°C and 37°C. Synaptosomes were collected by centrifugation and analysed via SDS PAGE and immunoblotting. Note that all vesicular proteins were digested in MβCD treated synaptosomes incubated at 37°C.

Pronase application on isolated SVs

Since pronase respects the plasma membrane, pronase was directly applied on SVs at 4°C and 37°C to check how efficiently pronase digests SV proteins. SVs were isolated from whole adult rat brain and followed by incubation with pronase as described in section 4.1.3. Equal amounts of protein were loaded on SDS PAGE and analysed by immunoblotting. As shown in Fig 8, direct access of pronase to SVs led to more than 80% digestion of all vesicular proteins including Rab3 (negative control) at both temperatures.
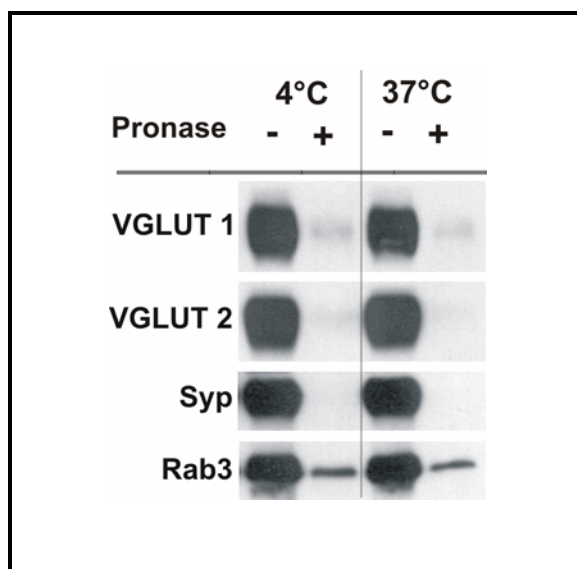
Figure 8: Direct access of pronase to SVs

SVs prepared from whole rat brain were incubated with or without pronase for 30 min at 4°C and 37°C. After pronase treatment, control and pronase treated samples were analysed via SDS PAGE and immunoblotting. The blots were analysed using antibodies against the indicated proteins. Note that direct access of pronase to SVs led to 90% digestion of all vesicular proteins at both temperatures.

4.2.2 Quantification of vesicular proteins at the plasma membrane under resting conditions in rat and mouse synaptosomes

To identify vesicular proteins which are translocated to the plasma membrane during/after exocytosis, pronase assay was done on synaptosomes prepared from rat (crude as well as purified synaptosomes) and mouse whole brain. SVs orient themselves in an inside out fashion on plasma membrane. Translocation of SVs to the plasma membrane exposes the inside of the vesicle membrane thus allowing the exposed protein molecules for protease cleavage. After incubation for 30 min at 37°C, the synaptosomes were collected by centrifugation and analysed via SDS PAGE and immunoblotting. The blots were analysed using antibodies against the respective proteins.

Quantification was performed by calculating % of digestion of all indicated proteins. Significant amounts of vesicular proteins VGLUTs and synaptotagmin (Syt), and plasma membrane proteins NMDA-R and Na^+/K^+-ATPase were digested compared to the proton pump (116 kDa subunit) and Rab3 which are used as internal references. Considerable amounts (30-40%) of VGLUT1 and VGLUT2 were digested while Rab3 and the proton pump digestion was about 10% and 5%, respectively. No increase in digestion of Syp could be observed compared to both standards. On the other hand, Syt digestion was enhanced suggesting its presence at the plasma membrane under resting conditions. As expected, the plasma membrane associated NMDA-receptor and the Na^+/K^+-ATPase were digested by more than 60% (Fig. 9B). Comparable data were obtained when using mouse synaptosomes (Fig. 10). All statistical values were performed in a combination of three individual experiments (n=3). These results for crude as well as purified rat and mouse synaptosomes indicate considerable amounts of VGLUTs and Syt occurring at the plasma membrane under resting conditions during/after exocytosis.
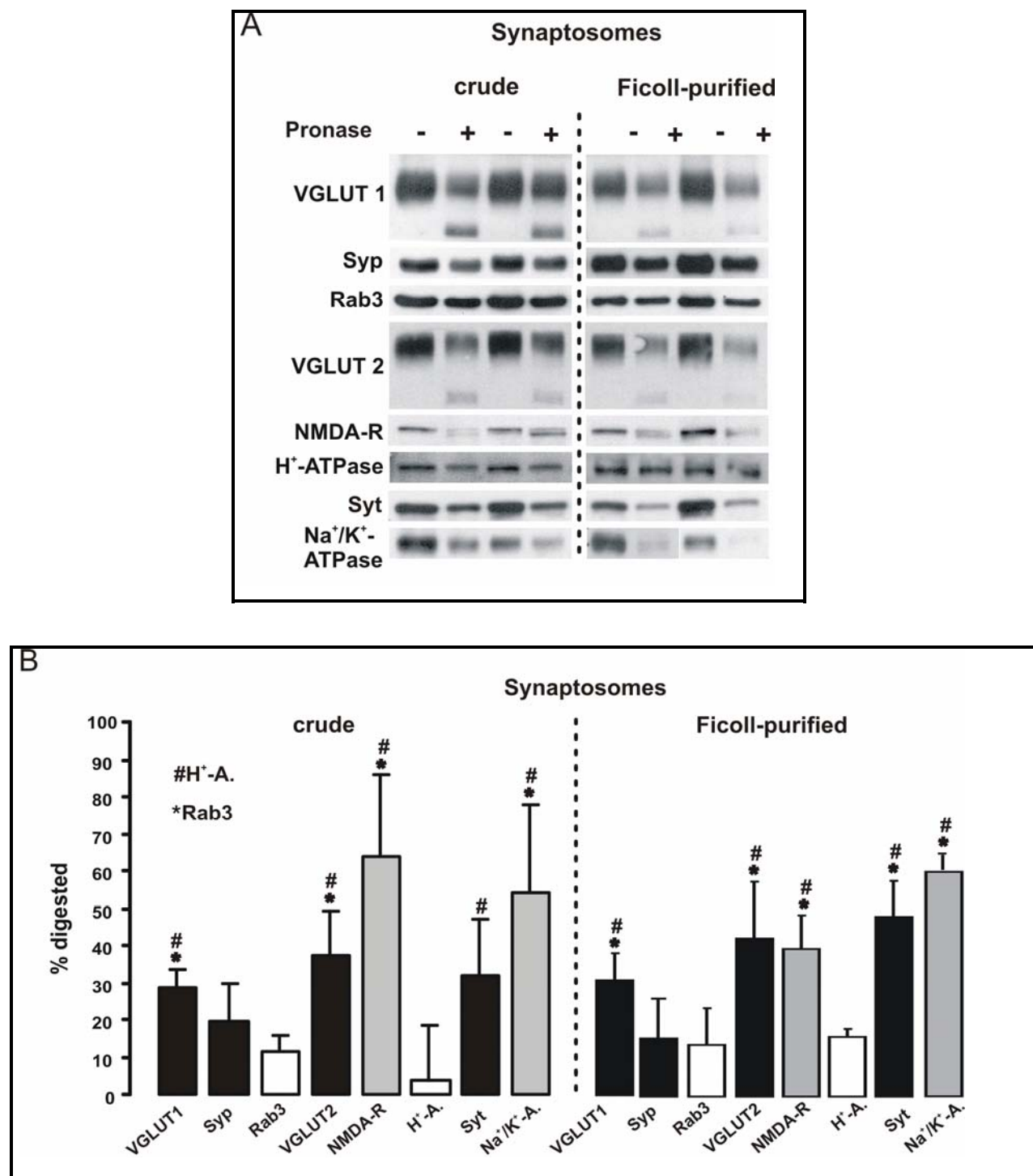
Figure 9: Quantification of the amount of proteins digested by pronase in rat synaptosomes

A. Crude and purified synaptosomes, prepared from whole rat brain, were incubated with pronase. Control and pronase treated samples were analysed via SDS PAGE and immunoblotting. Antibodies against the indicated proteins were used for analysis. Immunoblots represent the two different experiments.

B. Quantification was performed as % of digestion and amounts of digested proteins given in % of untreated samples. Values are obtained from 6 different experiments of crude rat synaptosomes and 3 different experiments of purified rat synaptosomes. * and **#** indicate significant (p < 0.05, according to Student´s t-test) digestion with respect to Rab3 and proton pump (H$^+$-A) which were used as internal standards.

Mouse synaptosomes
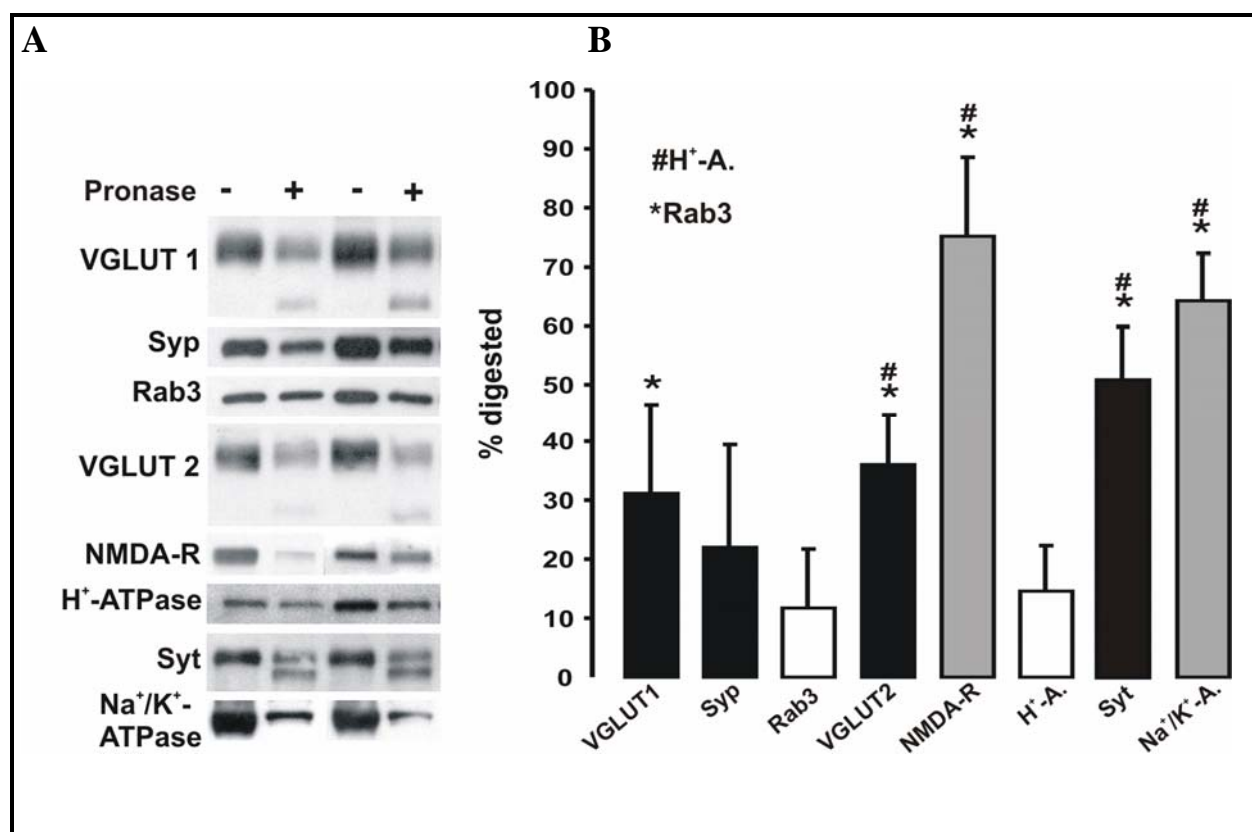
Figure 10: Quantification of the amount of proteins digested by pronase in mouse synaptosomes

A. Crude synaptosomes, prepared from whole mouse brain, were treated with pronase. Control and pronase treated samples of two different experiments were analysed via SDS PAGE and immunoblotting. Antibodies against the indicated proteins were used for analysis.

B. Amounts of digested proteins given in % of untreated samples. Values are obtained from 6 different experiments of mouse synaptosomes. * and **#** indicate significant (p < 0.05, according to Student´s t-test) digestion with respect to Rab3 and proton pump (H$^+$-A) which were used as internal standards.

4.3 Stimulation dependent translocation of vesicular proteins to the plasma membrane in rat and mouse synaptosomes

To see whether dynamic switching of vesicular proteins is distinguishable, stimulation of synaptosomes was performed. Following stimulation an increase in the digestion of most of the vesicular proteins should occur. Synaptosomes were stimulated with different substances like 0.5 M sucrose, α-latrotoxin and ionomycin prior to pronase treatment. Typically, stimulation of synaptosomes should increase the amount of vesicular proteins appearing at the plasma membrane. It was imperative to know whether digestion of vesicular proteins could be enhanced upon stimulating synaptosomes prior to pronase treatment.

4.3.1 Translocation due to stimulation by sucrose

Sucrose stimulates exocytosis of not only the readily releasable pool but also the reserve pool of SVs. The harsh stimulation causes considerable amounts of synaptic vesicles to fuse with the plasma membrane making the luminal domains of their transmembrane proteins accessible to plasma membrane and more susceptible to pronase digestion.

Synaptosomes, prepared from adult rat and mouse brain, were stimulated with 0.5 M sucrose for 15 min at 37°C prior to pronase treatment. After pronase treatment, all fractions were analysed for the indicated proteins. As can be seen from Fig. 11, sucrose stimulation increased considerably the digestion of VGLUTs, Syp and Syt compared to resting condition. In contrast, amounts of Rab3, which is an internal standard for digestion, and amounts of plasma membrane associated NMDA-receptor and the Na^+/K^+-ATPase digestion, positive controls for digestion, did not show any increase following stimulation. Similar results were obtained when mouse synaptosomes were used. These results confirmed that pronase digestion allows detecting changes of the translocation of vesicular proteins to the plasma membrane between resting conditions and after stimulation.
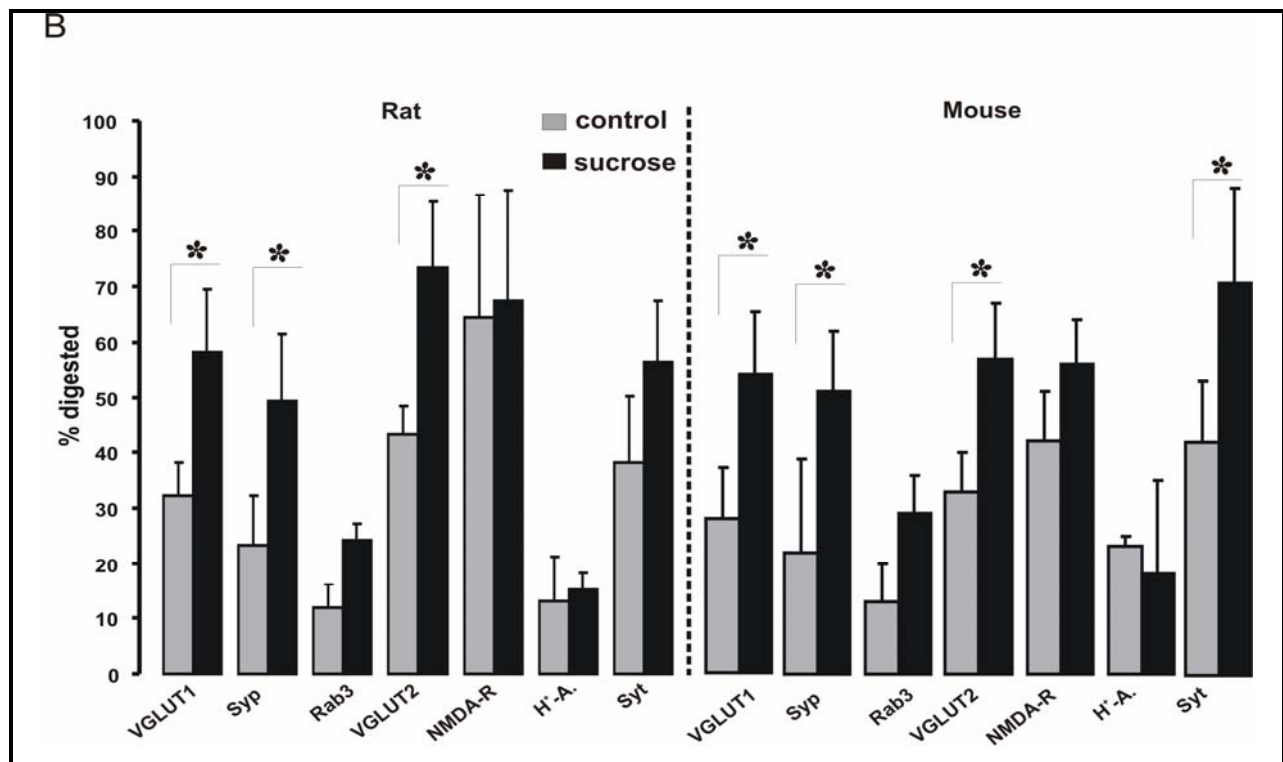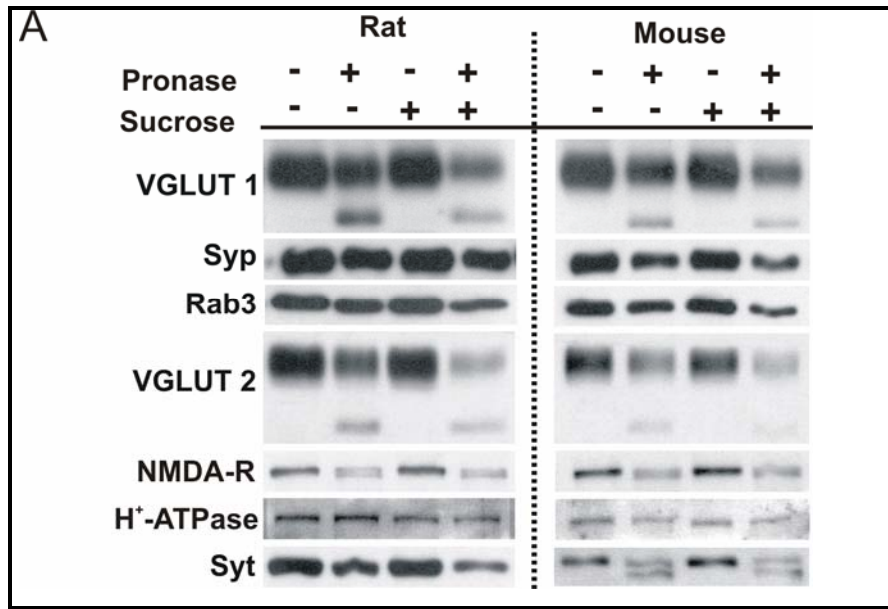
Figure 11: Effect of sucrose stimulation on translocation of VGLUTs in rat and mouse synaptosomes

A. Crude synaptosomes, prepared from whole rat and mouse brain, were stimulated with 0.5 M sucrose for 15 min at 37°C. The synaptosomes were collected by centrifugation and subjected to pronase digestion. After SDS PAGE and transfer to nitrocellulose, blots were analysed using antibodies against the indicated proteins.

