Studies of synapsin phosphorylation and characterization of monoclonal antibodies from the Würzburg Hybridoma Library in *Drosophila melanogaster*

Untersuchungen der Phosphorylierung von Synapsin und Charakterisierung monoklonaler Antikörper der Würzburg Hybridoma Library in *Drosophila melanogater*

Doctoral thesis for a doctoral degree
at the Graduate School of Life Sciences,
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Würzburg, 2014

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I. Summary

Synapsins are conserved synapse-associated phosphoproteins involved in the fine regulation of neurotransmitter release. The aim of the present project is to study the phosphorylation of synapsins and the distribution of phospho-synapsin in the brain of *Drosophila melanogaster*.

Three antibodies served as important tools in this work, a monoclonal antibody (3C11/α-Syn) that recognizes all known synapsin isoforms and two antisera against phosphorylated synapsin peptides (antiserum PSyn(S6) against phospho-serine 6 and antiserum PSyn(S464) against phospho-serine 464). These antisera were recently generated in collaboration with Bertram Gerber and Eurogentec.

Using these antibodies, phosphorylated synapsin isoforms were identified by Western blots of brain homogenates and their distribution in the brain was characterized by immunohistochemical stainings of whole mount and cryostat preparations. As a first step, the specificity of the antisera had to be tested. To this end various experimental parameters for tissue homogenization and fixation for immunohistochemical detection were optimized using wild-type flies and synapsin null mutants as negative controls.

It could be demonstrated that the PSyn(S6) and PSyn(S464) antisera are specific, whereas control antisera (Syn(S6) and Syn(S464)) against the corresponding unphosphorylated peptides apparently mainly crossreact with unrelated proteins.

Next, the effect of the Sap47 null mutation on synapsin expression and phosphorylation was investigated. The results indicate that in Sap47 null mutants an additional synapsin isoform is expressed but that phosphorylation at serine 6 (S6) is reduced, whereas S464 phosphorylation seems to be unaffected.

Since vertebrate synapsins are substrates for protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), the role of PKA and CaMKII in phosphorylation of synapsins of *Drosophila* was investigated. Preliminary experiments with transgenic flies expressing a knock-down construct for the major catalytic PKA subunit (DCO) or mutated forms of this subunit did not indicate a significant influence of

PKA on synapsin phosphorylation at S6 or S464 in wild-type flies. However, a transgenically expressed non-edited isoform with the conserved PKA target site appears to be hyperphosphorylated at S6. Transgenic expression of a peptide inhibiting CaMKII or overexpression of CaMKII with a mutation in the autophosphorylation target site at threonine 287 significantly lowers the apparent molecular weight of major synapsin isoforms and reduces synapsin phosphorylation at S6 (but not at S464). This indicates that synapsin is a substrate for CaMKII also in *Drosophila*. A function of S6 phosphorylation by CaMKII in olfactory short-term habituation of *Drosophila* has been demonstrated in a collaboration.

Immunohistochemical experiments using whole mount or cryosectioned preparations demonstrate that synapsin isoforms phosphorylated at S6 are prominent in ellipsoid body ring neurons of the central complex but are also present at much lower concentration in all other neuropil regions. The distribution of isoforms phosphorylated at S464, on the other hand, is similar to the staining pattern obtained with 3C11 antibody that recognizes synapsins irrespective of their phosphorylation status.

In the second part of this work monoclonal antibodies (mAbs) from the Würzburg hybridoma library were investigated by immunohistochemistry using whole mount brain preparations and by confocal microscopy in comparison with cryosections made earlier by Alois Hofbauer. The antibody nb168 showed the same staining pattern as mAb nb33 which binds to cells containing the pigment dispersing factor (PDF). The antibody nb169 shows a specific staining pattern with two large cell bodies in the central brain and two clusters of small cells near the optic lobes. Certain features similar to anti-serotonin staining suggested that the antibody nb169 might recognize a protein involved in the serotonergic pathway but double staining revealed no cellular co-localization. Janelia farm Gal4 lines (employing regulatory sequences of neuronal genes) were screened for similarities between Gal4 driven GFP expression and the monoclonal antibody staining patterns. Antibodies nb169, ab47 and nc24 selectively stained specific subsets of GFP-expressing cells observed in different Gal4 lines. Hence, the split Gal4 technique might become an important tool to investigate and functionally characterize these subsets of cells.

II. Zusammenfassung

Synapsine sind konservierte, Synapsen-assoziierte Phosphoproteine, die an der Feinregulation der Neurotransmitterfreisetzung beteiligt sind. Das Ziel des Projektes ist, die Phosphorylierung der Synapsine und die Verteilung des Phospho-Synapsins im Gehirn von *Drosophila melanogaster* zu untersuchen.

Aus diesem Grunde wurden drei bestimmte Antikörper in dieser Arbeit verwendet: Ein monoklonaler Antikörper (3C11/α-Syn), der alle bekannten Isoformen von Synapsin erkennt, und zwei Antiseren gegen phosphorylierte Synapsinpeptide (das Antiserum PSyn(S6) gegen Phosphoserin 6 und das Antiserum PSyn(S464) gegen Phosphoserin 464). Diese Antiseren wurden unlängst in Zusammenarbeit mit Bertram Gerber und Eurogentec hergestellt.

Durch Verwendung dieser Antikörper wurden die phosphorylierten Isoformen in Homogenaten adulter Gehirne mittels Western Blot identifiziert und ihre Verteilung im Gehirn durch immunhistochemische Färbungen von wholemount Präparaten sowie Cryostatschnitten charakterisiert. Als erstes wurde die Spezifität der Antiseren getestet. Um diese zu gewährleisten, wurden verschiedene experimentelle Parameter für die Homogenisierung und Fixierung des Gewebes für den immunhistochemischen Nachweis durch Verwendung von wildtypischen Fliegen und Synapsin-Nullmutanten als Negativkontrolle optimiert.

Es konnte gezeigt werden, dass die Antiseren PSyn(S6) und PSyn(S464) spezifisch sind, wohingegen die Kontrollantiseren (Syn(S6) und Syn(S464)), die gegen die entsprechenden unphosphorylierten Peptide gerichtet sind, scheinbar vor allem mit nicht verwandten Proteinen kreuzreagieren.

Des Weiteren wurde die Auswirkung der Sap47-Nullmutation auf die Expression und die Phosphorylierung von Synapsin untersucht. Die Ergebnisse zeigen, dass in Sap47-Nullmutanten eine zusätzliche Synapsin Isoform exprimiert wird und die Phosphorylierung des Serins 6 (S6) reduziert ist, wohingegen die Phosphorylierung von S464 nicht beeinflusst zu sein scheint.