B. Amounts of digested proteins given in % of untreated samples. Values are obtained from 4 different experiments. * indicates significant ($p < 0.05$, according to Student´s t-test) digestion of VGLUT1, VGLUT2, Syp and Syt proteins after sucrose stimulation compared to non stimulated samples. Note that along with VGLUTs and Syt, significant digestion of Syp was also observed under stimulation condition compared to resting conditions.

4.3.2   Translocation following stimulation by α-latrotoxin and ionomycin

Subsequent to confirmation of increased the translocation of vesicular proteins following sucrose stimulation; attempts were made to know the effects of other stimulating conditions like α-latrotoxin and ionomycin prior to pronase treatment. α-Latrotoxin binds to high affinity receptors in nerve terminals and causes massive stimulation of neurotransmitters release by exocytosis (Frontali et al; 1976; Tzeng et al; 1978). Ionomycin acts as a Ca^{+2} ionophore by inserting into the plasma membrane and allows direct calcium entry and neurotransmitter release independent of endogenous ion channel-mediated mechanisms.

Synaptosomes, prepared from adult rat brain, were stimulated with 5 nM α-latrotoxin and 5 µM ionomycin in KR-Hepes buffer for 15 min at 37°C prior to pronase treatment. After pronase incubation for 30 min at 37°C, all fractions were analysed for respective proteins. As can be seen from Fig. 12, there was a slight increase in the digestion of VGLUTs and Syp in synaptosomes stimulated with α-latrotoxin and ionomycin compared to control conditions. In contrast to sucrose stimulation, VGLUT digestion was not significantly increased in synaptosomes stimulated with α-latrotoxin and ionomycin. Since these experiments were carried out without calcium in KR-Hepes buffer, further experiments will be needed to optimise the conditions for stimulation with α-latrotoxin and ionomycin in presence of calcium.
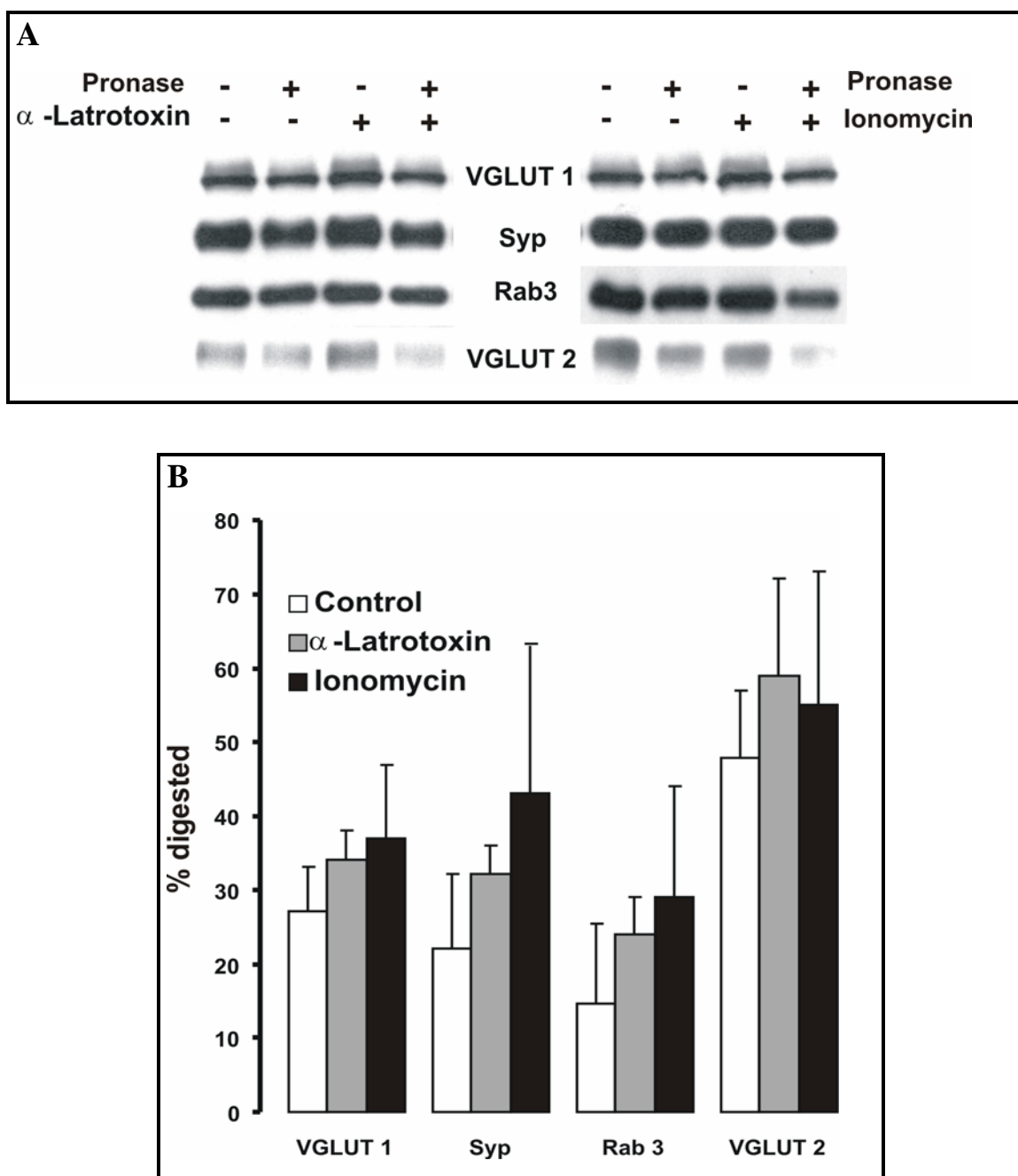
Figure 12: Effect of α-latrotoxin and ionomycin stimulation on translocation of vesicular proteins

A. Crude synaptosomes, prepared from whole rat brain, were stimulated with 5 nM α-latrotoxin and 5 µM ionomycin separately for 15 min at 37°C. The synaptosomes were collected by centrifugation and subjected to pronase treatment. After SDS PAGE and transfer to nitrocellulose, the blots were analysed using an antibody against the indicated proteins.

B. Amounts of digested proteins given in % of untreated samples. Values are obtained from 3 different experiments. In contrast to sucrose stimulation, there was no significant change in the digestion of vesicular proteins after pronase treatment in synaptosomes followed by α-latrotoxin and ionomycin stimulation.

4.3.3 Translocation of vesicular proteins following application of db-cAMP

Db-cAMP is an analogue of cyclic AMP and is believed to enter cells more readily on account of its greater hydrophobicity. It has been shown that cAMP increases translocation of Na-K-2Cl cotransporter (NKCC2) in kidney by a mechanism involving vesicle-associated membrane protein (Syb) and that NKCC2 trafficking to the apical membrane is involved in the stimulation of thick ascending limb of Henle's loop (TAL) NaCl absorption by cAMP (Ortiz, 2005). Under these conditions, it is of significance to know whether cAMP dependent signal transduction pathways play any role in the translocation of VGLUTs to the plasma membrane. Accordingly, experiments were carried out to know whether the cell-permeable cAMP analogue db-cAMP would increase translocation of vesicular proteins to the plasma membrane.

Crude rat synaptosomes, prepared in KR-Hepes buffer, were incubated with 2 mM db-cAMP for 2 hrs at 37°C prior to pronase treatment. The synaptosomes were collected by centrifugation and subjected to pronase treatment. All fractions were analysed for the respective proteins. As shown in Fig. 13, there was no significant digestion of vesicular proteins in db-cAMP treated fractions compared to control conditions. These results indicate that cAMP dependent pathways appear not to be involved in the translocation of VGLUTs to the plasma membrane.
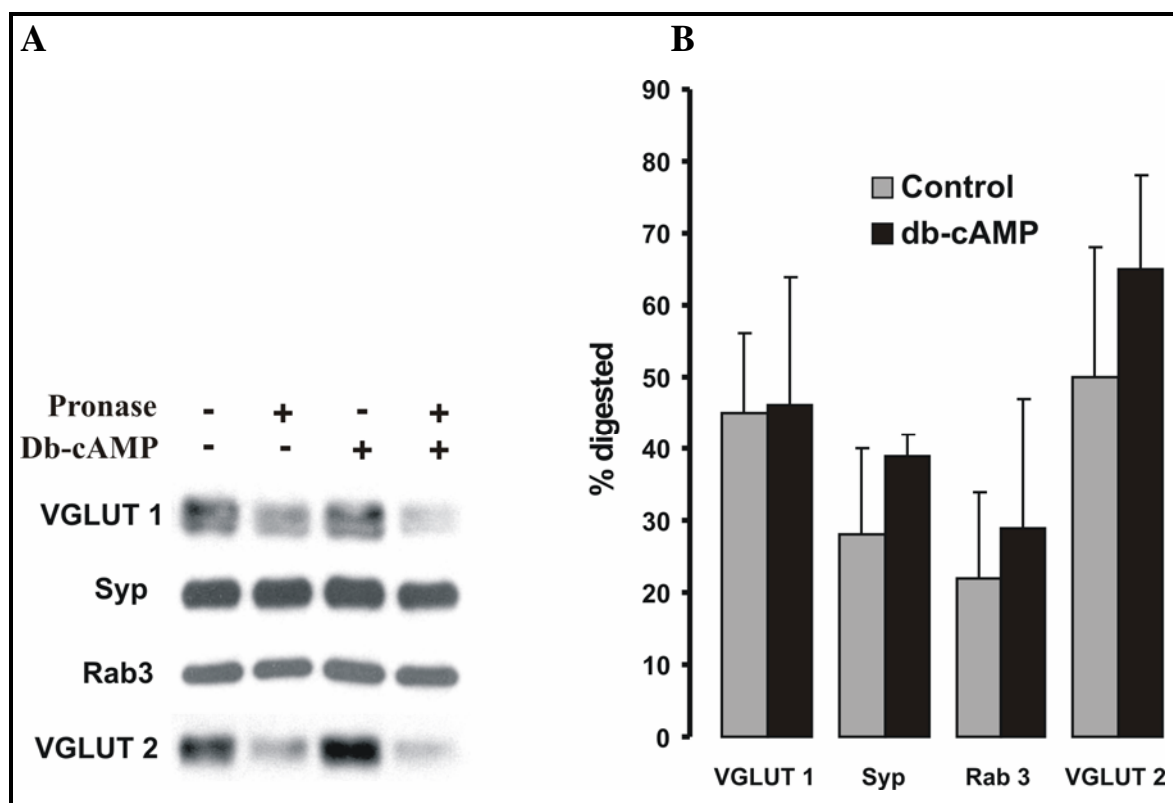
Figure 13: Effect of db-cAMP treatment on translocation of VGLUTs in rat synaptosomes

A. Crude synaptosomes, prepared from whole rat brain, were stimulated with 2 mM db-cAMP for 2 hrs at 37°C. The synaptosomes were collected by centrifugation and subjected to pronase treatment. After SDS PAGE and transfer to nitrocellulose, the blots were analysed using an antibody against the indicated proteins.

B. Amounts of digested proteins given in % of untreated samples. Values are obtained from 3 different experiments. In contrast to sucrose stimulation, there was no significant digestion of vesicular proteins after pronase treatment in synaptosomes followed by db-cAMP treatment.

4.3.4   Translocation of vesicular proteins in the presence of BFA and dynasore

Subsequent to confirmation of appearance of VGLUTs and Syt at the plasma membrane by sucrose stimulation, experiments were done in an effort to find any difference in translocation of VGLUTs to the plasma membrane by inhibiting different endocytic pathways using BFA (AP3 pathway inhibitor) and dynasore (dynamin inhibitor). Since dynasore arrests the formation of endocytic clathrin-coated pits and vesicles (Macia et al., 2006), and VGLUT1 levels are reduced

in the synaptic-like microvesicles of PC12 cells after treatment with BFA (Salazar et al; 2005), an increase of synaptic vesicular proteins could be expected at the plasma membrane after exocytosis. Accordingly, experiments were planned to find out whether the digestion of vesicular proteins is enhanced after pronase treatment in the presence of BFA and dynasore.

Purified rat synaptosomes, prepared in KR-Hepes buffer, were incubated with 10 µM BFA and 80 µM dynasore with or without 0.5 M sucrose for 30 min at 37°C prior to pronase treatement. After pronase treatment, all fractions were analysed for the indicated proteins. As Fig. 14 depicts, there was no further increase in digestion of vesicular proteins including VGLUTs in the presence of BFA and dynasore in synaptosomes under rest as well as in stimulation conditions. Although, these results were obtained from two different experiments, further investigation needed to check the translocation of vesicular proteins in different stimulation conditions (ionomycin or α-latrotoxin) in the presence of BFA and dynasore.
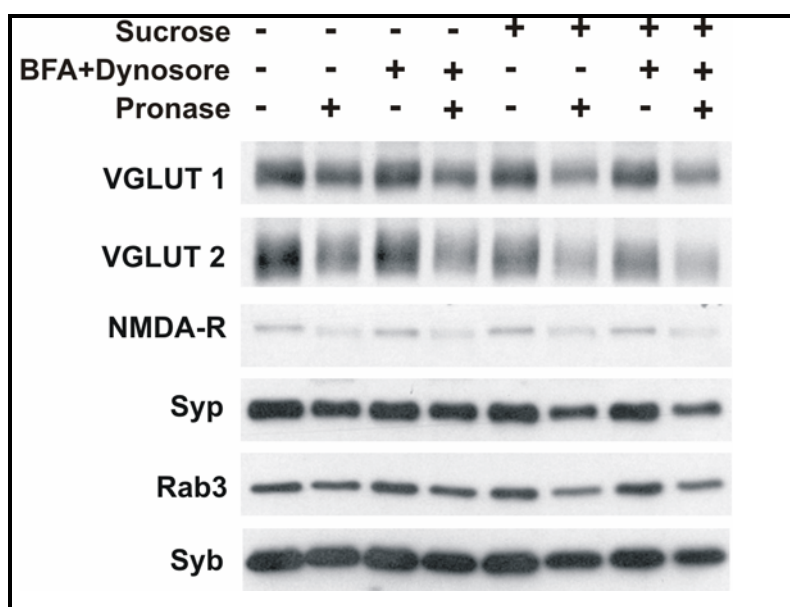
Figure 14: Translocation of vesicular proteins in the presence of BFA and dynasore

Purified synaptosomes, prepared from adult rat brain, were stimulated with or without sucrose in the presence or absence of BFA and dynasore prior to pronase treatment for 15 min at 37°C. After pronase treatment for 30 min at 37°C, synaptosomes were collected by centrifugation and analysed via SDS PAGE and immunoblotting. Note that there was no further increase in the digestion of VGLUTs after inhibiting dynamin by dynasore and AP3 pathway by BFA in synaptosomes followed by sucrose stimulation.

4.4   Distribution of VGLUTs in lipid raft like domains

A lipid raft is a cholesterol-enriched microdomain in cell membranes. Cholesterol is an abundant lipid present in SVs and is not evenly distributed but enriched in membrane patches referred to as detergent resistant membranes (DRMs). It is implicated in the regulation of certain signal transduction and membrane traffic pathways. A recent report indicated that glutamate transporters are associated with cholesterol-rich lipid raft microdomains of the plasma membrane (Butchbach et al, 2004) and such association is of great importance for their function. In this background, it gains credence to know if VGLUTs distribute in cholesterol enriched lipid rafts and influence changes in the trafficking pathways of VGLUTs.

To test this distribution of VGLUTs in lipis rafts, lipid rafts were isolated using triton from purified synaptosomes with or without depletion of cholesterol by MβCD treatment. These detergents isolate multimeric complexes present on the SVs. In order to differentiate between lubrol rafts and triton rafts a comparable study was made and we were able to isolate rafts successfully with lubrol detergent. Detergent insoluble fractions from synaptosomes were prepared as described in section 3.6. When samples were centrifuged, DRMs (because of their inherent low density) migrate up the sucrose gradient leaving the bulk of the solubilised membrane proteins at the end of the gradient. A 5-30% (w/v) sucrose gradient was used in our method where the majority of cellular proteins remain in the 40% sucrose; further rest of the protein is located in the insoluble pellet. Different markers were used to differentiate raft from non-raft fractions. Flotilin was used as a raft marker which encodes a caveolae associated, integral membrane protein and Na^+/K^+-ATPase as a non-raft marker as it also serves as an internal control since it is a plasma membrane protein and thus differentiates plasma membrane proteins from vesicle proteins. As seen in Fig. 15, synaptosomal fractions extracted with lubrol show that part of VGLUT1 and Syp are present on the raft fraction (8 fractions from the top) and Syb was found on the non raft fractions. In contrast, in MβCD treated synaptosomal fractions, all indicated proteins, except part of the VGLUT1, had shifted to non raft fractions (9-13 fractions). However, these results were based on a single experiment and further standardisations are necessary to optimise the conditions for the isolation of lipid rafts with or without MβCD treatment.
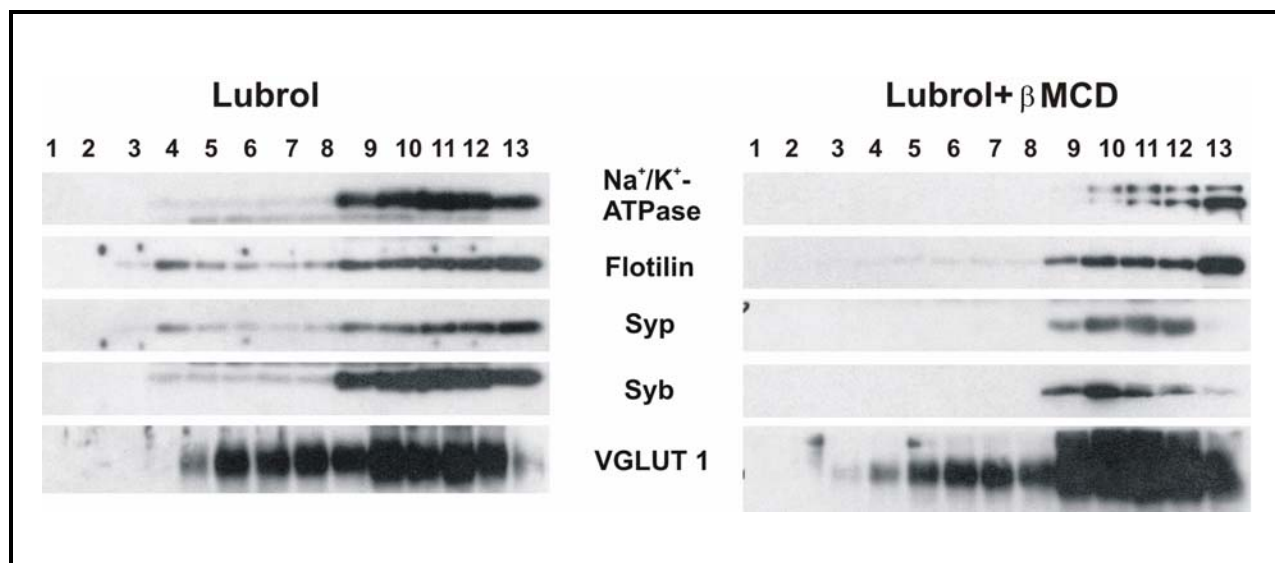
Figure 15: Lipid raft fractions from synaptosomes extracted with 2% Lubrol

Synaptosomal fractions from adult rat brain were treated with or without MβCD prior to extraction with 2% Lubrol. The extracts were loaded on 5-30% sucrose gradients supplemented with 0.2% Lubrol in each fraction. 1ml fractions were harvested from top of the gradients and samples were analysed via SDS PAGE and immunoblotting using antibodies against the non-raft marker Na$^+$/K$^+$-ATPase, raft marker flotilin, and other vesicular proteins Syp, Syb and VGLUT1.

4.5   Time-dependent trafficking of vesicular proteins in wild type mice entrained in 12 hr light-12 hr dark cycle (LD cycle)

4.5.1   Analysis of synaptic vesicle proteins in wild type and *Per2^Brdm1* mutant mice entrained in LD cycle

Previously, experiments were done to study the physiological aspects of synaptic vesicle proteins and transporters by using time-dependent wild type and *Per2^Brdm1* mutant models (Yalamanchilli et al., 2006). *Period (Per)* genes are involved in the regulation of the circadian clock and modulate several brain functions. SVs were prepared from wild type and *Per2^Brdm1* mutant mice at various time points over 24 hrs entrained in LD cycle (ZT24/ZT0, ZT6, ZT12 and ZT18) (see section 3.1.4). Synaptic vesicular fractions were analysed via SDS PAGE and immunoblotting

for synaptic vesicle proteins Syb, Syt, Syp and for vesicular transporter VGLUT1. All statistical values were performed in a combination of three individual experiments (n=3). All vesicular proteins analysed were not significantly altered in their expression patterns in between various time points or between the genotypes (Yelamanchili et al., 2006). However, the expression patterns of the transporter VGLUT1 were observed to be strikingly rhythmic in wild type animals which is absent from *Per2^{Brdm1}* mutants (Fig. 16A). High expression of VGLUT1 was seen at ZT0 and ZT12 whereas significantly lower expression was seen at ZT6 and ZT18. The significant expression of VGLUT1 at ZT0 and ZT12 in wild type animals was lost in *Per2^{Brdm1}* mutant mice implicating a possible physiological role of clock genes in the regulation of vesicular glutamate transporters (Fig. 16B). Time-dependent cycling of VGLUT1 was not seen when analysing homogenate or synaptosomes, the starting fractions for vesicle preparation. Time-dependent cycling of VGLUT1 was also not reflected at the mRNA level *(personal communication: Yelamanchili thesis).*
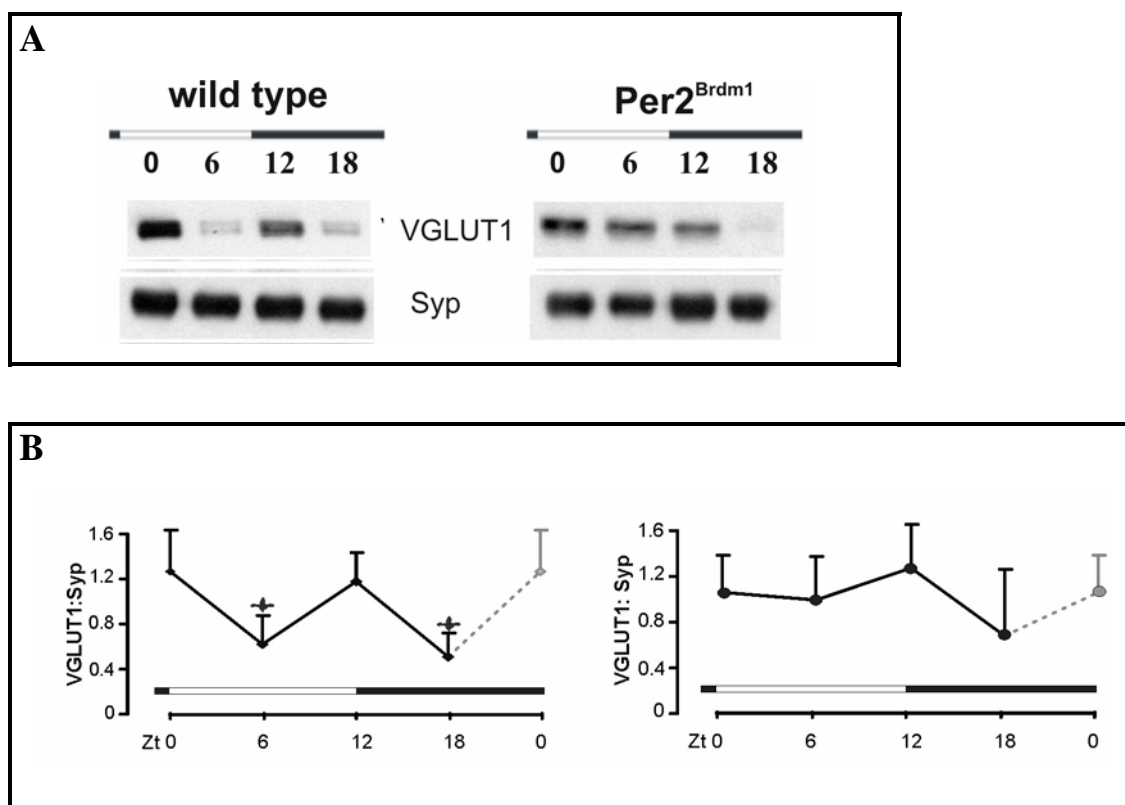
Figure 16: Oscillation of VGLUT1 amount in SVs under light/dark condition

A. SVs were prepared at the indicated time points from wild type and *Per2^Brdm1^* mutant mice. ZT24/0 represents dawn at 6.00 am, before the light was switched on, ZT6 represents noon at 12.00 pm, ZT12 dusk at 6.00 pm (before the light was switched off) and ZT18 night at 12 am. The vesicular membrane fractions were analysed via SDS PAGE and immunoblotting for the indicated proteins. Syp is used as internal reference.

B. Quantification was performed by calculating the ratio of VGLUT1 to Syp. Values are obtained from three to five different sets of experiments. * denotes significance (p< 0.05 according to Student´s t-test) between time points ZT24/0 or ZT12 and ZT6 or ZT18, respectively (Yelamanchili et al., 2006).

Since transmitter uptake strongly depends on the presence of the vacuolar proton pump (H^+-ATPase) which is involved in the acidification of various intracellular organelles including SVs, expression of vacuolar proton pump was analysed in homogenate and SVs prepared from wild type and *Per2^Brdm1^* mutant mice entrained in LD cycle (Fig. 17). There was no difference in the ratio between the vacuolar proton pump (identified by an antibody against its 116 kDa subunit) and Syp between time points ZT24/0 and ZT6, and ZT12 and ZT18 in SVs of wild type and *Per2^Brdm1^* mutant mice under LD conditions. In addition, as shown in Fig. 17B, the ratios between VGLUT1 or proton pump and Syp did not vary in the homogenate fractions but the ratio between VGLUT1 and Syp was more at ZT0 compared to ZT6 in SVs, which confirmed earlier observations (Fig. 16). As expected, Syp and VGLUT1 were enriched in the SVs fraction compared to homogenate, while the enrichment in the vacuolar proton pump due to its occurrence on a variety of organelles was less pronounced.

These results indicate that VGLUT1 could be sorted into a special compartment or pool.
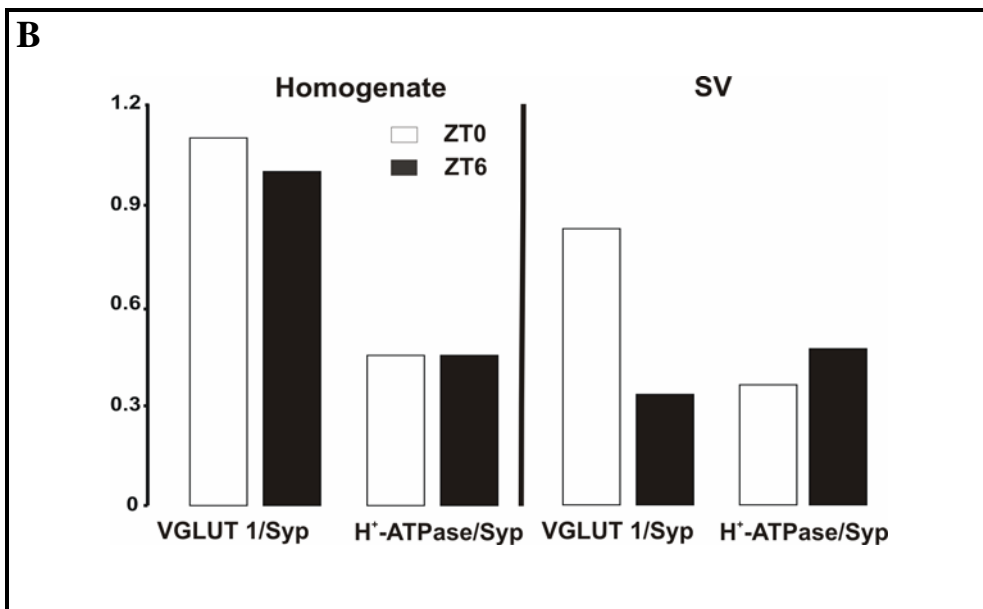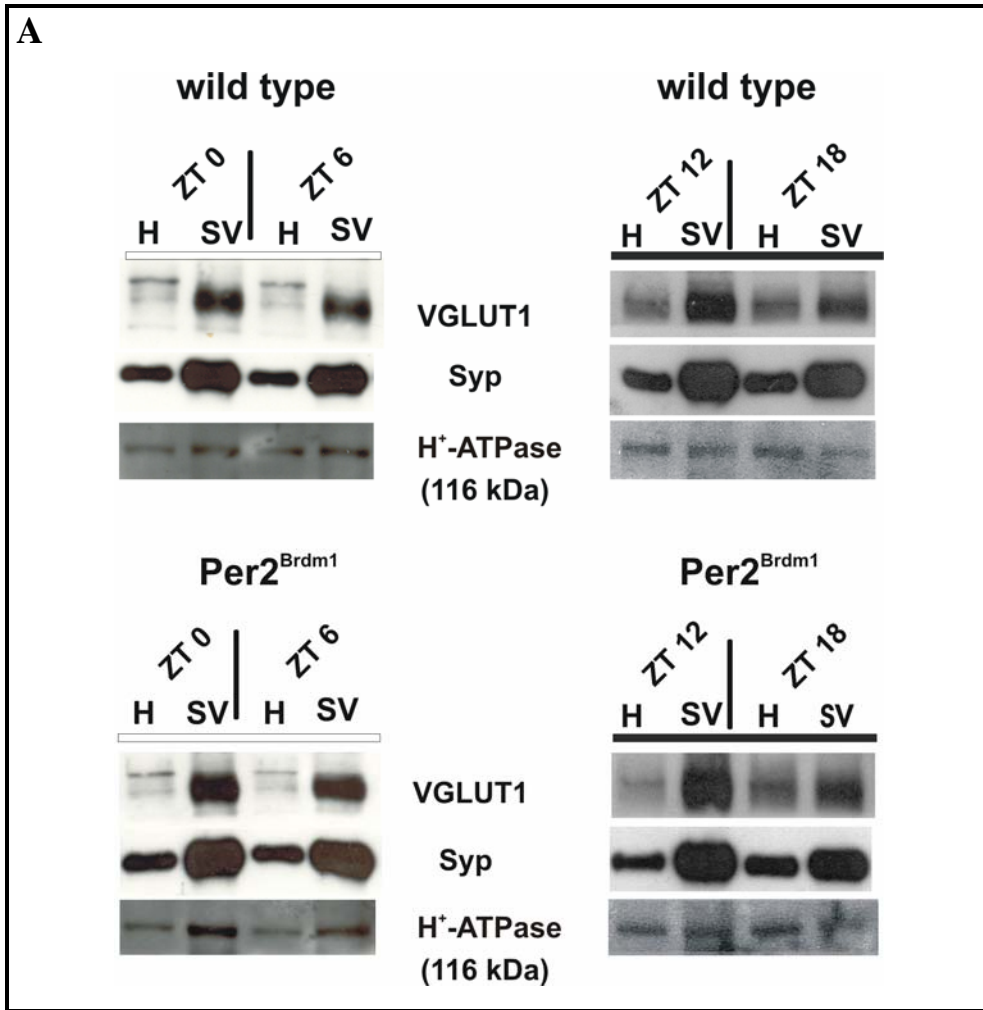
Figure 17: The vacuolar proton pump, synaptophysin and VGLUT1 in homogenate and SVs under LD condition

A. Homogenate or SVs were prepared at the given ZT time points and analysed for the presence of VGLUT1, Syp and H^+-ATPase. The light phase (ZT0 and ZT6) reflected by the white bar heading the blot and dark phase (ZT12 and ZT18) reflected by the black bar heading the blot. All homogenate fractions were extracted with triton X-114.

B. VGLUT1/Syp and H^+-ATPase/Syp values were obtained from two different sets of experiments. The graph represents the ratios at time points ZT0 and ZT6.of wild type mice. Note that VGLUT1/Syp ratio did not change in homogenate but in SVs. In contrast, H^+-ATPase/Syp ratio did not change in homogenate as well as in SVs (Yelamanchili et al., 2006).

Hypothesis for sorting of VGLUTs to the plasma membrane

Strong diurnal cycling of VGLUT1 on synaptic vesicles were restricted to synaptic vesicle preparations and not seen when analysing synaptosomes (Yelamanchili et al, 2006), which are the starting material of vesicle preparation. It was assumed that VGLUT may be sorted out to the plasma membrane in a time-dependent way. More over, VGLUTs were initially identified as members of type I Na^+- dependent inorganic phosphate transporter family (Ni et al., 1994; Aihara et al., 2000). If VGLUT1 is imagined to be translocated to the plasma membrane at time point ZT6 and ZT18, where VGLUT1 expression is minimum, then more transporters would be exposed to the plasma membrane leading to increased protease cleavage. The contrary scenario could happen at time point ZT24/0 and ZT12, where VGLUT1 expression is maximum, then there should be less transporter molecules translocated to the plasma membrane. To test this assumption, a pronase assay was done on intact synaptosomes prepared at time points ZT0, ZT6 and ZT12 from wild type mice entrained in LD cycle.

4.5.2 Analysis of vesicular/plasma membrane trafficking of VGLUTs by pronase assay in wild type mice entrained in LD cycle

As shown in previous experiments (see sections 4.2.2 and 4.3.1), pronase digestion indicated the translocation of synaptic vesicle proteins including VGLUTs to the plasma membrane under rest

as well as following stimulation conditions. Taking this as a cue, further experiments were done to test for a putative specific translocation of VGLUTs under physiological conditions i.e., during diurnal light dark cycles. Accordingly, wild type mice were entrained under a constant LD cycle (ZT conditions). Synaptosomes from whole brains were prepared at the indicated time points and immediately incubated with or without pronase. Control and pronase treated synaptosomes taken at ZT24/0 and ZT6 were first analysed for the proportional amount of digested VGLUT1, VGLUT2, Syp, Syt and Rab3. As shown in Fig. 18, the pattern of digestion of vesicular proteins as well as plasma membrane associated proteins at both time points followed the one observed in the initial experiments (see sections 4.2.2). As expected, most digestion was obtained for the plasma membrane associated NMDA-receptor and the Na^+/K^+-ATPase and higher amounts of VGLUTs and Syt were digested compared to Rab3 and the proton pump.