Da Synapsine von Vertebraten als Substrate der Proteinkinase A (PKA) sowie der Ca^{2+}/Calmodulin-abhängigen Proteinkinasen (CaMKs) bekannt sind, wurde die Bedeutung von PKA und CaMKII für die Phosphorylierung der Synapsine von *Drosophila* untersucht. Vorversuche mit transgenen Fliegen, die ein knock-down-Konstrukt für die wichtigste katalytische PKA-Untereinheit (DCO) oder mutierte Formen dieser Untereinheit exprimieren, zeigten im Vergleich zu wildtypischen Fliegen keinen signifikanten Einfluss von PKA auf die Synapsin-Phosphorylierung an S6 oder S464. Dagegen scheint eine transgen-exprimierte nicht editierte Isoform mit der konservierten PKA Zielsequenz an S6 hyperphosphoryliert zu sein. Die transgene Expression eines Peptids, das die CaMKII hemmt, oder von CaMKII mit einer Mutation in der Autophosphorylierungsdomäne am Threonin 287 senkt das scheinbare Molekulargewicht von prominenten Synapsin Isoformen signifikant und reduziert die S6, nicht aber die S464 Phosphorylierung. Dies zeigt, dass Synapsin auch in *Drosophila* ein Substrat für die CaMKII ist. Eine Funktion der S6-Phosphorylierung durch CaMKII bei olfaktorischen Kurz-Zeit-Habituation wurde in einer Kooperation nachgewiesen.

Immunhistochemische Versuche mit wholemount Präparaten und Cryostatschnitten zeigen, dass die Isoformen des Synapsins, die an S6 phosphoryliert sind, hauptsächlich in den Ringneuronen des Ellipsoidkörpers des Zentralkomplexes, aber auch in geringer Konzentration in allen Regionen des Neuropils vorhanden sind. Dagegen weist der Nachweis die Verteilung der Isoformen, die an S464 phosphoryliert sind, das gleiche Färbemuster auf wie Färbungen mit dem Antikörper 3C11, der alle Synapsin-Isoformen unabhängig vom Phosphorylierungszutand erkennt.

Im zweiten Teil Arbeit wurden Antikörper der Würzburg Hybridoma Library immunhistochemisch, durch Verwendung von wholemount Gehirn Präparaten und Konfokalmikroskopie in Vergleich zu den früher angefertigten Cryostatschnitten von Alois Hofbauer untersucht. Der Antikörper nb168 zeigte das gleiche Färbemuster wie der monoklonale Antikörper nb33, der an Zellen bindet, die pigment dispersing factor (PDF) enthalten. Der Antikörper nb169 weist ein spezifisches Färbemuster auf, bestehend aus zwei großen Zellkörpern im Zentralhirn und zwei weiteren Clustern kleiner Zellen nahe der optischen Loben. Gewisse Ähnlichkeit mit der Färbung gegen Serotonin führte zu der Spekulation, dass nb169 möglicherweise ein Protein erkennt, das

im serotonergen Signalweg beteiligt ist, aber Doppelfärbungen ließen keine zelluläre Kolokalisation erkennen. Janelia Farm Gal4-Linien (die regulatorische Sequenzen von neuronalen Genen verwenden) wurden nach Ähnlichkeiten der Gal4 getriebenen GFP-Expression mit Färbemustern der monoklonaler Antikörper durchgemustert. In einigen Gal4-Linien zeigte die GFP-Expression in bestimmten Zellgruppen Kolokalisation mit den Antigenen der monoklonalen Antikörper nb169, ab47 und nc24 auf. Die Split Gal4-Technik kann deshalb möglicher Weise ein wichtiges Werkzeug darstellen, um diese Zellgruppen zu untersuchen und funktionell zu charakterisieren.

1.Introduction

1.1 The synapse

The brain of mammals is made up of over 100 billion nerve cells, or neurons, that communicate with each other through some specialized contacts called synapses. A single neuron typically can receive signals from up to 1000 other neurons, through synapses that are found mostly on the dendrites of the neuron, multi-branching tree-like projections originating from the cell body. The axon, a thin and long filament that grows out from the cell body of the neuron, usually makes synaptic contacts with numerous other neurons (Südhof, 1995; Schoch and Gundelfinger, 2006). Thus, the signal passes through an intricate network of neurons which forms an extensive and complicated neuronal circuit.

Some synapses can excite the neuron and generate a signal called action potential (AP), a change of voltage across the neuronal membrane that is propagated down the axon. Sodium channels are important for the depolarization of the membranes, whereas in the repolarization and hyperpolarization potassium channels are involved. The resting potential (RP) of -70 mV needs to be elevated to about -40 mV to trigger an AP. When an action potential arrives at the synapse, the nerve terminal excretes small quantities of chemicals called neurotransmitters. They diffuse over a short distance through the synaptic cleft and bind to special receptors, called postsynaptic receptors, which are located in the dendrite that receives the action potential (Fernández-Chacón and Südhof, 1999). Synapses have the ability to initiate or inhibit the triggering of APs. An excitatory synapse releases transmitter molecules that bind to specific receptors which leads to an EPSP (excitatory postsynaptic potential) through sodium influx, elevates the membrane potential (MP) and therefore increases the probability of an AP firing. If the summation of several EPSPs from different synapses on the neuron occurs, and -40 mV is reached, the neuron fires. In contrast, inhibitory synapses induce IPSPs (inhibitory postsynaptic potentials) that causes the MP to become more negative. The action potential is propagated down the axon to the points where the axon has made synapses with the dendrites of other nerve cells, muscles or glands. There are two major types of synapses, conveying electrical or chemical signals.

- Electrical synapses:

An electrical synapse is an electrically conductive link between two abutting neurons that is formed at a point of contact between the pre- and postsynaptic neurons known as a gap-junction. Each gap-junction consists of numerous channels composed of two hemichannels, which are themselves each composed of six connexin molecules. The current that influences the membrane potential of the postsynaptic cell is generated directly by the voltage difference of the two cells. The gap-junction channels that connect both terminals (pre- and postsynaptic terminal) constitute a high conductance to make sure that the electric current between the presynaptic terminal and the postsynaptic terminal influences the membrane potential of the postsynaptic cell. If this current is large enough, the voltage-gated ion channels of the postsynaptic cell may open and generate an action potential (Meier and Dermietzel, 2006).

- Chemical synapses:

No continuity between the pre- and postsynaptic neurons can be found at chemical synapses. The function of a chemical synapse depends on the release of neurotransmitters from the presynaptic terminal. A neurotransmitter is a chemical substance that binds to its specific receptor at the postsynaptic membrane of the synapse. The presynaptic terminal contains a collection of synaptic vesicles filled with thousands neurotransmitter molecules. The synaptic vesicles bind to the specialized release regions of the presynaptic membrane called active zones. When the propagating action potential reaches the presynaptic membrane, Ca^{2+} ions enter the presynaptic terminal through the voltage-gated Ca^{2+} channels in the active zones. The increase of intracellular Ca^{2+} causes the vesicle fusion with the presynaptic membrane and thus the release of their neurotransmitters into the synaptic cleft, a process called exocytosis. The neurotransmitter molecules diffuse through the synaptic cleft and bind to the receptors at the postsynaptic membrane. The receptor is activated and, either directly or indirectly through metabolic step specific ion channel will open or close. The resulting ion flux changes the conductance of the membrane and also the potential of the postsynaptic cell (Murthy and De Camilli, 2003).

Chemical synapses can be either excitatory or inhibitory. Glutamate is the most prominent excitatory neurotransmitter in the mammalian central nervous system while γ-aminobutyric acid (GABA) usually works as an inhibitory neurotransmitter. Proteins assembled at the pre- and postsynaptic membrane form electron-dense structures called pre- and postsynaptic densities, respectively, and are essential to control the structure and plasticity of the synapses (Kim and Sheng, 2004).

The pre- and postsynaptic membranes are joined by interactions between cell adhesion molecules (CAMs). The major group of CAMs is the immunoglobulin (Ig) family of the adhesion molecules: neurexins, neuroligins, and ephrins as well as ephrins receptors (Eph). They contribute to the specifity of the synapses and play a role in the organization of the ion channels at the post-synaptic density. Neurexins are a calcium-dependent family that is expressed from three different genes. Neurexins are located in the presynaptic membranes and bind to the extracellular domain of the neuroligins at the postsynaptic density. They are found at excitatory or inhibitory synapse (Nguyen and Südhof, 1997). Ephrins are ligands for the ephrin receptors, which constitute a large family of 13 receptor tyrosine kinases. They can trigger bi-directional signaling cascades and are very important in the formation and maturation of the synapses (Dalva *et al.,* 2007).

Cadherins and synaptic cell-adhesion molecules (SynCAMs) have an Ig extracellular domain and are classified as single-pass transmembrane proteins (Shapiro *et al.,* 2007). Cadherins keep the pre- and postsynaptic membranes connected by Ca^{2+}-dependent homophilic interactions. However, there are some controversial theories about the SynCAMs. Some authors conclude that they remain connected by homophilic interactions (Biederer *et al.,* 2002), while some argue they are joined by heterophilic adhesions (Fogel *et al.,* 2007). Nonetheless, they all agree that the SynCAM interactions, in contrast to cadherins, are Ca^{2+}- independent.

1.1.1 Synaptic vesicles

Synaptic vesicles (SVs) are formed by lipids and proteins and carry small hydrophilic proteins. SVs store the neurotransmitters and are responsible for the release of the neurotransmitters at the actives zones (Bonanomi *et al.,* 2006). Synaptic vesicles measure 40 nm in diameter and there are at least nine classes of transmembrane proteins that are found in the synaptic vesicles. Upon the arrival of an action potential stimulus, vesicles fuse with the plasma membrane and release their content (Dittman *et al.,* 2009). Furthermore, these vesicles contain transport proteins involved in the neurotransmitter uptake and trafficking proteins that are involved in synaptic vesicle exo- and endocytosis, and recycling (Südhof, 2004).

1) **Mobilization**

Synaptic vesicles represent a reserve of neurotransmitters when they are far from the active zones. They are not able to move freely through the terminal. Normally, they are anchored to some cytoskeletal filaments by synapsins, a family of ten proteins (Ia, Ib, IIa, IIb, IIIa-f) (review Cesca *et al.,* 2010). Synapsins Ia and Ib are substrates for both the protein kinase A (PKA) and the Ca^{2+}/calmodulin-dependent kinases (CaMKs). When synapsin I is not phosphorylated, it is thought to immobilize synaptic vesicles by linking them to actin filaments and other components of the cytoskeleton. Phosphorylation of synapsin frees the vesicles from the cytoskeletal constraint, allowing them to move to the active zone (De Camilli *et al.,* 1983).

2) **Docking**

Docked vesicles are the ones found closely to the plasma membrane. Synaptic vesicles can be recruited by Rab3A and Rab3C, they belong to the superfamily of the ras protooncogens that bind and hydrolyze GTP to GDP and inorganic phosphate. They bind to the synaptic vesicles that contain a hydrophobic hydrocarbon group that attaches covalently to the carboxy terminal of the Ras protein. The hydrolysis of GTP can be important for the synaptic vesicles to reach the docking point. During exocytosis, the Rab protein can be released to the cytoplasm. α-RIM (rab-3-interacting molecule) contains Munc13 and Rab3 binding sites. These three proteins form the ternary Rab3/RIM/Munc13 complex. Due to this interaction, the priming machinery can be activated (Dulubova *et al.,* 2005).

3) **Priming**

Priming is the process by which the vesicles become release competent. ATP-dependent reactions are characteristic of this process. Only primed vesicles can fuse with the plasma membrane when the Ca^{2+} concentration increases (Verhage and Sorensen, 2008). Priming steps include synthesis of phosphatidylinositol (4,5)-bisphosphate and disassembly of assembled soluble NSF attachment protein receptor (SNARE) complexes by the AAA+-ATPase N-ethylmaleimide-sensitive fusion protein (NSF) and its co-factor α-soluble NSF attachment protein (αSNAP).

4) **Fusion**

The proteins responsible for the fusion event are the Sec1/Munc18-1 (SM) protein Munc18-1; N-ethylmaleimide sensitive factor (NSF); soluble NSF attachment proteins (SNAPs); the SNAP receptors (SNAREs) synaptobrevin (known also as VAMP, vesicle-associated membrane protein), syntaxin-1 and SNAP-25; and small GTPases from the Rab3 family (Rizo and Rosemmund, 2008).