As explained in our hypothesis, if VGLUT1 translocates to the plasma membrane at time point ZT6, then more amount of transporter would be exposed to the plasma membrane and therefore be susceptible to protease cleavage and reverse would be the case at time point ZT0 when less transporter molecules are translocated to plasma membrane. Interestingly, digestion of VGLUT1 and VGLUT2 was selectively enhanced at ZT6 compared to ZT24/0 while digestion of the NMDA-receptor and the vesicular proteins Syp, Syt and Rab3 remained unchanged between the time points (Fig. 18B), confirming our initial hypothesis.
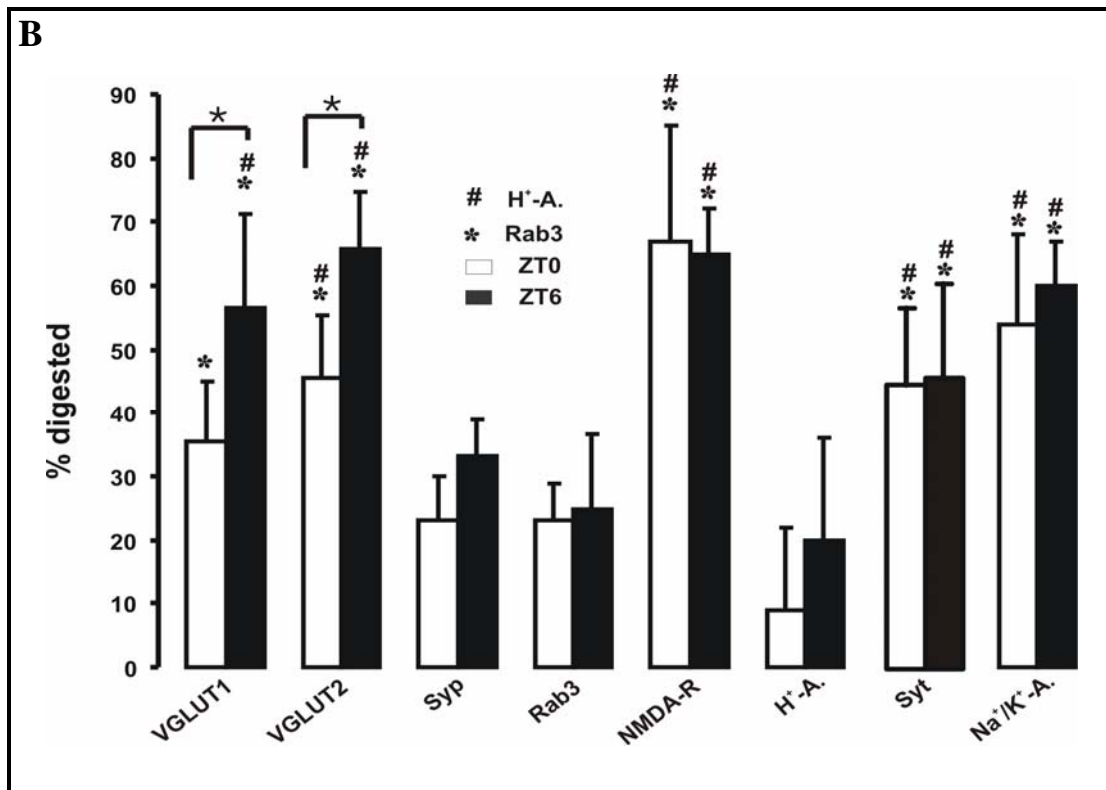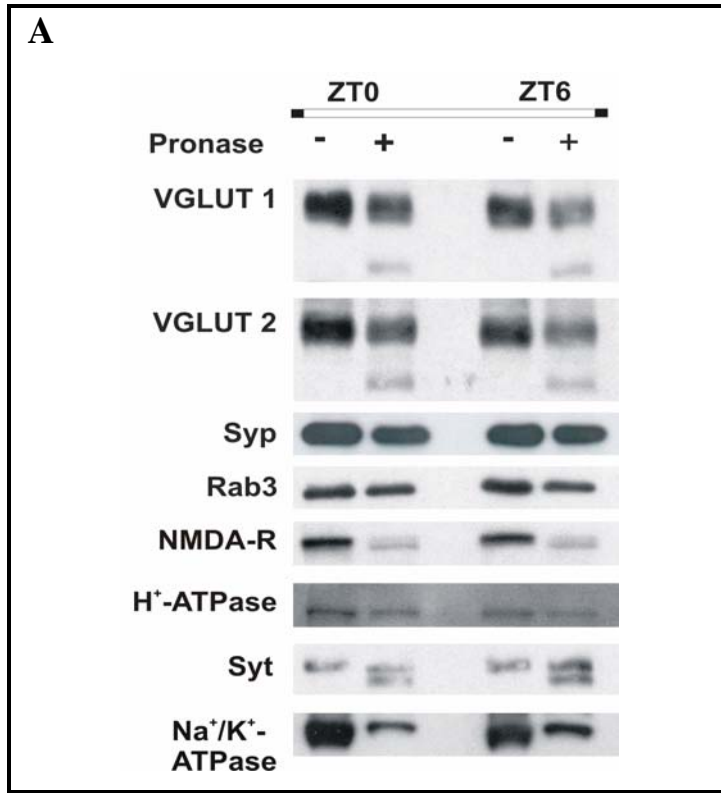
Figure 18: Pronase digestion of vesicular and plasma membrane proteins from mouse synaptosomes prepared at different times of the day (ZT0 and ZT6)

A. Synaptosomes were prepared from wild type mice sacrificed at time points ZT0 and ZT6. Synaptosomes were treated with pronase for 30 min at 37°C. The non-treated and treated samples were analysed via SDS PAGE and immunoblotting. Antibodies against the indicated proteins were used for analysis. H^+-ATPase and Rab3 were used as internal standards.

B. Amounts of digested proteins are given in % of non-digested samples from six different preparations. White and black bars indicate time points ZT0 and ZT6, respectively. * and **#** indicate significant (p< 0.05, according to Student´s t-test) digestion of VGLUT1 and VGLUT2 proteins with respect to Rab3 and the proton pump, respectively. * indicates significant digestion of VGLUT1 and VGLUT2 at ZT6 compared to ZT0.

In the next step, relative amounts of vesicular proteins based on ratios to the amounts of Syp or Rab3 as abundant vesicle proteins were taken. Quantification over 6 independent preparations revealed a significant decrease at ZT6 in the ratio of both VGLUT isoforms to Syp or Rab3 following pronase digestions (Fig 19B). Controls run in parallel, where pronase was omitted, the VGLUTs to Syp or to Rab3 ratio were unchanged at all time points confirming earlier observations (Yelamanchili et al., 2006). These results indicating that the selectively reduced amount of VGLUT1 on synaptic vesicles is indeed due to a specific translocation of VGLUT transporters to the plasma membrane where they are digested by pronase.
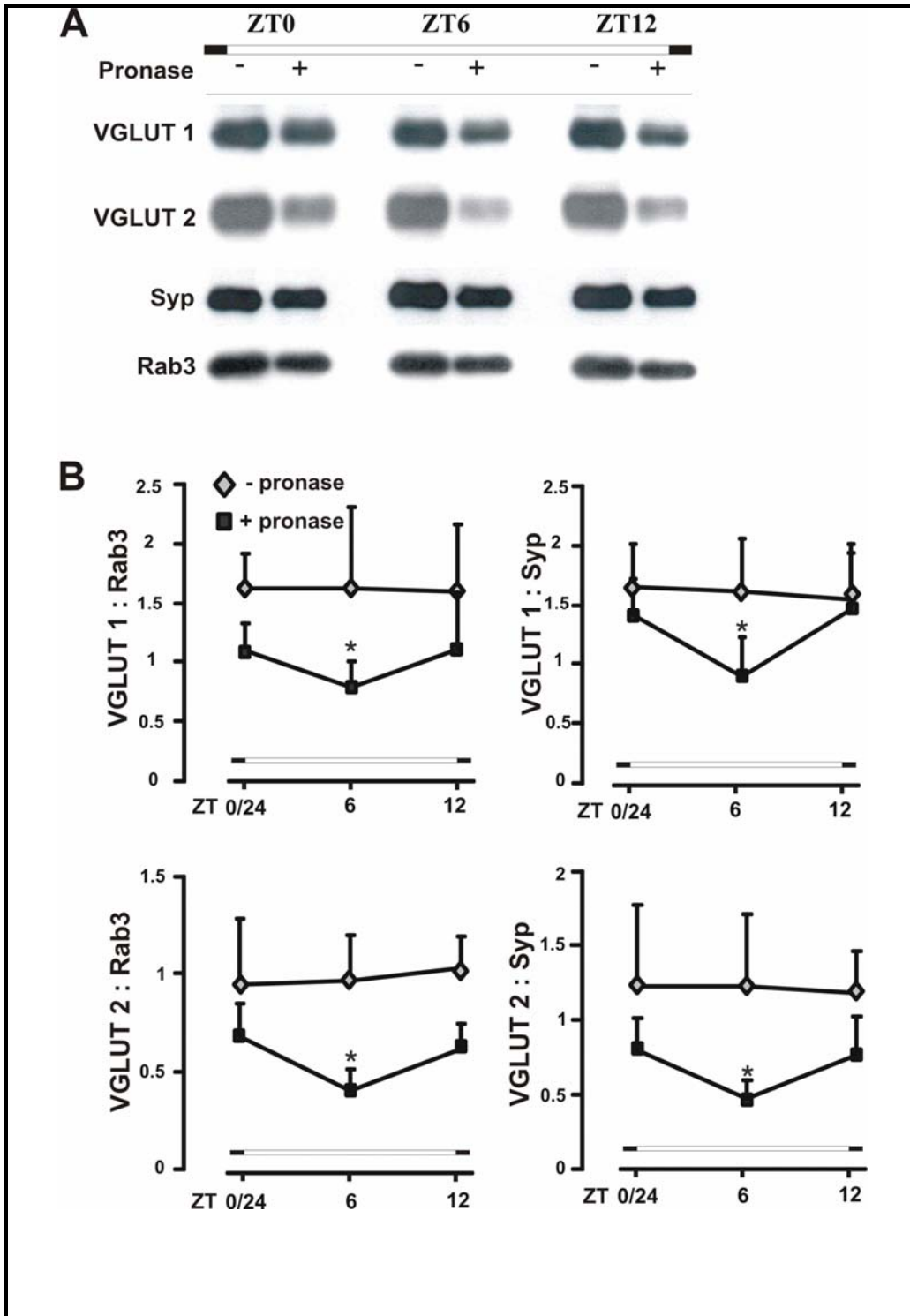
Figure 19: Oscillation of VGLUT amounts in pronase digested synaptosomes under light/dark conditions

A. Synaptosomes were prepared from wild type mice sacrificed at time points ZT0, ZT6 and ZT12. Synaptosomes were treated with pronase for 30 min at 37°C. Control and pronase treated samples were analysed via SDS PAGE and immunoblotting. Antibodies against the indicated proteins were used for analysis. Syp and Rab3 were used as internal standards.

B. Quantification was performed by calculating the ratio between the amount of Syp and Rab3, internal standards, to the respective transporters VGLUT1 and VGLUT2. The relative ratios are indicated for untreated samples (gray line) and pronase treated samples (black line). Statistical analysis was made from 6 individual sets. Note that the expression pattern of VGLUT1 and VGLUT2 is significantly less at ZT6 when compared to time points ZT0 and ZT12. * indicates significant (p <0.05) when ZT6 was compared to ZT0 and ZT12.

4.5.3 Analysis of proteins involved in endocytosis of VGLUTs (AP2, AP3 and endophilin) in synaptosomes and SVs from wild type and *Per2^{Brdm1}* mutant mice entrained in LD cycle

As described in section 4.5.2, VGLUTs are sorted to the plasma membrane in synaptosomes prepared from wild type mice entrained in LD cycle at time point ZT6 than ZT0. Moreover, it was shown that during prolonged, high frequency stimulation deletion of the endophilin binding domain slows endocytosis of VGLUT1 (Voglmaier et al., 2006). Surprisingly, inhibition of the AP3 recycling pathway with BFA rescues this defect, suggesting that during prolonged high frequency stimulation, the interaction with endophilin redistributes VGLUT1 from a slower AP3 dependent pathway to a fast recycling pathway, presumably mediated by AP2. These studies raised the question whether proteins AP2, AP3 and endophilin of endocytotic pathways of VGLUTs are involved in this sorting mechanism. Hence, it is of importance to know whether any difference could be observed in the expression of endophilin, AP2 and AP3 proteins at different times of the day in wild type and *Per2^{Brdm1}* mutant mice entrained in LD cycle.

SVs and synaptosomes were prepared from wild type and *Per2^{Brdm1}* mutant mice entrained in LD cycle at different times of the day (ZT0, ZT6, ZT12 and ZT18). All synaptosomal and synaptic vesicular proteins were analysed via SDS PAGE and immunoblotting and checked for the expression of endophilin, AP2, AP3 and Syp, which is internal standard. As shown in Fig. 20, there was no difference in the expression of endophilin, AP2 and AP3 proteins in synaptosomes (Fig. 20C) as well as in SVs (Fig. 20A) between different times of the day and

different genotypes. However, when the total amount of AP2 in SVs is calculated during the dark period (ZT0+ZT18) and the light period (ZT6+ZT12) of the day separately, it seems that there is more AP2 in the light period (ZT6+ZT12) compared to the dark period (ZT0+ZT18) in *Per2^Brdm1* mutant mice entrained in LD cycle (Fig. 20B). This phenomenon is not seen in wild type animals.
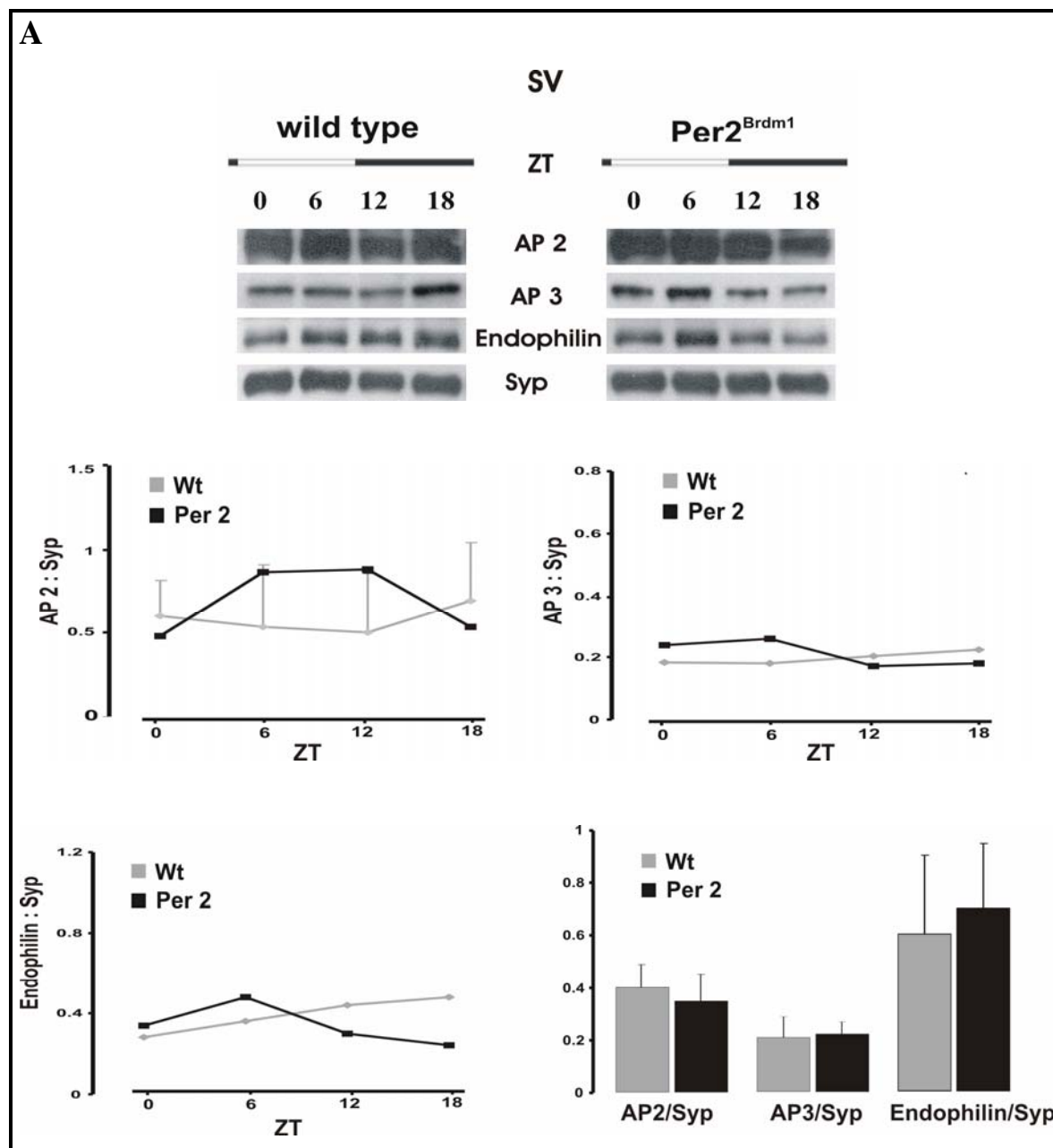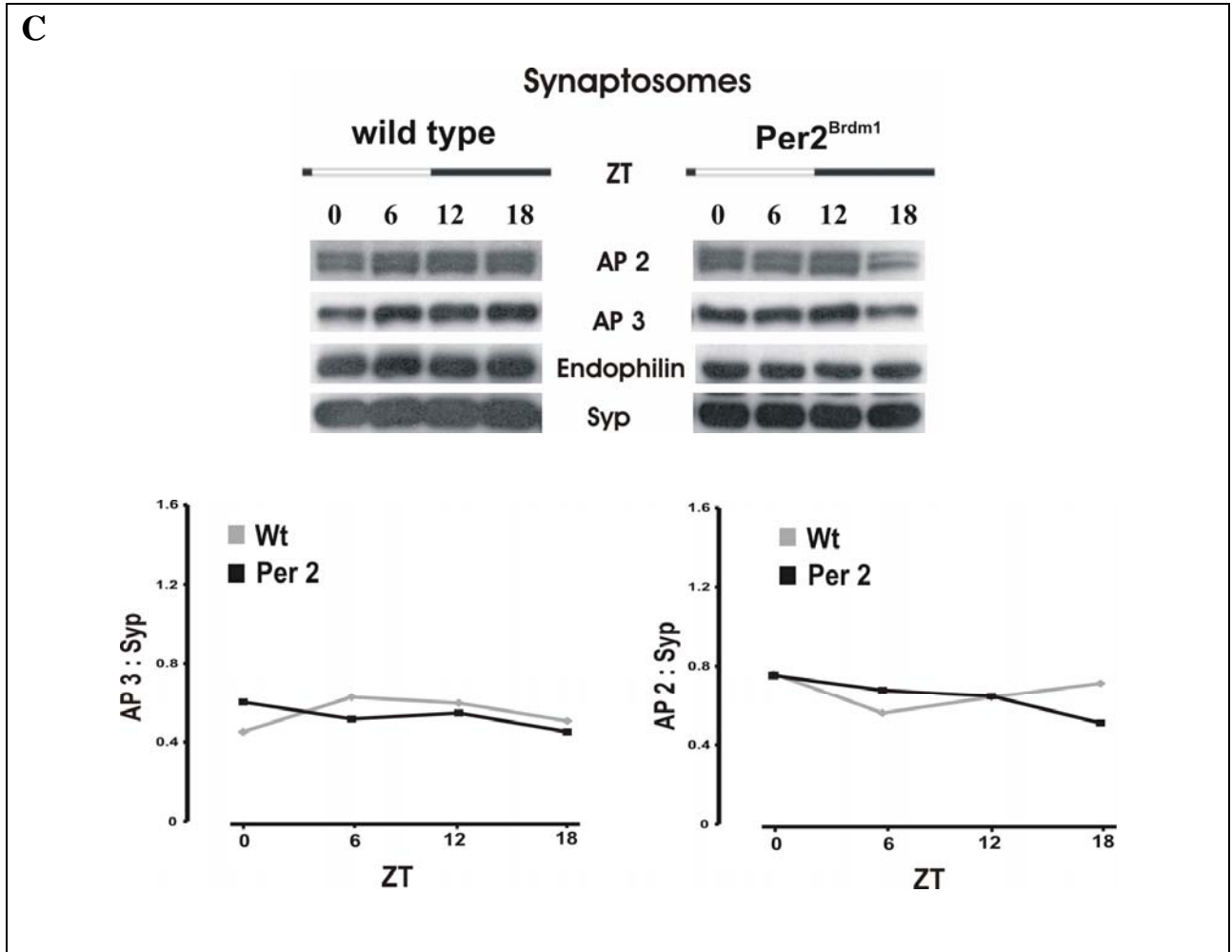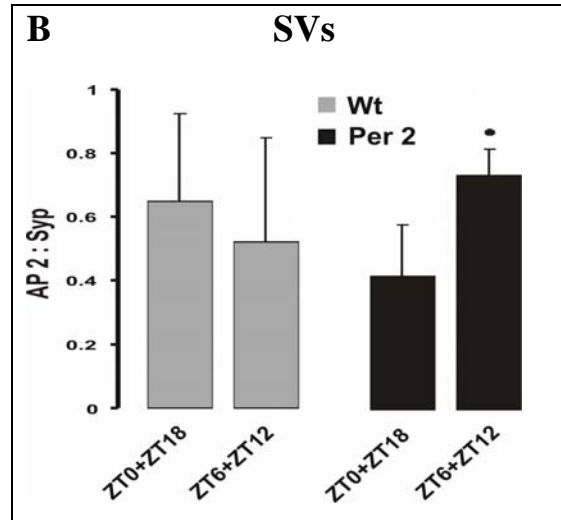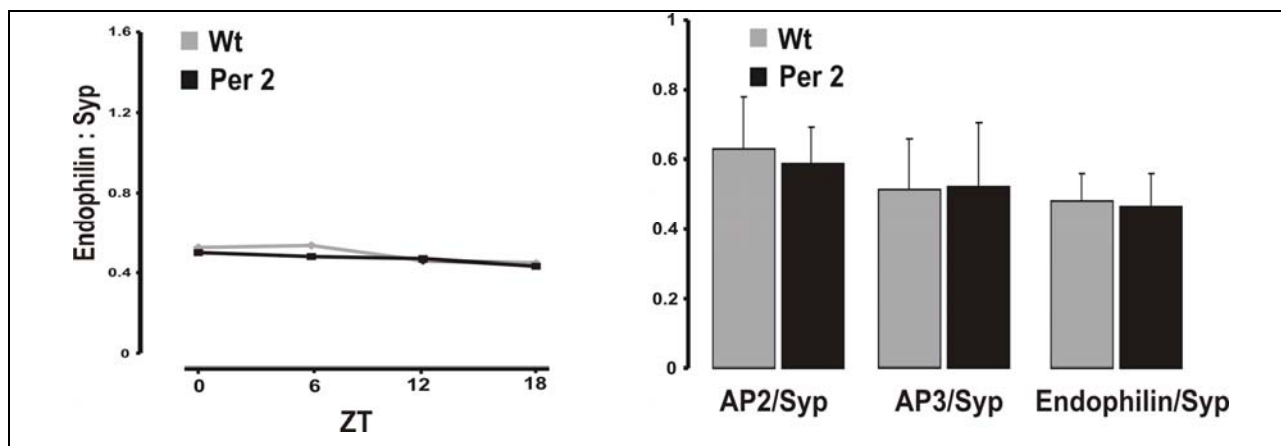
Figure 20: Expression profile of AP2, AP3 and endophilin in SVs and synaptosomes isolated from wild type and *Per2^Brdm1^* mutant mice entrained in LD cycle.