The SNARE complex is the key in membrane fusion with the SNARE protein of the synaptic vesicle synaptobrevin and the plasma membrane SNAREs synatxin-1 and SNAP-25. Thus, these three proteins form the SNARE complex which can be disassembled upon binding to SNAPs and NSF through ATPase activity of NSF (Söllner *et al.,* 1993). The SNARE complex is responsible to bring the vesicle and the plasma membrane into close proximity.

One of the prerequisites for the fusion event is the formation of a trans-SNARE complex between two membranes. However, some SNARE complexes are not able to lead to fusion. One of the possible reasons could be the insertion of flexible sequences in the synaptobrevin linker that inhibits Ca^{2+}-triggered exocytosis (Deak *et al.,* 2006). In addition, synaptobrevin can be attached to synaptophysin which inhibits the SNARE complex. On the other hand, Munc18-1 regulates the fusion machinery. It binds to the closed conformation of syntaxin-1 (Dulubova *et al.,* 1999) and blocks the SNARE motif from participating in the SNARE complex. Munc18-1 has to be released for SNARE complex formation. However, Munc18-1 can also be involved in SNARE complex assembly interacting with the heterodimer synatxin-1-SNAP-25 (Guan *et al.,* 2008).

Munc13 regulates the conformational transition of synatxin-1 allowing the conversion of syntaxin-1 from a closed conformation to an open conformation (Richmond *et al.,* 2001). The MUN domain of the Munc13 binds to synatxin-1-SNAP-25 heterodimer as well as to membrane-anchored SNARE complexes (Guan *et al.,* 2008). Besides, RIM shows interactions with other active zones proteins suggesting its role in organizing the active zone (Koushika *et al.,* 2001). RIM is functionally linked to Munc-13 and both form the Munc13-RIM complex. Furthermore, RIM also shows interaction with Rab3. These two interactions can occur simultaneously and lead to a tripartite complex. Thus, this tripartite complex may help to recruit the synaptic vesicles in the active zone (Duludova *et al.,* 2005).

Synaptotagmin-1 and complexins are the key factors for the Ca^{2+}-triggering step of release. Synaptotagmin-1 is a synaptic vesicle protein which contains two homologous Ca^{2+}-binding domains (C2A and C2B). The C2A domain binds to SNAP-25 and the C2B domain is capable to bring two membranes together through a Ca^{2+}-dependent mechanism and may help to open the fusion pore. Thus, synaptotagmin-1 binds simultaneously to the phospholipids in the membranes and the SNARE complexes once they are anchored to the membrane forming a quaternary complex (Dai *et al.,* 2007). Moreover, complexin binds to already formed complexes. It binds tightly to the SNARE complex forming an α-helix that interacts with synaptobrevin and syntaxin-1. Taken together, synaptotagmin-1 and complexin bind to SNAREs after Ca^{2+} influx and the Ca^{2+} rearranges the interactions leading to the vesicle fusion and the neurotransmitter release (McMahon *et al.,* 1995).

5) **Endocytosis and recycling**

Synapses are able to reuptake the synaptic vesicle membranes with very high efficiency after the exocytosis from the presynaptic terminal. A fast regeneration of the functional synaptic vesicles is required for synapses that are highly active during long periods of time. Three vesicle recycling pathways are proposed: the kiss-and-stay pathway in which the vesicles remain in the active zone to be refilled; kiss-and-run pathway in which the vesicles are recycled locally without clathrin-mediated endocytosis and the endosomal recycling, a slower pathway in which clathrin is involved (Südhof, 2004).

Clathrin-mediated endocytosis starts with the binding of the coat proteins to proteins from the vesicular membrane and requires the presence of phosphatidylinositol-(4,5)bisphosphate, which is found at the plasma membrane. There are four different internalization signals present in the cytoplasmic tail of the transmembrane proteins, which can be recognized by AP-2, ARH, Db2, β-arrestin, numb, epsin, Eps15, AP180/CALM and HIP1/R adaptors. Cargo-bound AP2 stimulates PIPK1γ, which increases local phosphatidylinositol-(4,5)bisphosphate concentration, thereby facilitating recruitment of more adaptators that, in turn, recruit clathrin to assemble the clathrin coat. The invagination of the membrane is mediated by the coordinate actions of clathrin and proteins of the epsin N-terminal homology (ENTH) family and EFC/F-BAR family.

Dynamin is important in vesicle scission and dissociation. It binds to the neck of the nascent vesicle and constrains it by a GTP-dependent reaction. Also contractin, N-WASP, Arp 2/3, actin, endophilin and SJ1 are recruited, since actin is important for scission. Actin connects with clathrin via HIP1/R. The actin motors myosin-VI and myosin-1E bind Dab2 and phosphatidylinositol-(4,5)bisphosphate, dynamin and synaptojanin, respectively, and might generate opposing forces that induce the pulling of the neck and the freeing of the vesicle into the cell interior (Ungewickell and Hinrichsen, 2007). Disassembly of the clathrin coat is initiated by several independent events, such a desphosphorylation of phosphatidylinositol-(4,5)bisphosphate. Thus, synaptic transmission leads to the activation of important signaling cascades involving phosphorylation of various substrates by intracellular protein kinases.

1.2 Cyclic AMP-dependent protein kinase A (PKA)

The cAMP-dependent protein kinase (PKA) is a highly conserved kinase in vertebrates and invertebrates but it is not present in plants. It is found in every mammalian tissue including the brain (Kuo and Greengard, 1969). PKA plays an important role in synaptic transmission in certain types of neurons (Greengard and Kuo, 1970). The holoenzyme is composed of a tetramer of two regulatory subunits and two catalytic subunits and it can be autophosphorylated (Ueda and Greengard, 1977). When cAMP binds to the regulatory subunit of the holoenzyme, this binding affects the affinity between the regulatory and the catalytic subunit. It causes the dissociation into a dimer of two regulatory subunits and two independent and active catalytic subunits with the peptide

substrate-binding site free (Granot *el al.,* 1980). PKA consists in a N-terminal part dominated by β-sheets and a C-terminal part with helical lobe (Taylor *et al.,* 2013).

1.2.1 The catalytic subunit

The active catalytic subunit of PKA is responsible for modulating the physiological responses in the cells. In mammals, three forms for the catalytic subunit can be found: C_α, C_β and C_γ with C_α being the most abundant form. On the other hand, C_γ is only present in testis (Uhler *et al.,* 1986; Beebe *et al.,* 1990). The catalytic subunit is highly conserved between animal species. The ATP-binding site is localized at the amino terminal of the catalytic subunit (review Taylor *et al.,* 1990). In the catalytic subunit a loop of glycine is found at the amino terminal (Hanks *et al.,* 1988). The structure of the ATP-binding site begins with a β-strand followed by a glycine-rich loop and ending with an α-helix. A lysine (Lys 72), located at the end of the β-strand, is essential for the function of the catalytic subunit (Hanks *et al.,* 1988; Taylor *et al.,* 1989, 1990). Moreover, the catalytic subunit has some carboxyl groups which are responsible for the binding of Mg^{2+} to the ATP and can also participate in the recognition of the peptide substrate (Yoon *et al.,* 1987). The catalytic subunit can suffer posttranslational modifications like myristoylation (Bastidas *et al.,* 2012). The mammalian catalytic subunit has two phosphorylation sites at Thr 197 and Ser 338 (Shoji et al., 1979). The first phosphate is added at Ser 388 while the protein is still attached to the ribosome. This phosphorylation step is essential for the maturation of the kinase (Taylor *et al.,* 2013).

1.2.2 The regulatory subunit

The principal function of the regulatory subunit is to inactivate the catalytic subunit in the absence of cAMP. There are two types of regulatory subunits (Type I and Type II). The type II contains an autophosphorylation site which is absent in the type I (Takio *et al.,* 1984). The regulatory subunit is structured in a heterodimer, type I differs in ionic properties, molecular weight and primary structure from the type II subunit (Zoller *et al.,* 1979). The dimer interaction takes place in the N-terminal region. This region is also important for protein interaction and mediates the membrane fraction association. On the other hand, the catalytic subunit binding site on the regulatory subunit is found at the C-terminal. The regulatory subunit occupies the peptide-binding site of the catalytic subunit and makes it inactive. There are two high-affinity binding sites for cAMP

on each regulatory subunit localized at the C-terminus (review Taylor *et al.,* 1990). Both cAMP-binding sites must be occupied before the dissociation of the holoenzyme occurs (Smith *et al.,* 1981).

1.2.3 Drosophila PKA

In *Drosophila*, the conservation in sequence, compared to human orthologs, of the PKA gene is 71% amino acid identity for the regulatory subunit type I (RI), 32% for the regulatory subunit type II (R2) and 82% for the catalytic subunit (C). *Drosophila* RI has two carboxy-terminal cAMP-binding domains homologs to mammalian RI subunit. In contrast to mouse, the *Drosophila* genome only contains a single RI gene which can suffer splicing within the coding region (Kalderon and Rubin, 1988). Thus, three major transcripts derive from different promoters. In addition, three genes were found in *Drosophila* for catalytic subunits, PKA-C1 (DCO) (Kalderon and Rubin, 1988), PKA-C2 (Bettencourt-Dias et al., 2004), and PKA-C3 (Bettencourt da Cruz *et al.,* 2008). Furthermore, PKA and cAMP signaling are involved in associative learning in *Drosophila*. Modifications in the gene for the major catalytic subunit PKA-C1 (DCO) as well as in the *rutagaba* gene (encodes the calcium-dependent form of adenylate cyclase) and the *dunce* gene (encodes a cAMP-specific phosphodiesterase (PDE)) impair associative learning (Drain *et al.,* 1991; Kiger *et al.,* 1999). Recently, the dynamics of PKA activity in the mushroom body, a brain center for olfactory learning and memory, has been investigated *in vivo* using an optogenetic probe (Gervasi *et al.,* 2010).

1.3 Calcium/calmodulin-dependent protein kinase II (CaMKII)

Calcium/calmodulin protein kinase II is a relatively abundant Ser/Thr protein kinase of the brain (Schulman and Greengard, 1978, Hanson *et al.,* 1992). It regulates glutamatergic synapses. The two predominant isoforms in the brain are α and β (McGuinness *et al.,* 1985). The α isoform is found predominantly in the forebrain and exclusively expressed in neurons, whereas the β isoform is predominant in the cerebellum and it is also present in glia cells (Miller and Kennedy, 1985). However, CaMKII is a family of 28 isoforms that are derived from four genes (α, β, γ and δ) (Soderling *et al.,* 2001).

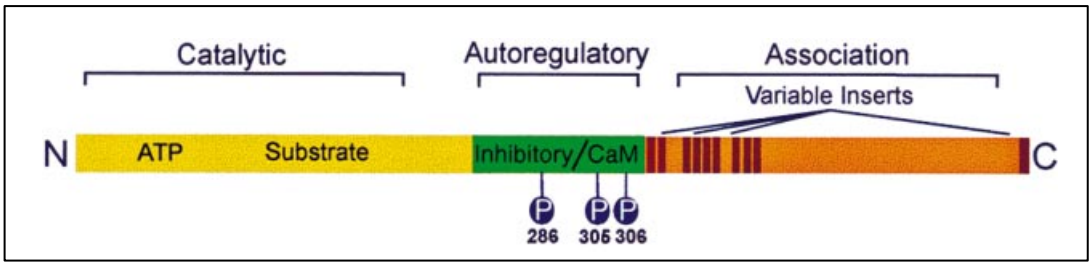


Figure 1.1. Diagram of CaMKII subunit structure (Hudmon and Schulman, 2002). Schematic visualization of all CaMKII domains. The phosphorylation sites are also indicated in the figure. A region of multiple alternative spliced sequences that can be found in the association domain contributes the different isoforms.

Each isoform consists of: (a) a catalytic domain with the ATP- and substrate-binding domains and interaction sites with anchoring proteins; (b) an autoinhibitory domain which binds to the catalytic domain at the substrate-binding site; (c) a Ca^{2+}/CaM binding motif; (d) a variable segment and (e) a C-terminal association domain which allows the assembly to a non-dissociable holoenzyme of 12 subunits (Figure 1.1) (Lisman *et al.,* 2002). The kinase functional domains are grouped into two clusters of six. The pseudosubstrate region (interaction between the catalytic domain and the autoinhibitory domain) blocks protein substrate binding but also contributes to the blocking of the ATP-binding site (Hudmon and Shulman, 2002). Stabilized contacts between Ca^{2+}/calmodulin and the kinase are due to the hydrophobic residues at the N- and C-terminal ends of the Ca^{2+}/calmodulin-binding domains (Putkey and Waxham, 1996). The gate opens when Ca^{2+}/calmodulin binds to the region that overlaps with the pseudosubstrate region. Then, the exposure of Thr 286 (Thr 287 in β, γ and δ isoforms) makes it suitable for phosphorylation. Once this site is phosphorylated the gate cannot close even in the absence of Ca^{2+}/calmodulin (Figure 1.2). It is in this state when the enzyme becomes Ca^{2+}/calmodulin independent (Griffith, 2004).