A. SVs were prepared at the indicated time points ZT either from wild type (Wt) or *Per2^Brdm1^*mutant mice. The light/dark condition is reflected by the white/black bar heading the blot documentation. All fractions were resolved on a SDS PAGE and immunoblotted with antibodies against Syp, AP2, AP3 and endophilin. Quantification was performed by calculating the ratio of Syp to AP2, AP3 and endophilin. Although, the values were obtained from two individual experiments, note that there were no differences in the expression patterns observed between wild type and *Per2^Brdm1^*mutant mice, and between different times of the day. Syp used as a internal reference.

B. Values represent the sum of amounts of AP2 in SVs during dark phase (ZT0+ZT18) and during light phase (ZT6+ZT12) under ZT condition and were obtained from two different experiments. Note that there is more amount of AP2 during night than day time in wild type mice and vice versa in *Per2^Brdm1^*mutant mice. * denotes significance (p< 0.05) according to Student´s t-test.

C. Synaptosomes were prepared at the indicated time points ZT either from wild type or *Per2^Brdm1^*mutant mice. The light/dark condition is reflected by the white/black bar heading the blot. All fractions were resolved on a SDS PAGE and immunoblotted with antibodies against Syp, AP2, AP3 and endophilin. Note that there were no differences in the expression patterns observed between wild type and *Per2^Brdm1^*mutant mice and between different times of the day. Syp used as an internal reference.

4.5.4 Analysis of Syntaxin isoforms in SVs from wild type and $Per2^{Brdm1}$ mutant mice entrained in LD cycle

A large number of Syn isoforms have been identified and are associated with specific target membranes and organelles within the cell. They are thought to contribute to the specificity of vesicular trafficking between different organelles and subcellular compartments (Bennett et al., 1992 and Bennett et al., 1993). Syn 6 primarily localised on the trans-golgi network (TGN) along with its adapter protein AP1 on clathrin-coated membranes and regulates the delivery of microdomain-associated lipids and proteins to the cell surface, which are required for caveolar endocytosis (Choudhury et al., 2006). Syn 13 is primarily found in tubular early and recycling endosomes and plays a role in membrane fusion events during the recycling of plasma membrane proteins (Prekeris et al., 1998). Syn 16 may play a role in neurite outgrowth and perhaps other specific dendritic anterograde/retrograde traffic (Lin Chua and Tang, 2007). Playing a role in different trafficking pathways enabled us to test any time or light dependent change in the expression of these Syn isoforms in SVs prepared at different times of the day from wild type and $Per2^{Brdm1}$ mutant mice.

SVs were prepared from wild type and $Per2^{Brdm1}$ mutant mice entrained in LD cycle at different times of the day. All fractions were analysed via SDS PAGE, immunoblotting and checked for Syn 16, Syn 13, Syn 6 and Syp. There was no significant difference in their expression pattern during different times of the day in wild type and $Per2^{Brdm1}$ mutant mice. However, when total amount of each Syn isoform in a whole day was calculated, significantly higher amounts of Syn 16 was found in $Per2^{Brdm1}$ mutant mice compared to the wild type mice (Fig. 21B). In contrast to Syn 16, there was no significant difference in the amount of Syn 13 and Syn 6 in $Per2^{Brdm1}$ mutant mice compared to wild type mice.
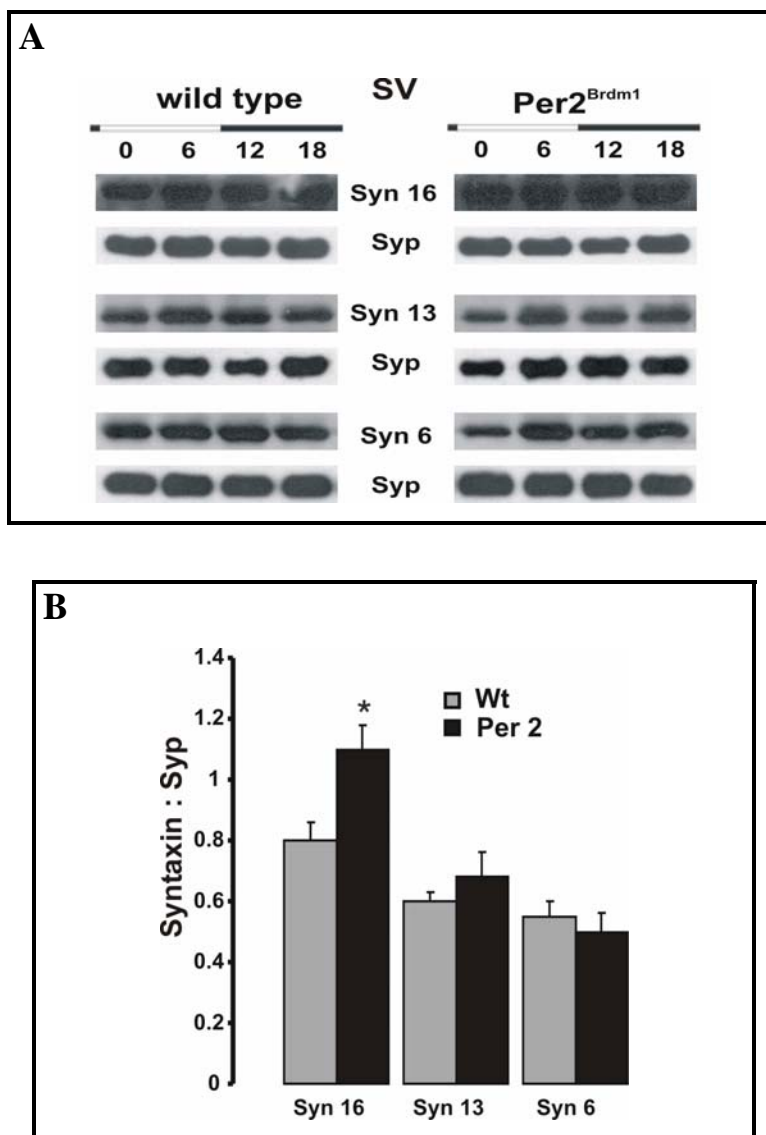
Figure 21: Expression profile of syntaxin isoforms in SVs prepared from wild type and *Per2*[Brdm1] mutant mice

A. SVs were prepared at the indicated time points ZT either from wild type or *Per2*[Brdm1]mutant mice. The light/dark condition is reflected by the white/black bar heading the blot documentation. All fractions were resolved on a SDS PAGE and immunoblotted with antibodies against Syn 16, Syn 13, Syn 6 and Syp as internal standard.

B. Quantification was performed by calculating the ratio of Syp to Syn 16, Syn 13 and Syn 6. Values represent the sum of all time points of the day under ZT condition and were obtained in one to three sets of different experiments. Note that Syn 16 expression is more in *Per2*[Brdm1]mice and is significant. * denotes significance (p< 0.05) according to Student´s t-test. In contrast to Syn 16, Syn 13 and Syn 6 expressions were not changed in *Per2*[Brdm1]mutant mice compared to wild type mice.

4.6   Analysis of VGAT expression in synaptosomes and SVs from wild type and *Per2^Brdm1* mutant mice entrained in LD and DD (12 hr dark-12 hr dark, CT condition) cycle

Diurnal oscillation of VGLUTs in SVs at different times of the day (Yelamanchili et al; 2006) and time-dependent translocation of VGLUTs to the plasma membrane (see section 4.5) raises the question whether VGAT is also regulated in a time or circadian dependent manner. SVs were prepared from wild type and *Per2^Brdm1* mutant mice at various time points over 24 hrs entrained in LD and DD cycle, and checked for VGAT and Syp expression. In contrast to the data for VGLUTs, as can be seen from Fig. 22B, there was no significant difference between different time points of the day and between wild type and *Per2^Brdm1* mutant mice entrained in LD as well as in DD cycle.

Figure 22: Expression profile of VGAT in SVs isolated from wild type and *Per2^Brdm1^* mutant mice entrained in LD and DD cycle

A. SVs were prepared at the indicated time points ZT and CT (circadian time) either from wild type or $Per2^{Brdm1}$ mutant mice. The light/dark condition is reflected by the white/black bar heading the blot documentation and the dark/dark condition is also reflected by the black bar heading the blot documentation. The vesicular membrane fractions were analysed for VGAT and Syp which is a internal standard.

B. Quantification was performed by calculating the ratio of Syp to VGAT. Values are obtained in three to five different sets of experiments. Note that there was no significant difference in the VGAT expression at different ZT and CT time points in both wild type and $Per2^{Brdm1}$ mutant mice.

Similar results were obtained when synaptosomes (prepared from wild type and $Per2^{Brdm1}$ mutant mice entrained in LD and DD cycle) were used. However, the VGAT/Syp ratio in SVs as well as in synaptosomes appeared to be decreased in both genotypes when kept in complete darkness (CT). To check this, total amount of VGAT in a whole day in synaptosomes and in SVs prepared from mice entrained in LD cycle (ZT condition) is compared to mice with those kept under complete darkness (DD cycle, CT condition), the latter showed a reduction in VGAT in SVs. This reduction was also clearly seen in synaptosomal preparations and seems to be independent from the presence of *Per2* (Fig. 23B). Probably light is required to increase or sustain the expression of VGAT which get reduced when mice were kept in complete darkness.
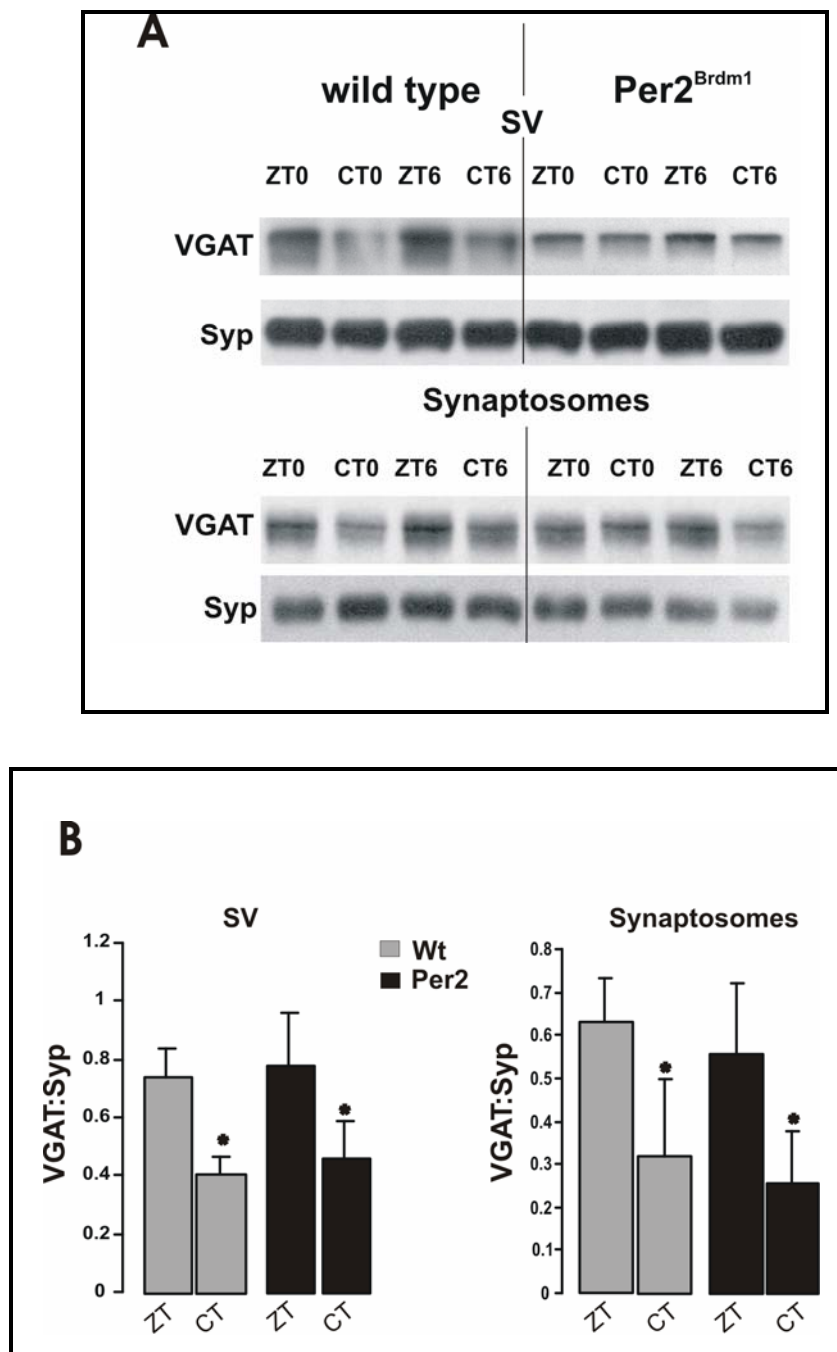
Figure 23: Light dependent expression of VGAT in P2 and SVs

A. Synaptosomes and SVs were prepared from wild type or *Per2^Brdm1^* mutant mice kept either under light/dark (ZT) and dark/dark (CT) condition. Immunoblots represent the vesicular and synaptosomal fractions prepared at two time points (ZT0, ZT6 and CT0, CT6) from wild type or *Per2^Brdm1^* mutant mice.

B. Quantification was performed by calculating the ratio of Syp to VGAT. Values represent the sum of all time points under either ZT or CT condition, respectively and were obtained in three to six sets of different experiments. Note that VGAT expression is more in SVs and synaptosomes prepared from wild type and *Per2*[Brdm1] mutant mice entrained in light/dark cycle than in mice entrained in dark/dark conditions. * denotes significance (p< 0.05) according to Student´s t-test.

4.7 *Per2* dependent expression of VMAT2 in SVs and synaptosomes isolated form wild type and *Per2*[Brdm1] mutant mice entrained in LD and DD cycle

The vesicular monoamine transporter (VMAT) 2 is a transport protein located within the pre-synaptic cell. Substrates for the transporter are mainly noradrenaline, adrenaline, dopamine, 5-HT, and isoprenaline. Recently it was shown that monoamine oxidase (MAO a) acitivity is reduced and dopamine levels, concentrated in the vesicles by VMAT2, are elevated in *Per2*[Brdm1] mutant mice (Hampp et al., 2008) prompted us to check the VMAT 2 levels in SVs. SVs prepared from wild type and *Per2*[Brdm1] mutant mice entrained in LD and DD cycle and checked for VMAT2 and Syp expression. As Fig. 24A depicts, there was no difference of VMAT2 expression between different times of the day in wild type and *Per2*[Brdm1] mutant mice but it seems that there is a difference in the total amount of protein between wild type and *Per2*[Brdm1] mutant mice. When the total amount of VMAT2 in a whole day was calculated (Fig. 24B), there was more VMAT2 protein in SVs prepared from *Per2*[Brdm1] mutant mice compared to wild type mice entrained in LD as well as DD cycle. These results indicate that mice lacking *Period 2* gene might be involved in the regulation of VMAT2.
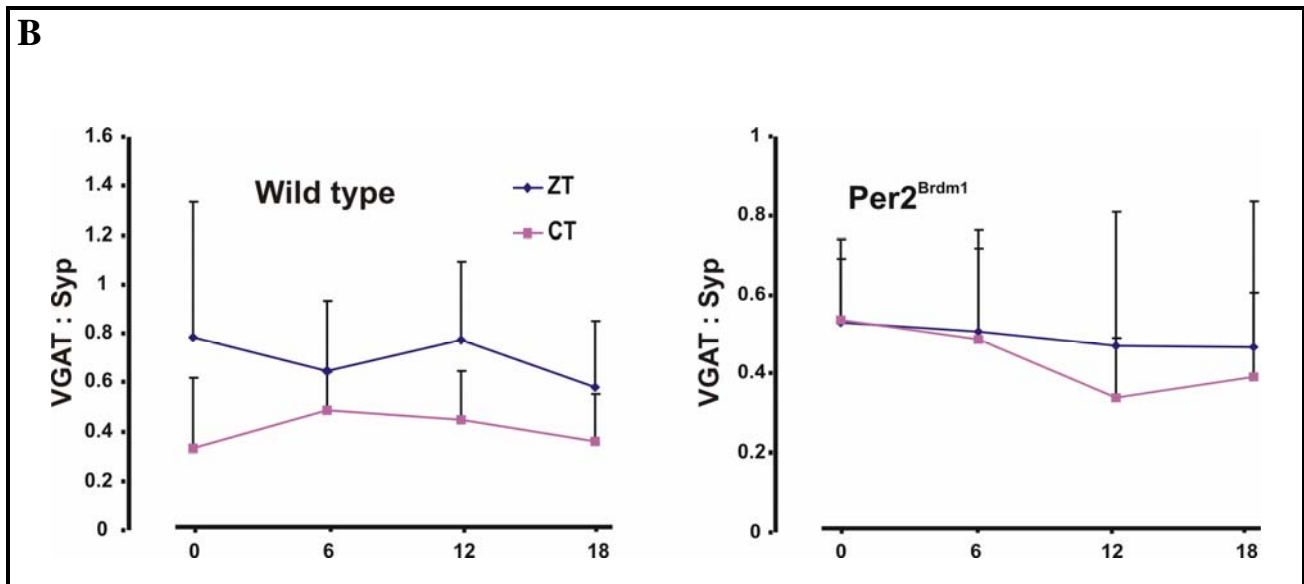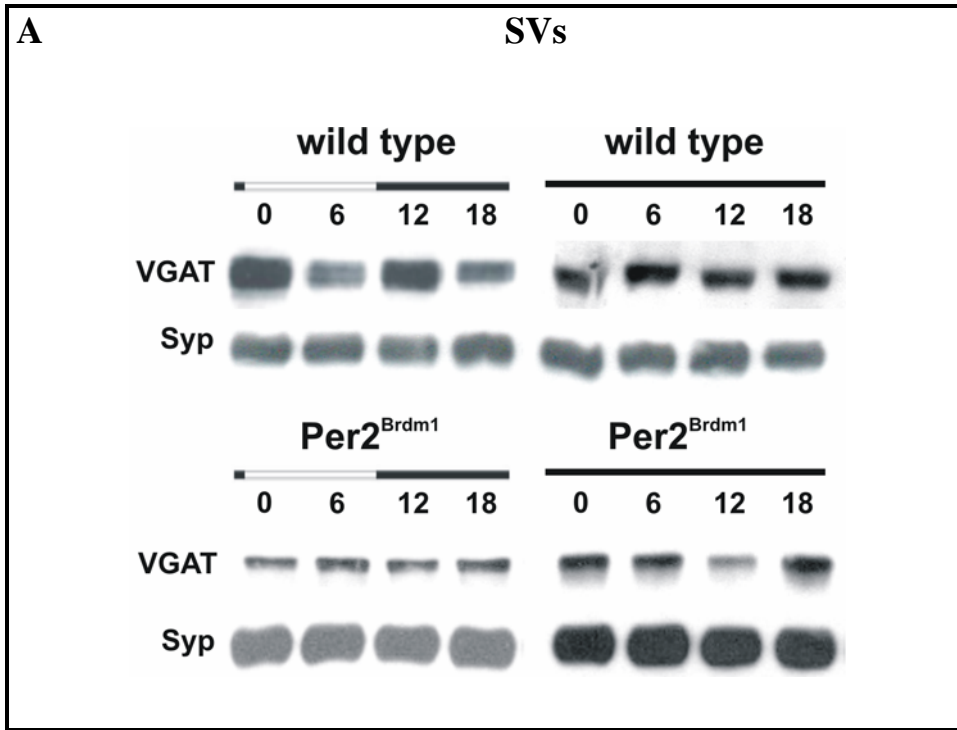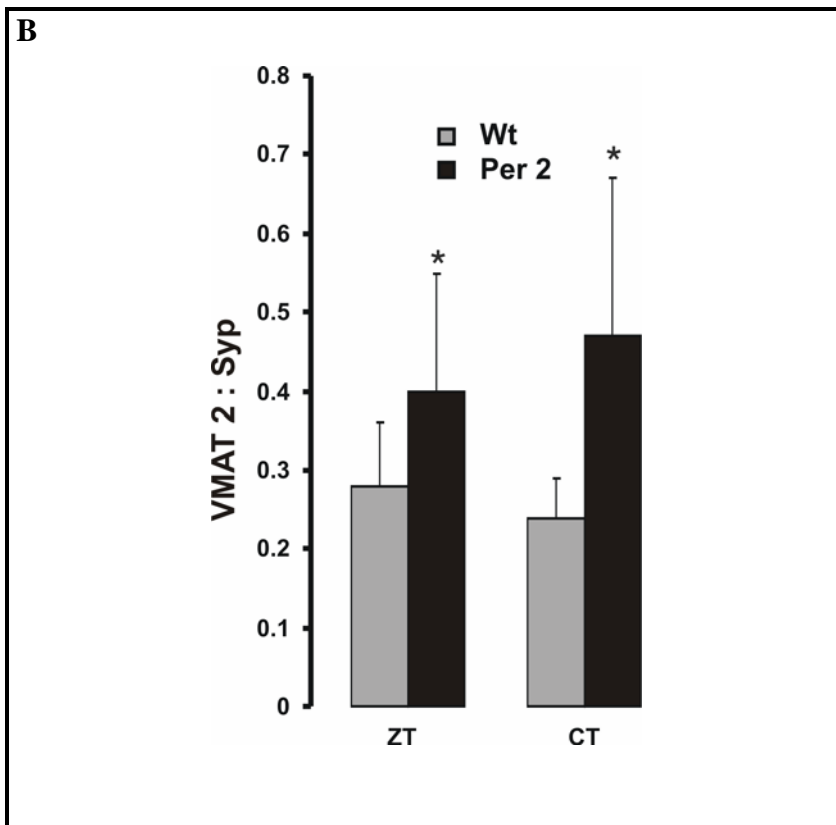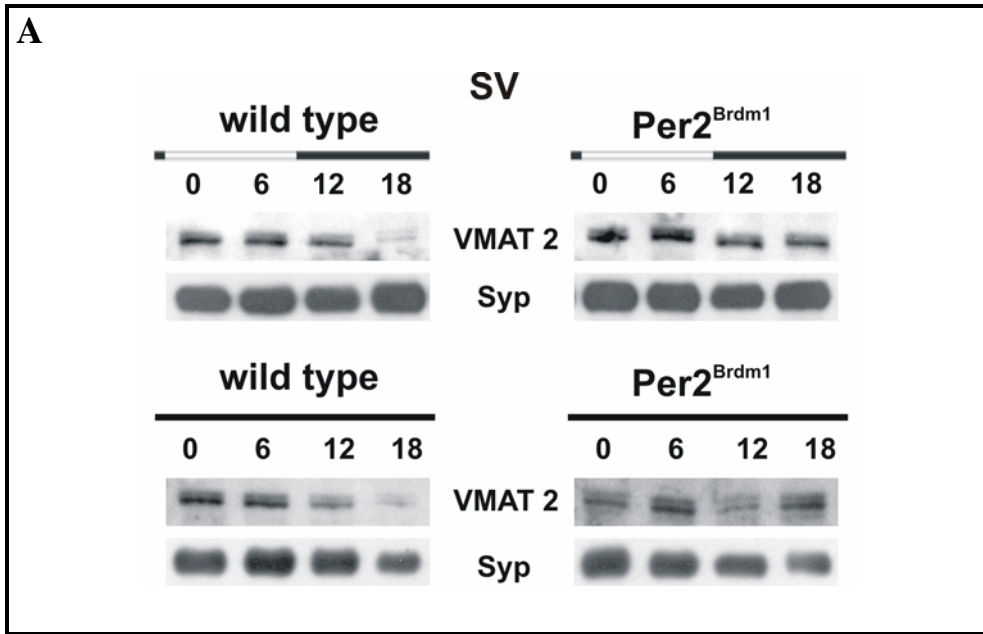
Figure 24: Expression profile of VMAT2 in SVs prepared from wild type and *Per2Brdm1* mutant mice entrained in LD and DD cycle.

A. SVs were prepared at the indicated time points ZT and CT either from wild type Wt or *Per2^{Brdm1}* mutant mice. The light/dark condition is reflected by the white/black bar heading the blot documentation and the dark/dark condition is also reflected by the black bar heading the blot documentation. The vesicular membrane fractions were analysed for VMAT2 and Syp, which is an internal standard.

B. Quantification was performed by calculating the ratio of Syp to VMAT2. Values represent the sum of all time points under either ZT or CT condition, respectively and ZT values were obtained from three and CT values from two different experiments. Note that VMAT2 expression is more in *Per2^{Brdm1}* mutant mice entrained in LD as well as in DD conditions compared to wild type mice. * denotes significance (p< 0.05) according to Student´s t-test.

5   DISCUSSION

5.1   Identification of vesicular proteins at the plasma membrane by pronase assay

5.1.1   Establishment and characterisation of pronase treatment

Synaptic transmission is mediated by neurotransmitters that are stored in synaptic vesicles and released by exocytosis upon activation. The vesicle membrane is then retrieved by endocytosis, and synaptic vesicles are regenerated and re-filled with neurotransmitter. The vesicular fusion and membrane recycling are well understood, however the fate of the vesicles after fusion is still unclear. It is still not clear, whether the vesicular components diffuse on to plasma membrane, or remain together. This issue is complicated by the fact that synaptic vesicles are too small (approximately 40 nm in diameter) and are densely packed to be resolved by the available fluorescence microscopes. In recent past, new wave of endocytosis assays using optical imaging techniques were used to track vesicle proteins before, during, and after exocytosis (reviewed in Kavalali, 2006). However, these techniques were restricted to vesicular proteins at the plasma membrane in transfected and primary hippocampal neurons obtained from embryonic brain. These assays cannot be generally extrapolated to SV proteins like VGLUTs that are upregulated during development. On the other hand, there are no known techniques that identify vesicular proteins at the plasma membrane of mature adult neurons. To overcome this problem, we employed synaptosomes from adult rat or mouse whole brain and subjected them to pronase treatment. Although synaptosomal preparations could be contaminated by some free synaptic vesicles and glial-derived membranes, this method identifies vesicular proteins at the plasma membrane by pronase digestion. Pronase is a mixture of proteolytic enzymes that cleaves specifically the peptide chains of membrane proteins facing the extracellular space.

Initially, some control experiments were done to identify concentration and incubation time for pronase. Pronase treatment of rat synaptosomes resulted in a partial digestion of Syp and VGLUTs while the NMDA-receptor signal almost completely changed to lower mobility due to proteolytic cleavage. A complete digestion of the synaptic vesicle proteins Rab3, Syp, VGLUT1 and 2 was observed when synaptosomes were permeabilized by streptolysin O (SLO) which allows the proteolytic enzymes to seep into the cytosolic compartment or when isolated SVs were directly treated with pronase. Similarly, complete digestion was obtained using synaptosomes

extracted with Triton X-100 prior to pronase treatment. These experiments clearly showed that pronase can not enter intact synaptosomes and does not non-specifically cleave synaptic vesicle proteins when applied to intact synaptosomes. This illustrates the efficacy of pronase digestion of synaptosomes as an ideal tool to identify vesicular proteins at the plasma membrane of mature neurons in general.

5.1.2 Quantification of vesicular proteins at the plasma membrane under resting conditions in rat and mouse synaptosomes

To get an idea of the occurrence of vesicular proteins at the plasma membrane, pronase was applied on crude, purified rat and mouse synaptosomes. This was followed by quantification of vesicular proteins like VGLUTs, Syp, Syt, the 116 kDa subunit of the proton ATPase and Rab3 along with plasma membrane proteins like NMDA-receptor and Na^+/K^+-ATPase. Significant digestion of vesicular proteins like VGLUTs, Syt, and plasma membrane proteins like NMDA-receptor and the Na^+/K^+-ATPase was observed compared to Rab3 and the proton ATPase, used as internal references. A proportion of 5 and 10% digestion of the total amount was observed for Rab3 and proton pump, respectively. The choice of Rab3 and proton pump ATPase as internal references was based on the following assumption. Normally, Rab3 is not accessible for digestion, and the observed 5% digestion could have been from the contamination of synaptic vesicles in the preparations. The proton pump was identified by its 116 kDa unit having several transmembrane domains. Moreover, these proton pumps are present on the endosomal membranes besides synaptic vesicles, where as the latter presence only one or two copies per vesicle (Takamori et al., 2006). These few copies per vesicles if digested will not contribute much to the overall amount of proton pump.

The obtained digestion of Syp was not significantly higher compared to both references and it was between 5 and 10% after subtracting the background. It was shown that 8% of Syp was localised at the plasma membrane of boutons of hippocampal neurons in culture by using a synaptopHluorin, a pH sensitive GFP fused to Syp, which is in the range of the average amount of Syp found at the plasma membrane in our pronase assay (Granseth et al., 2006)

On the other hand, pronase digestion revealed presence of Syt to an extent of 25 to 28% on the plasma membrane under resting conditions when normalised to Rab3. Analysis using an

antibody against luminal domain of Syt identified at least 15% of the total amount to be present at the plasma membrane under resting conditions in postnatal day 1-3 derived hippocampal neurons (Fernandez-Alfonso et al., 2006). These authors also mentioned that the amounts of Syt and Syb fused pHluorin on the plasma membrane were comparable. In contrast, comparing pHluorin fusion proteins of Syp and Syb showed that Syb at the plasma membrane exceeded Syp by 2.5 to 3-fold (Granseth et al., 2006). Thus, the data reported for the plasma membrane pools of Syt and Syp in transfected neurons are somewhat lower but in the range of the amounts found by pronase digestion using synaptosomes from whole brain. Since Syb antibody detects only cytosolic part, Syb was not included in our studies and moreover, has only 2-3 aminoacids on luminal part of plasma membrane exposed during exocytosis.

VGLUT1 and VGLUT2 were found to be digested to an extent of 20-30% (corrected for Rab3 reference). The amounts of VGLUTs accessible to pronase and thus presumed to be present at the plasma membrane were found to be higher than those reported from transfection studies using hippocampal neurons and VGLUT1pHluorin (Voglmaier et al., 2006). This may be due to the differences in the systems used, i.e. adult neurons from whole brain versus primary hippocampal neurons obtained from embryonic brain. Significantly, studies with VGLUT1 pHluorin focussed on boutons and referred to transfected VGLUT1 fusion proteins in embryonic neurons while pronase digestion estimates endogenous VGLUT present in the total plasma membrane pool of a great variety of adult neurons. In addition, the good correlation between the amounts for Syp and Syt by both experimental approaches (see above) strengthens the data obtained for VGLUTs. VGLUT1 is developmentally up-regulated leading to a different distribution in adult neurons in the brain compared to embryonic or postnatal neurons developing in culture.

As expected the plasma membrane associated NMDA-receptor and the Na^+/K^+-ATPase were digested by more than 60%. This partial digestion could be attributed to their presence also on the endomembranes. A complete digestion was observed, however when a pronase digestion was applied to triton extracts. Similar data were obtained with mouse synaptosomes. In summary, pronase digestion of synaptosomes reflects the overall distribution of vesicular proteins at the plasma membrane.

5.2 Study of the translocation of vesicular proteins to the plasma membrane following stimulation

5.2.1 Stimulation-dependent translocation of vesicular proteins to the plasma membrane

Stimulation-dependent studies were made to see the dynamics of the vesicular protein translocation in synaptosomes prepared from rat and mouse whole brain. First, translocation of different vesicular proteins to the plasma membrane was analysed during /after exocytosis. The pronase assay was used to identify vesicular proteins that were translocated to the plasma membrane after exocytosis. The results indicated more Syt and VGLUTs present at the plasma membrane compared to other vesicular proteins. Next, the experiments were performed to study - firstly, if the translocation to plasma membrane involves any other vesicular proteins other than Syt and VGLUTs; secondly, any increase in the amount of Syt and VGLUTs translocation following stimulation compared to resting conditions. In this regard, synaptosomes were stimulated with 0.5 M sucrose, α-latrotoxin and ionomycin.