The activation of the enzyme starts with the binding of two Ca^{2+}/calmodulin molecules to two subunits of the same enzyme. ATP-binding enhances Ca^{2+}/calmodulin-binding (Yamauchi *et al.,* 1993). Once CaMKII is phosphorylated, it remains activated even if the Ca^{2+} levels decrease. The phosphorylation of Thr 286 (in the α subunit) or Thr 287 (in the β subunit) disengages the autoinhibitory domain and the enzyme becomes autonomous, i.e. independent of its stimulus (Thiel *et al.,* 1988). When the Ca^{2+} levels

decline, Ca^{2+}/calmodulin is dissociated from the enzyme. CaMKII is known to have lower affinity for Ca^{2+}/calmodulin than other kinases. However, the phosphorylation of Thr 286 changes the affinity of CaMKII for Ca^{2+}/CaM by more than three orders of magnitude (Meyer *et al.,* 1992). Phosphorylation of Thr 286 exposes the binding site for NMDA-subtype of glutamate receptor. Therefore, Ca^{2+} influx recruits CaMKII to the postsynaptic site increasing the association with the NMDA receptor (Leonard *et al.,* 1999). In addition, once the enzyme is autophosphorylated, the phosphorylation of Thr 305 and Thr 306 blocks the rebinding of Ca^{2+}/calmodulin but only Thr 306 acts as the regulatory autophosphorylation site in the basal reaction (Colbran, 1992). CaMKII can reverse its phosphorylation state in the presence of ADP (review Hudmon and Schulman, 2002). CaMKII is also found at the presynaptic site associated with synaptic vesicles (reviewed by Wang, 2008) and has been shown to phosphorylate mammalian synapsin I in domain D that is not conserved in *Drosophila* (see below). CaMKII preferentially phosphorylates substrates with the motif: Hyd-X-Arg-NB-X-Ser*/Thr*-Hyd, where X corresponds to any amino acid, NB to non-basic amino acids and Hyd to hydrophobic amino acids, respectively (White *et al.,* 1998).

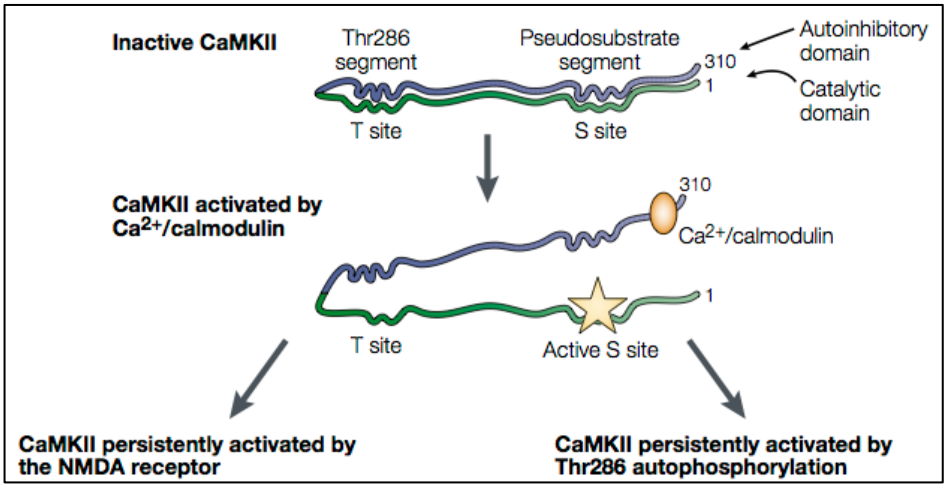


Figure 1.2. Activation and regulation of CaMKII (adapted from Lisman *et al.,* 2002). Schematic visualization of the catalytic and autoinhibitory domain binding sites. The enzyme activity is inhibited when the gate is closed. When Ca^{2+}/calmodulin binds to the enzyme, it becomes active.

1.3.1 Drosophila CaMKII

Drosophila contains only one CaMKII gene that leads to four highly similar protein isoforms. The CaMKII cDNA encodes an amino acid sequence that shows 75-80% homology including all known autophosphorylation sites with the rat α isoform. The

CaMKII expression is most abundant in the brain of the fly (Cho *et al.*, 1991). In *Drosophila*, the autophosphorylation occurs at Thr 287 position making the enzyme independent of Ca^{2+}/calmodulin. On the other hand, the phosphorylation of Thr 306 and Thr 307 occurs only in the absence of calcium and it is capable to inhibit the binding of more Ca^{2+}/calmodulin (Wang *et al.,* 1998). *Drosophila* CaMKII has a role in behavior and synaptic plasticity. Expression of an inhibitory peptide for CaMKII (Ala), a peptide corresponding to the rat autoinhibitory domain (amino acids 273-302) with a T287A substitution, or a threonine inhibitory peptide corresponding to the unmodified domain shows impaired behavior and synaptic plasticity (Griffith *et al.,* 1993, 1994). Thus, CaMKII can modulate physiological and morphological plasticity of neurons (Wang *et al.,* 1994). When CaM kinase activity is blocked in the mushroom bodies or parts of the central brain, flies show a memory defect (Joiner and Griffith, 1999). Furthermore, CaMKII is involved in light adaptation in photoreceptors (Lu *et al.,* 2009). In the present work we show that also in *Drosophila* one of the important substrates for CaMKII is synapsin.

1.4 Synapsins

1.4.1 Cellular and subcellular distribution of vertebrate synapsins

Synapsins are phosphoproteins located in the presynaptic terminal of neurons (reviewed by Cesca *et al.,* 2010). They are localized to synaptic vesicles (SVs) both in the distal pool and in the proximal pool of the synaptic terminal (De Camilli *et al.,* 1979, 1983) and are differentially expressed in different parts of the brain indicating their complementary and not exclusive function. Thus, synapsins are expressed mostly in the central and peripheral nervous system labeling specific neuronal structures associated with synaptic junctions; in contrast, they are not present at significant concentrations in non-neuronal cells. The distribution of the synapsins varies within the neuron types (Bloom *et al.,* 1979). The only cells which do not express synapsin are the cells of the sensory organs such photoreceptors and bipolar cells of the retina, hair cells or the inner ear and taste cells (Finger *et al.,* 1990; Favre *et al.,* 1986; Mandell *et al.,* 1990).