Stimulation of synaptosomes from either rat or mouse brain with 0.5 M sucrose increased the amount of digested Syp, VGLUT1 and VGLUT2 without changing the reference protein Rab3. As explained in the scheme (Fig. 25), this extensive stimulation caused considerable amounts of synaptic vesicles to fuse with the plasma membrane making the luminal domains of their transmembrane proteins accessible to the pronase digestion. Hence, an increase in digested amounts of VGLUT1, VGLUT2, Syp and Syt were observed. As shown in Fig. 25, a difference of increased % of digestion after stimulation was 25-30% for VGLUTs and Syp, 20% for Syt and 3-10% for proton ATPase and Rab3 which were used as internal references.

There was also a significant digestion of Syp following stimulation compared to the resting condition. The reason could be that Syp is one of the most abundant proteins in SVs so that more Syp molecules are translocated to the plasma membrane under harsh stimulation. About 40-50% (corrected for Rab3 reference) of Syt was found on plasma membranes in our pronase studies following stimulation, correlated with other studies (Fernandez-Alfonso et al., 2006), where around 50% of pHluorin-tagged synaptotagmin on the vesicle was replaced during stimulus that depletes the releasable pool. Further, considerable increase in the digestion of VGLUTs following stimulation indicates presence of higher amounts of VGLUTs at the plasma membrane

compared to other vesicular proteins. However, a significant difference of digestion of vesicular proteins including VGLUTs was not seen following stimulation with α-latrotoxin and ionomycin in the absence of Ca^{+2}. The reason could be that the vesicular membrane fusion following stimulation with α-latrotoxin and ionomycin may not be enough to detect the change in the translocation of vesicular proteins to the plasma membrane.

These experiments demonstrate that pronase digestion enables to track changes in the translocation of synaptic vesicle proteins to the plasma membrane between resting and stimulated conditions.
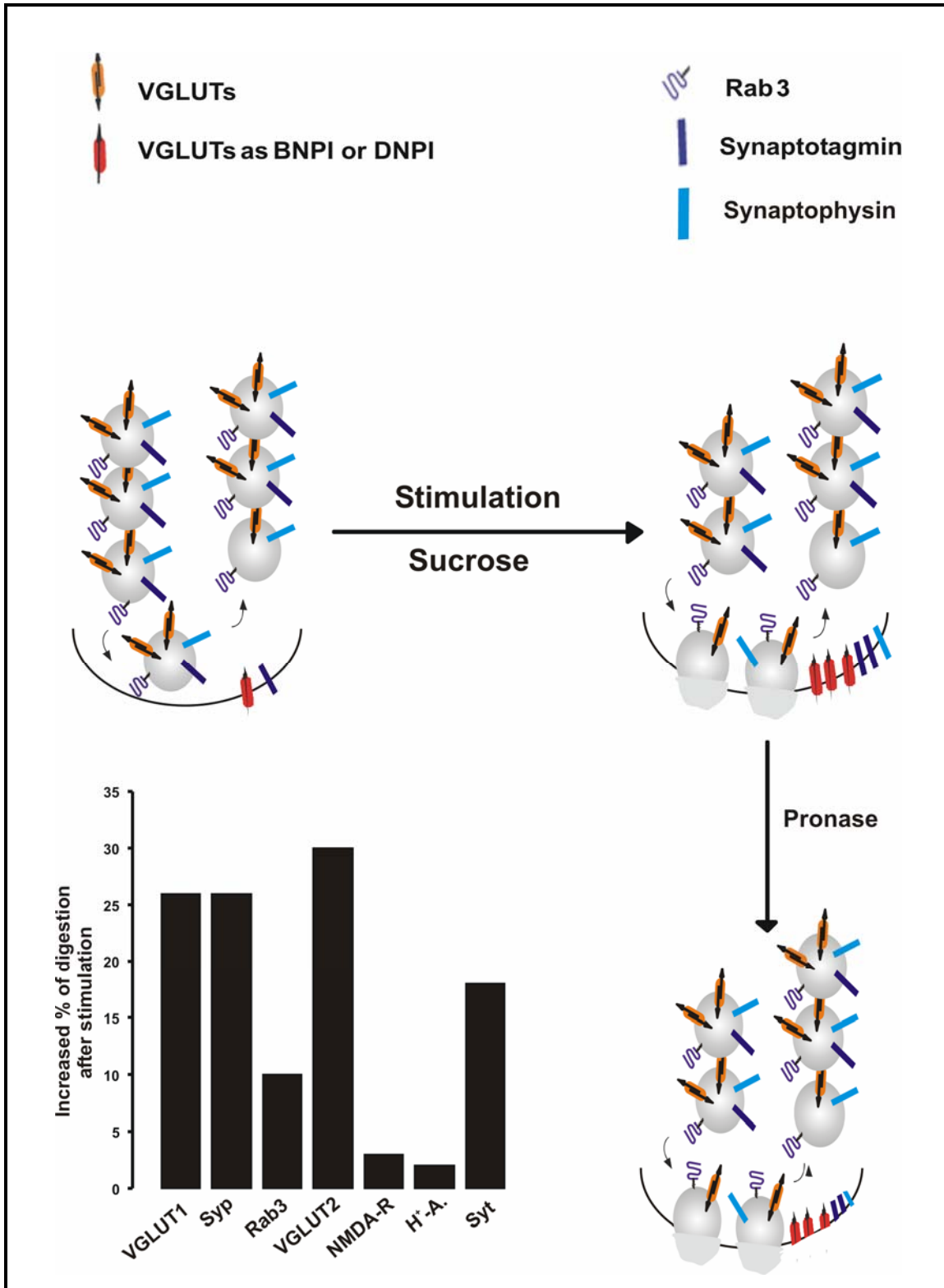
Figure 25: Scheme of stimulation dependent translocation of vesicular proteins to the plasma membrane

The scheme represents the fusion of more SVs and their proteins at the plasma membrane following stimulation compared to the resting conditions and lower panel represents the digestion of vesicular proteins after pronase digestion following stimulation. The graph represents the difference of increased % of digestion of vesicular proteins followed stimulation. Note the significant difference of increase of % of digestion of VGLUTs, Syp and Syt compared to Rab3 and proton pump, which are internal references, followed stimulation.

5.2.2   Translocation in the presence of BFA and dynasore

Vesicular proteins like Syt and VGLUTs in resting conditions, and Syp, Syt and VGLUTs following stimulation are translocated to the plasma membrane. Next, studies were made to check the changes in the translocation of vesicular proteins by inhibiting AP3 and dynamin dependent endocytosis pathways using BFA and dynasore, respectively, with or without sucrose.

Previously, it has been reported that dynasore rapidly inhibits the GTPase activity of dynamin with high specificity (Newton et al., 2006). Endocytosis after sustained or brief stimuli was completely and reversibly blocked by dynasore in cultured hippocampal neurons expressing the fluorescent tracer synaptopHluorin. In contrast, dynasore had no effect on exocytosis. In the presence of dynasore, low-frequency stimulation led to sustained accumulation of synaptopHluorin and other vesicular proteins on the surface membrane. These vesicular components remained on surface membrane even after the stimulus was terminated, suggesting that all endocytic events rely on dynamin during low-frequency activity as well as in the period after it. Further, BFA inhibits the AP3 pathway, which is one of the mechanisms of endocytosis of SVs through endosomal intermediate. It was also shown that during prolonged stimulation, BFA diverts VGLUTs recycling from the slower AP3 pathway to a much faster pathway involving AP2 to which VGLUT1 is normally targeted by endophilin (Voglmaier et al., 2006). This accumulation of vesicular proteins at the plasma membrane after exocytosis in the presence of dynasore and BFA might increase the digestion of vesicular proteins by pronase digestion. Since our preliminary results indicated no significant difference in the digestion of vesicular proteins in the presence of BFA and dynasore, further standardizations are necessary to optimise the conditions for dynasore and BFA treatment, and also stimulation conditions.

5.3   Time- and light-dependent regulation of neurotransmitter transporters and other proteins in wild type and *Per2^Brdm1* mutant mice

5.3.1   Time-dependent trafficking of VGLUTs to the plasma membrane in wild type mice

Previously, the effect of circadian rhythm on SV proteins was studied. In our initial studies, VGLUT1 expression was rhythmic in SVs prepared from wild type mice entrained in LD cycle. Expression was high at ZT24 and ZT12 and significantly lower at ZT6 and ZT18. The significant expression pattern of VGLUT1 at ZT24 and ZT12 in wild type animals was lost in *Per2^Brdm1* mutant mice. No changes in LP1, P2 or in whole brain homogenates were observed. Moreover, no changes in the expression of H^+-ATPase in SVs providing an evidence for VGLUT1 sorting from SVs to different compartment.

The efficiency of transmitter transport into the cytosolic compartment may be regulated by sorting more or less transporter molecules to the plasma membrane. It might be that transmitter transporters reside on vesicles beneath the plasma membrane from where they are rapidly recruited by membrane fusion and so modulate the duration of postsynaptic response. This has been recently shown for GAT1, which is rapidly recycled upon Ca^{2+}- dependent exocytosis and stored in a distinct class of vesicles, lacking Syp and VGAT (Deken et al., 2003). VGLUT1 was initially identified as a Na^+-dependent inorganic phosphate transporter (Ni et al., 1994; Aihara et al., 2000). It has been hypothesized that the vesicular transporter could be incorporated into the plasma membrane during exocytosis thereby mediating transport of inorganic phosphate (Takamori et al., 2000). But no conclusive data is available so far. We presumed that if VGLUT1 is translocated to the plasma membrane then pronase assay should detect the change in the digestion of VGLUT1, since translocation of vesicular proteins to the plasma membrane by pronase assay was seen in previous experiments.

To study this time-of-the-day dependent translocation of VGLUTs, pronase assay was performed on synaptosomes prepared at ZT24/0, ZT6 and ZT12. The digestion pattern at both time points ZT0 and ZT6 was similar to the digestion pattern obtained in initial control experiments with the most digestion for NMDA-receptor and a higher digestion of VGLUTs compared to Rab3. Interestingly, the digestion of VGLUT1 and VGLUT2 was selectively

enhanced at ZT6 compared to ZT 24/0 while digestion of the NMDA-receptor and the vesicular proteins Syp, Syt and Rab3 remained unchanged between these time points.
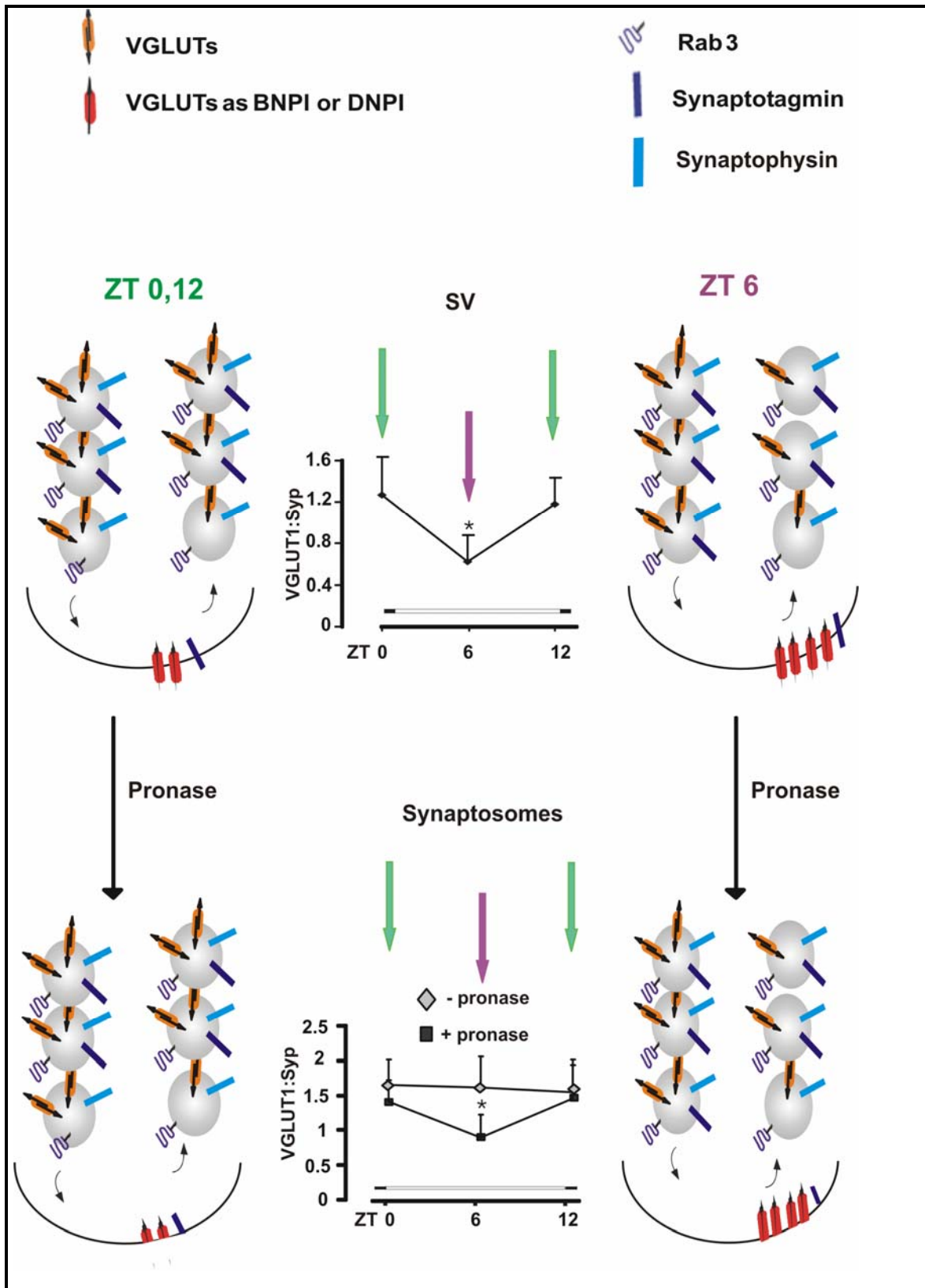
Figure 26: Scheme of the time-dependent translocation of VGLUTs to the plasma membrane

Upper panel represents the expression of VGLUT1 in SVs (graph) and at the plasma membrane at ZT0 and ZT12, and ZT6 time points. Lower panel represents the respective digestion of VGLUTs at the plasma membrane after pronase treatment on synaptosomes.

Further, significant decrease was observed at ZT6 in the ratio of both VGLUT isoforms to Syp or Rab3 after pronase digestion. No change was observed in the non-digested controls taken at the indicated time points and run in parallel, confirming our earlier observations (Yalamanchili et al., 2006). In our initial experiments with SVs, expression of VGLUT1 was higher before beginning of light phase (ZT24/0) and dark phase (ZT12), respectively, and lowers during the middle of the day (ZT6). A similar pattern, as shown in scheme (Fig. 26), was observed using synaptosomes treated with pronase indicate that the selective reduction of VGLUT on SVs at ZT6 is due to its specific translocation to the plasma membrane. Such a translocation was absent in the synaptic vesicle proteins Syp and Syt.

This diurnal switch between the vesicular and the plasma membrane compartment applies to both VGLUT1 and VGLUT2. Synaptic terminals of glutamatergic neuronal subpopulations may differ in their cytoplasmic matrix resulting in changes in the amount of vesicles freed by a hypoosmotic shock used to prepare SVs under standard conditions. So it may well be that diurnal changes in the amount of VGLUT2 have escaped detection in our initial analysis. Indeed a tendency towards a diurnal oscillation has been observed (Yelamanchili et al., 2006). Since the amount of VGLUT per vesicle is crucial for transmitter filling, the observed switch may represent a diurnal change between high and low activity of subpopulations of glutamatergic neurons. Probably the time-dependent membrane traffic allows the pre-synaptic terminal to replenish during physiological resting periods. How the membrane trafficking of VGLUT is regulated at the pre-synaptic level is not clear so far. In this respect vesicular glutamate transporters appear to be special i.e., for VGLUT1 a highly specific reuptake way has been described not shared by other vesicular proteins (Voglmaier et al., 2006).

Currently it is unclear whether VGLUT at the plasma membrane indeed represent a physiologically relevant transporter i.e., for Na/PO_4 or only a storage pool. The interpretation is further complicated by the fact that the ionic basis of VGLUT transport activity for either Na/PO_4 or glutamate is far from being understood. However, the occurrence of high amounts of VGLUT at the plasma membrane and the diurnal regulation of its traffic are in line with observations from

other transmitter transporters. An activity dependent traffic in and out of the plasma membrane has been described for monoamine transporters like the serotonin transporter SERT (Steiner et al., 2008). While these transporters reside on endocytotic vesicles the choline transporter which returns choline to the presynaptic terminal for acetylcholine synthesis is directly localized to acetylcholine storing synaptic vesicles. This transporter is functional at the plasma membrane and appears to be inhibited when localized to synaptic vesicles (Iwamoto et al., 2006). Probably transmitter transporters of the plasma membrane are functionally linked to transporters of synaptic vesicles by regulating traffic between both membrane compartments. Whether in this respect VGLUT has a dual transport function remains unclear.

It is possible to reflect on how such diurnal modulation of glutamatergic transmission is achieved in the context of whole brain. Probably, at each switch, either dark/light or light/dark, different populations of neurons enhance their VGLUT equipment to be prepared for the specific task of the coming light or dark period, respectively. Those neurons which are more active just before the start of the light period, may decline their vesicular VGLUT content by sorting some copies to the plasma membrane during the middle of the day. This may probably occur even before the neurons responsible for the dark period tasks, start to replenish their vesicular VGLUT. This explanation may also apply for the low amounts of VGLUT1 seen in the middle of the night (Yelamanchili et al., 2006).

5.3.2   Are AP2, AP3 and endophilin involved in the trafficking of VGLUTs at the plasma membrane?

VGLUTs, Syb and Syt contain sorting signals which direct them to the endocytic machinery, which may mediate their roles in synaptic vesicle recycling (Grote and Kelly, 1996, Blagoveshchenskaya et al., 1999, Haucke and De Camilli, 1999, Grass et al., 2004, Poskanzer et al., 2006, Voglmaier et al., 2006). Some proteins may be targeted to synaptic vesicles by interacting with other proteins that contain sorting signals but little is known about the signals required for sorting and endocytosis of synaptic vesicle proteins (Prado and Prado, 2002, Voglmaier and Edwards, 2007). However, many synaptic vesicle proteins contain dileucine or tyrosine-based sequences thought to interact with adaptor proteins such as AP2 and AP3. These differences could affect the rate or pathway of recycling, or targeting to different vesicle pools.