1.4.2 Interaction with synaptic vesicles and cytoskeletal components

Synapsins are phylogenetically conserved between the species as well as their phosphorylation sites for cAMP-dependent protein kinase (PKA) and calcium-calmodulin dependent protein kinase II (CaMKII) (Kao *et al.,* 1999). Synapsins are associated with the cytoplasmic surface of synaptic vesicles. The lipid-binding activity to acidic phospholipids of synapsin resides in the N-terminal and a second vesicle-binding region regulated by phosphorylation was identified in the C-terminal. The binding of synapsin to synaptic vesicles appears to consist of multiple interactions of distinct sites of synapsin to various components of synaptic vesicles (Benfenati *et al.,* 1989).

Synapsins are involved in the regulation of neurotransmitter release (De Camilli *et al.,* 1983). Upon excitation of the nerve terminal, when synapsin is phosphorylated, neurotransmitter release is enhanced (review Cesca *et al.,* 2010). However, this does not mean that all synapsins are dissociated from synaptic vesicles after depolarization of the terminal. Intense anti-synapsin staining can be observed near the plasma membrane when synaptic vesicles are in the releasable pool. Additionally, synapsins are involved in the synaptic vesicle endocytosis process; it seems likely that synapsin reassociates to a certain extent with the vesicle membrane following its endocytosis (Torri Tarelli *et al.,* 1992; Jovanovic *et al.,* 2001). Furthermore, synapsins are involved in both glutamatergic and GABA-ergic neurotransmission. In excitatory and inhibitory synapses, they regulate the number of vesicles in the reserve pool and also in the readily releasable pool (Gitler *et al.,* 2004). Taken together, synapsins are required for normal synaptic vesicle biogenesis, trafficking and immobilization of synaptic vesicles (Vasileva *et al.,* 2012).

Synapsin binds all known cytoskeletal components such as actin, spectrin, microtubules and neurofilaments, and acts as a cross-linking element between synaptic vesicles and actin filaments. At least three actin binding sites were identified; two in the N-terminal and a third one in the C-terminal region of synapsin (review Cesca *et al.,* 2010). The actin bundling activity is reduced upon phosphorylation of synapsin by PKA and mostly abolished upon phosphorylation by CaMKII. Dephosphorylated synapsins are able to inhibit the polymerization of the actin filaments (Bähler and Greengard, 1987).

When depolarization occurs, the association of synapsin with actin filaments decreases whereas the association with microtubules increases (Petruci and Morrow, 1987).

1.4.3 Mammalian synapsins

The synapsin family is composed of ten homologous proteins (Porton *et al.,* 1999). Phylogenetic analyses include the synapsin gene family in the list of genes that show gene duplication specific to the vertebrate lineage (Humeau *et al.,* 2011). Mammals have three synapsin genes (Syn I, Syn II and Syn III) that are localized on the X, 3rd and 22nd chromosomes, respectively (Hosaka *et al.,* 1998). Each primary transcript is alternatively spliced to generate the 'a'-type and 'b'-type isoforms of the protein (Figure 1.3) (Südhof *et al.,* 1989). They have a highly homologous N-terminal region and are more variable in the C-terminal region. Syn I represents approximately 0.4% of the total protein in the brain, whereas Syn II represents 0.2% (Goelz *et al.,* 1981). They are rich in glutamine and asparagine, which causes the negative charge of the protein (Ueda and Greengard, 1977). Syn I and Syn II are linked to the synaptogenesis and maturation of synaptic vesicles, whereas Syn III is expressed during the elongation of neuronal processes and is enriched in cell bodies and growth cones (Ferreira *et al.,* 2000). Furthermore, Syn I is involved in the latest stage of endocytosis and, on the other hand, Syn IIa is pointed at as the isoform responsible for the synapsin-dependent maintenance of the reserve pool of vesicles (review Bykhovskaia, 2011).

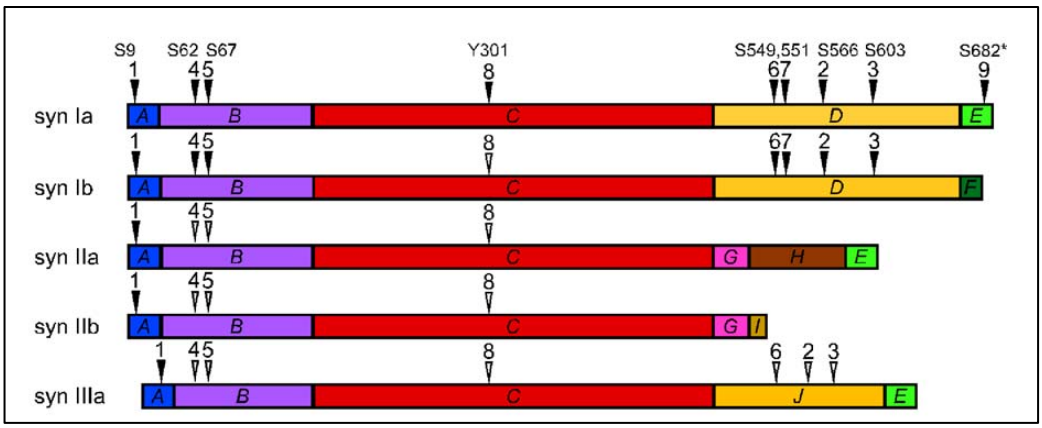


Figure 1.3. Synapsin genes and major isoforms in mammals (adapted from Cesca *et al.,* 2010). Schematic representation of the three synapsin genes and major isoforms including their phosphorylation sites and domains.

The raf gene family was found in close chromosomal vicinity to the synapsin genes and each synapsin gene is found in very close proximity with a TIMP gene. The

TIMP gene family consists of four known genes (TIMP 1, TIMP 2, TIMP 3 and TIMP 4), and represents a family of natural inhibitors of the matrix metalloproteinases involved in the regulation of the extracellular matrix. A close physical linkage can be found between the TIMP gene and the synapsin gene (Kao *et al.,* 1999).

1.4.3.1 Synapsin domain structure

The N-terminal region is composed by three domains (A, B and C) conserved across isoforms and species where the domain A contains phosphorylation site for PKA. The domain B, rich in short-chain amino acids, is less conserved evolutionarily in synapsin and it may serve as a link between domain A and C. The domain C is the most conserved and appears to contain the contact site for actin and synaptic vesicles (Südhof *et al.,* 1989). This domain also binds ATP but the ATP-binding is differentially regulated in Syn I, Syn II and Syn III. ATP-binding to Syn I is Ca^{2+}-dependent, whereas ATP-binding to Syn II is Ca^{2+}-independent and in Syn III Ca^{2+} acts as an inhibitor (Hosaka *et al.,* 1998). At the C-terminal next to domain C, the amino acid sequence starts to diverge in different isoforms.