Differential sorting of proteins during recycling also raises the possibility of their activity-dependent redistribution, generating synaptic vesicles with varying protein compositions and functional characteristics, which could influence the neurotransmitter release (Valtorta et al., 2001, Bonanomi et al., 2006, Voglmaier and Edwards, 2007).

VGLUTs contain dileucine-like internalization motifs, but the C-terminus of VGLUT1 also contains two polyproline domains not found in VGLUT2 or 3. At one of these polyproline domains, VGLUT1 interacts with endophilin, a protein that mediates membrane curvature and the recruitment of other endocytic machinery (De Gois et al., 2006, Vinatier et al., 2006, Voglmaier et al., 2006). Studies indicated activity dependent differences in targeting of VGLUT1 to the two pathways viz. AP2 and AP3 (Voglmaier et al., 2006).

Since the translocation of VGLUTs to the plasma membrane seems to be time-dependent, studies were made to identify the proteins involved in the VGLUTs endocytosis pathways between different times of the day. The results obtained from two individual experiments showed no difference in the expression pattern of endophilin, AP2 and AP3 in synaptosomes and as well as in SVs between different times of the day and between different genotypes. However, when the amount of AP2 was calculated during light phase (ZT0+ZT18) and dark phase (ZT6+ZT12) separately, there was more AP2 found during dark phase than during light phase in wild type mice entrained in LD cycle. This phenomenon was reversed in case of *Per2^{Brdm1}* mutant mice. These results indicate that under physiological conditions i.e., during light-dark phase, VGLUT recycling may divert from slower AP3 dependent pathway to a faster recycling pathway, presumably mediated by AP2. The change in the expression of AP2 in *Per2^{Brdm1}* mutant mice during night and light phase in ZT condition indicates a light dependent *Period2* gene may play a role in the regulation of VGLUTs through AP2 pathway. There was no change in the expression of endophilin during different times of the day which is consistent with previous studies, in which VGLUT1 interaction with endophilin has no effect on recycling of tagged VGLUT1 under moderate stimulation conditions (Voglmaier et al., 2006). Since AP3 mediates internalisation of VGLUT1 after the prolonged stimulation associated with bulk endocytosis, and bulk retrieval has been implicated in recycling of the reserve pool, it is possible that competition of AP2 and AP3 for recycling of VGLUT1 during stimulation diverts the transporter to different pools.

Like VGLUTs, many synaptic vesicle proteins have their own distinct patterns of expression and trafficking signals, which may regulate their sorting, affecting synaptic vesicle

protein composition. This may in turn determine physiological properties of a synapse, with implications for information processing and synaptic plasticity.

5.3.3 Light-dark and *Per2^{Brdm1}*dependent expression of VGAT and VMAT2 in wild type and *Per2^{Brdm1}* mutant mice entrained in LD and DD cycle

Light-dark dependent expression of VGAT: VGAT transports GABA and glycine into SVs. These transporters were more prominently observed in the ventral suprachiasmatic nucleus (SCN). In contrast to rhythmic oscillation of VGLUT1, less pronounced oscillation of VGAT was seen in SVs between different times of the day. A similar phenomenon was observed in synaptosomal fractions. However, the amount of VGAT in SVs as well as in synaptosomes prepared from mice kept under complete dark conditions is less compared to mice kept under light-dark conditions. This reduction was also seen in *Per2^{Brdm1}* mice. This provides evidence that the *Period2* gene might not affect the VGAT expression. In contrast to VGLUT1, the regulation of VGAT was seen in both fractions under complete dark conditions. This indicates that VGAT expression might be light-dark dependent but not related to the sorting or trafficking of VGAT to different SV pools or compartments. It was also shown that the level of the GABA concentration is high in the hypothalamus during light phase with two peaks: one at 7.00A.M. and the other at 11.00A.M (Cattabeni et al., 1978). To conclude, probably light/dark cycle is required to increase or sustain the expression of VGAT which get reduced when mice were kept in complete darkness.

*Per2^{Brdm1}*dependent expression of VMAT2: So far, time-dependent regulation of VGLUTs and light-dark dependent regulation of VGAT was shown in wild type and *Per2^{Brdm1}* mutant mice. Further studies were made to analyse VMAT2 expression on time and circadian dependent manner in wild type and *Per2^{Brdm1}* mutant mice. VMAT2 was more abundant in areas surrounding the SCN, which are densely innervated by histaminergic fibres. Surprisingly, there was more VMAT2 found in *Per2^{Brdm1}* mutant mice entrained in LD as well as DD conditions compared to wild type mice. These results indicate that *Per2* might play a role in the expression of VMAT2. This is consistent with recent studies about influence of *Period2* gene on monoaminergic system (Hampp et al., 2008). They have shown increased levels of dopamine, and reduced expression and activity of monoamine oxidase A (MAOA) in *Per2^{Brdm1}* mutant mice.

The increased VMAT2 expression in *Per2^Brdm1^* mutant mice may transport excessive cytosolic dopamine levels to SVs. Further, MAOA-deficient mice also show a modest down regulation of VMAT2. These findings suggest a role of circadian-clock component *Per2* play a role in the regulation of VMAT2 at the synapse.

6 SUMMARY

The main players involved in synaptic neurotransmission are the synaptic vesicles (SVs), their associated proteins and transporters. Differences in the trafficking of individual synaptic vesicle proteins could target them to different recycling pathways changing rates of recycling and delivering them to different vesicle pools or compartments which could determine their functional characteristics. The present study focuses on the association of SV proteins with the plasma membrane and the effect of circadian rhythm on regulation of the amount of neurotransmitter transporters per vesicle.

In the first part, studies were made to identify SV proteins at the plasma membrane during/after exocytosis. The pronase assay was developed to study which and how much SV proteins translocated to the plasma membrane in synaptosomes prepared from adult rat or mouse whole brain. Pronase is a mixture of proteolytic enzymes and respects the plasma membrane thereby avoiding cleavage of proteins on SV inside the synaptosome. The following results were obtained: 1) Considerable digestion of synaptotagmin and the vesicular glutamate transporters (VGLUTs) in synaptosomes followed by pronase digestion indicated occurrence of these proteins at the plasma membrane. 2) Increased digestion of the SV proteins synaptophysin, synaptotagmin and VGLUTs in synaptosomes followed by sucrose stimulation compared to resting conditions indicated that pronase digestion allows for tracking changes in the translocation of SV proteins to the plasma membrane between resting and stimulated conditions.

In the second part, experiments were performed to study the influence of circadian rhythm on the amount of VGLUTs, the vesicular GABA (VGAT) and monoamine (VMAT2) transporters per vesicle. Significant time-of-the-day-dependent oscillations in the amount of VGLUT1 in SVs prepared from wild type mice were observed while these oscillations were not seen in synaptosomes. Digestion of mouse synaptosomes prepared at different times of the day revealed that more VGLUT was digested at noon (ZT6) compared to the start of the light period (ZT0) while digestion of synaptophysin and synaptotagmin was independent from diurnal cycling. The pronase digestion data reflects the amounts obtained with isolated SVs indicating a diurnal cycling of VGLUTs to the plasma membrane. In contrast to VGLUTs, the amount of the vesicular GABA transporter VGAT did not vary diurnally in SVs as well as in synaptosomes prepared from wild type and *Per2*^{Brdm1} mutant mice. However, when comparing the amount of

VGAT in SVs and synaptosomes from mice kept in light-dark cycle with those of mice kept under complete darkness, a decrease in the amount of VGAT was observed. Thus, the presence of a light/dark cycle seems to be an additional signal for VGAT expression. Finally, higher amounts of VMAT2 was found in *Per2^Brdm1* mutant mice compared to wild type mice entrained in a light-dark cycle or kept in complete darkness indicating a *Per2* dependent regulation of VMAT2.

In summary, three important neurotransmitter transporters are regulated differently by circadian-dependent mechanisms 1) VGLUTs are sorted diurnally to the plasma membrane of the pre-synaptic terminal. 2) VGAT expression appears to depend at least partially on a light/dark cycle. 3) VMAT2 expression seems to be modulated by the *Per2* gene.

7   ZUSAMMENFASSUNG

Synaptische Vesikel (SVs) sind Schlüsselorganellen neuronaler Kommunikation. Allen SV ist eine Reihe von Transmembranproteinen und assoziierten Proteinen gemein. SV in Subpopulationen von Neuronen unterscheiden sich aber in ihrem jeweiligen vesikulären Transmittertransporter. Unterschiede in der Sortierung individueller synaptischer Vesikelproteine können die Proteinausstattung von SV-Subpopulationen verändern. Variationen in der Sortierung können daher die Funktion von SV-Subpopulationen beeinflussen. Die vorliegende Studie untersucht das Vorkommen von SV-Proteinen an der Plasmamembran und den Effekt zirkadianer Rhythmik auf die Umverteilung und Regulation vesikulärer Neurotransmittertransporter für Glutamat (VGLUT), GABA (VGAT) und Monoamine (VMAT2).

Im ersten Teil wurde das Vorkommen von SV-Proteinen an der Plasmamembran vor und nach Stimulation erfasst. Hierzu wurden Synaptosomen einem partiellen Verdau mit einem Gemisch aus proteolytischen Enzymen (Pronase) unterworfen. Dabei werden nur die in den Extrazellulärraum reichenden Peptidketten abgedaut, während die zytosolischen Anteile der SV-Proteine verschont bleiben. Es wurden zunächst Synaptosomen aus dem Ratten- oder Mäusegehirn angedaut. Dabei ergaben sich folgende Befunde: 1) Ein deutlicher Verdau von Synaptotagmin und den VGLUTs als Folge der Pronasebehandlung an Synaptosomen zeigte das Auftreten von diesen Proteinen an der Plasmamembran. 2) Der erhöhte Verdau der SV-Proteine Synaptophysin, Synaptotagmin und von VGLUTs an Synaptosomen nach einer Saccharosestimulation zeigte, dass die Pronasebehandlung geeignet ist, die Verlagerung von SV-Proteinen an die Plasmamembran nach Stimulation zu verfolgen.

Im zweiten Teil wurde der Einfluss zirkadianer Rhythmik auf ausgewählte synaptische Vesikelproteine und im Speziellen auf die Neurotransmittertransporter VGLUT1/2, VGAT und VMAT2 bezüglich deren Umverteilung und Regulation untersucht. Tageszeitlich abhängige Oszillationen wurden in der Menge von VGLUT1 in einer SV-Fraktion nicht aber in einer Synaptosomenfraktion, jeweils gewonnen aus Wildtypmäusen, beobachtet. Der Pronaseverdau von Maussynaptosomen zu verschiedenen Tageszeiten ergab ein tageszeitabhängiges Vorkommen der VGLUTs an der Plasmamembran. Die VGLUTs unterlagen mittags (ZT6) einem verstärkten Verdau verglichen mit dem Beginn der Lichtphase (ZT0), wohingegen der Verdau von Synaptophysin und Synaptotagmin unabhängig von der Tageszeit war. Die

Pronaseexperimente spiegeln dabei die im Tagesverlauf oszillierenden Mengen an VGLUT wie sie an SV-Fraktionen gefunden wurden wider und belegen, dass VGLUT an die Plasmamembran sortiert wird. Im Gegensatz zu den VGLUTs unterliegt der vesikuläre GABA-Transporter VGAT von Wildtypmäusen oder Per2^{Brdm1}-Mutanten keinen tageszyklischen Umverteilungen zwischen SVs und der Plasmamembran. Andererseits war bei Mäusen, die in kompletter Dunkelheit gehalten wurden, die VGAT-Menge in SV- und Synaptosomenfraktionen im Gegensatz zur Hell-Dunkel-Bedingung reduziert. Die Gegenwart eines Hell-Dunkel-Zyklus ist daher ein verstärkendes Signal für die VGAT-Expression. Schließlich wurde im Gegensatz zum Wildtyp eine erhöhte Menge des vesikulären Monoamintransporters (VMAT) 2 in der *Per2^{Brdm1}*-Mutante im Hell-Dunkel- und Dunkel-Dunkel-Zyklus gefunden. Zusammenfassend kann festgestellt werden, dass drei wichtige Neurotransmittertransporter unterschiedlich durch zirkadian-abhängige Parameter reguliert werden. 1) Die VGLUTs werden tageszeitabhängig auf der Ebene der präsynaptischen Terminalien umsortiert. 2) Die VGAT-Expression ist von einem Hell-Dunkel-Zyklus abhängig. 3) Die VMAT2-Expression wird durch das *Per2*-Gen moduliert.

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9 APPENDIX

Abbreviations

APS	ammonium persulfate
ATP	adenosine triphosphate
BCA	bicinchoninic acid –4,4-dicarboxy-2,2-bincincholin
Bmal1	brain-muscle Arnt-like protein 1
BNPI	brain-specific Na+-dependent inorganic phosphate cotransporter
Brdm1	Bradly mutation 1
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
Ca2+	intracellular calcium
CHO	chinese hamster cell line
Clock	circadian locomotor output cycles kaput
CNS	central nervous system
Cry	cryptochrome
CT	circadian time
C-terminal	carboxy terminal
Da	Dalton
DD cycle	dark-dark cycle
ddH2O	bi-distilled water
dH2O	distilled water
DMSO	dimethyl sulfoxide
DNPI	differentiation associated Na+-dependent inorganic phosphate cotransporter
DRM	detergent insoluble membrane
DTT	dithiothreitol
EAAT1/2	excitatory aminoacid transporters 1/2
ECL	Enhanced Chemiluminescence
EDTA	ethylene-diamine-tetra-acetic acid
EGTA	ethylenglycol bis (2-aminoethyl-)tetraacetate
ELISA	enzyme linked immuno sorbent assay

g	gram
GABA	g-amino butyric acid
GluR	glutamate receptor
h	hour
HEPES	N-2-Hydroxyethylpiperazin-N´-2-ethane-sulfonic acid
KO	knock-out
LD	cycle light-dark cycle
LP1	lysed pellet 1
LP2	lysed pellet 2
LS1	lysed supernatant 1
LS2	lysed supernatant 2
M	molar (mol/l)
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger RNA
nm	nano meter
NMDA	N-methyl-D-Aspartate
NSF	N-ethylmaleimide sensitive factor
NT	neurotransmitter transporter
OD	optic density
O/N	overnight
P2	synaptosomal pellet
PAGE	protein gel electrophoresis
PBS	phosphate buffered saline
PC12	pheocytochroma cell line 12
PCR	polymerase chain reaction
Per1	period circadian protein 1
Per2	period circadian protein 2
Per3	period circadian protein 3

PNS	post nuclear supernatant
pH	Neg. logarithm of the hydrogen ion concentration in moles per liter. p refers to power of 10 and H to hydrogen
ReP	resting pool
RHT	retinohypothalamic tract
RNA	ribonucleic acid
RP	reserve pool
rpm	rotations per minute
RRP	readily releasable pool
RT	room temperature
s	second
SCN	suprachiasmatic nucleus
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SLO	streptolysin-O
SNAREs	synaptosomal associated protein receptors
SNAP	soluble NSF attachment protein
Syt	synaptotagmin
SV	synaptic vesicle
Syb	synaptobrevin
Syn	synapsin
Syp	synaptophysin
Syp/Syb complex	synaptophysin/synaptobrevin complex
Syx	syntaxin
TEMED	N,N,N´,N´-Tetramethylethylendiamine
TRIS	tris-(hydroxymethyl)-aminomethane
t-SNAREs	target SNAREs
V	Volt
VAMP	vesicle associated membrane protein
VachT	vesicular acetylcholine transporter
VGLUT1/2/3	vesicular glutamate transporters 1/2/3

VGAT	vesicular GABA transporter
VMAT	vesicular monoamine transporter
VNT	vesicular neurotransmitter transporter
v-SNAREs	vesicular SNAREs
v/v	volume per volume
WT	wild-type
w/v	weight per volume
ZT	Zeitgeber time
μ	micro: prefix denoting 10-6
μg	microgram
μl	microliter
μmol	micromol
°C	Celsius degree centigrades

10 LIST OF PUBLICATIONS

1. Darna M, Schmutz I, Yelamanchili SV, Pendyala G, Albrecht U and Ahnert-Hilger G. Putative sorting mechanism involved in regulation of vesicular neurotransmitter transporters at presynapse **(submitted in august, 2008 to *J Biol Chem.*)**

2. Yelamanchili SV, Pendyala G, Brunk I, **Darna M**, Albrecht U, Ahnert-Hilger G. Differential sorting of the vesicular glutamate transporter 1 into a defined vesicular pool is regulated by light signaling involving the clock gene period 2. *J Biol Chem.* **(2006)** Jun 9;281(23):15671-79

3. Cotella D, Jost N, **Darna M**, Radicke S, Ravens U, Wettwer E. "Silencing the cardiac potassium channel Kv4.3 by RNA interference in a CHO expression system". *Biochem Biophys Res Commun*. **(2005)** May 6; 330(2):555-60.

Poster presentations at National and International Conferences

1. **Darna M**, Schmutz I,Yelamanchili SV, Pendyala G, Albrecht U, Ahnert-Hilger G. Putative sorting mechanism involved in regulation of vesicular neurotransmitter transporters, **Society for Neuroscience (SFN)**, November 2007, Sandiego, USA

2. **Darna M,** Yelamanchili SV, Pendyala G, Albrecht U, Ahnert-Hilger G. Circadian dependant sorting of Vesicular glutamate transporter (VGLUT) 1:may involve the plasma membrane, 7th meeting of the **German neuroscience society**, march 2007,Göttingen Germany.

3. **Darna M,** Cotella D, Jost N, Ravens U, Wettwer E. "Silencing the cardiac potassium channel Kv4.3 by RNA interference in a CHO expression system, 28th meeting of the **European working group in Cardiac Cellular Electrophysiology** (EWGCCE), september, 2004 - Szeged, Hungary

4. Attended the 5th **Forum of European Neuroscience meeting (FENS)** on July 8-12, 2006 in Vienna, Austria

5. Attended **Berlin Neuroscience Forum (BNF)** 2006, Berlin, Germany.

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12 ERKLÄRUNG

Hiermit versichere ich, die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt zu haben.

Bei der Verfassung der Dissertation wurden keine anderen als die im Text aufgeführten Hilfsmittel verwendet.

Ein Promotionsverfahren wurde zu keinem früheren Zeitpunkt an einer anderen Hochschule oder bei einem anderen Fachbereich beantragt.

Berlin, 29.08.2008

Mahesh Darna