The domain D, for example, is a collagenase sensitive region that contains two phosphorylation sites for CaMKII (Huttner *et al.,* 1981). The domain E is present in 'a'-type synapsin isoforms (Syn Ia, Syn IIa and Syn IIIa) but not in 'b'-type isoforms (Syn Ib and Syn IIb) (Porton *et al.,* 1999). Domains F, H and I represent results of alternative splicing. The different isoforms have different expression patterns.

1.4.3.2 Synapsin targeting

Three domains play a role in the targeting of synapsin: domains B, C and E. In contrast, the domain D has negative effects on targeting with an inhibitory function. This inhibitory effect of domain D can be offset by domain E in the Syn Ia isoform, but not by domain F in the Syn Ib isoform. On the other hand, heterodimerization is required for Syn Ib targeting and the domain involved is the domain C (Gitler *et al.,* 2004).

1.4.3.3 Phosphorylation of mammalian synapsins

Synapsins are a major substrate for several protein kinases including PKA and calcium-calmodulin protein kinase II (CaMKII). All synapsins contain only a single common phosphorylation site located in the N-terminal A domain. In the head region of

synapsin, the phosphorylation is carried out by PKA or CaMKI/IV, whereas in the tail region, CaMKII phosphorylates synapsin (Bähler and Greengard, 1987). The phosphorylation of serine 9 (site 1) partially reduces, and the phosphorylation of serine 566 and 603 (sites 2 and 3) abolishes the ability of synapsin to inhibit neurotransmitter release (review De Camilli *et al.,* 1990). The changes in phosphorylation of synapsin can be detected at some distance from the phosphorylation sites by conformational changes (Benfenati *et al.,* 1990).

When depolarization occurs, serine 9 (site 1 in domain A) becomes a good substrate for phosphorylation by PKA and Ca^{2+}/calmodulin-dependent protein kinase I or IV (CaMKI/IV). The amino acid sequence surrounding site 1 is Arg-Arg-X-Ser (Czernik *et al.,* 1987). PKA can dissociate the union between synaptic vesicles and synapsins. This domain has all the information required for synaptic-vesicle clustering and for dissociation dependent on phosphorylation. PKA was also shown to mediate the recycling of synaptic vesicles upon depolarization, but it has no role in the dissociation of actin filaments (Hosaka *et al.,* 1999).

In a Ca^{2+}-dependent manner, the domain D (serines 566 and 603) becomes a good substrate for CaMKII phosphorylation. CaMKII consensus sequence on these sites is Arg-Gln-Ala/Thr-Ser (Czernik *et al.,* 1987). The amount of phosphate incorporated by CaMKII is five times the amount of phosphate incorporated by PKA. This phosphorylation breaks the association with the actin filaments and synaptic vesicles (Bähler and Greengard, 1987). The entrance of Ca^{2+} into the nerve terminal activates CaMKII causing the phosphorylation of synapsin and, consequently, the dissociation from the synaptic vesicles, which now are ready to be released (Llinás *et al.,* 1985). Hence, CaMKII regulatory domain binds to the C-terminal fragment of Syn I inhibiting its association with the synaptic vesicles (Benfenati *et al.,* 1992). Syn II is not a good substrate for CaMKII phosphorylation due to the fact that the domain D is missing (Südhof *et al.,* 1989). However, a new phosphorylation site at serine 546 at the E domain in Syn IIa was recently identified (John *et al.,* 2007).

The domain B and D (serines 62, 67, 549 in rat and 551 in mouse), located in the N-terminal region (sites 4 and 5) and in the C-terminal region (sites 6 and 7), can also be phosphorylated by MAPK. Upon an increase of BDNF, MAPK phosphorylation has

only an effect on the binding between actin filaments and synapsin. It is not involved in the binding with synaptic vesicles. Alterations in the actin-based cytoskeleton due to MAPK phosphorylation of synapsin affect the action of neurotrophins in the central nervous system. Furthermore, serine 551 (site 7) is also phosphorylated by cdk-5 (Jovanovic *et al.,* 1996).

1.4.3.4 Synapsins and human disorders

Since synapsins are involved in the maintenance of the excitability of the neuronal network, several studies regarding its implication in human diseases have been performed. Autism spectrum and human epileptic disorders carry missense or non-sense mutations in the Syn I gene that can be associated with learning disabilities or aggressive behavior due to mRNA degradation (Garcia *et al.,* 2004; Fassio *et al.,* 2011). Furthermore, mutations in the Syn II gene also contribute to the epileptic disease. The lack of synapsin can involve distinct populations in different brain regions causing a complex epileptic mechanism (Lakhan *et al.,* 2010). The gene mostly linked to schizophrenia is Syn II. In schizophrenic brains the amount of synapsin is reduced. Treatments that increase the amount of Syn II are used in the schizophrenia patients (Chong *et al.,* 2006; Dyck *et al.,* 2009). Alzheimer is another disease which involves synapsin. Alzheimer patients show unaltered amounts of synapsins but less susceptibility to phosphorylation (review Cesca *et al.,* 2010).

1.4.4 Drosophila synapsins

In *Drosophila* only one gene for synapsin exists and it is located on the third chromosome. Alternative splicing of the primary transcript and partial UAG-read through produces at least four proteins of 70, 74, 80 and 143 KDa apparent molecular weight (see Results and Discussion). The homology found between synapsin of *Drosophila* and vertebrates is prominent in the C domain (Figure 1.4) (Klagges *et al.,* 1996). In *Drosophila*, a TIMP gene is also closely associated with the synapsin gene (Pohar *et al.,* 1999; Kao *et al.,* 1999).

Experiments using optical detection of the dye FM1-43 and focal recording on the larval neuromuscular junction of *Drosophila* suggest that synapsins maintain the reserve pool and the peripheral distribution of the recycling pool of vesicles in the synaptic

boutons (Akbergenova and Bykhovskaia, 2007). These conclusions were supported by electron microsocopy, and electrophysiology indicated that synapsin also has a role in the vesicle-recycling process. Thus, synapsins are required for activation of the recycling pathways via endocytosis. In addition, an influence of synapsin on quantal size has been described (Akbergenova and Bykhovskaia, 2009 a,b; 2010).

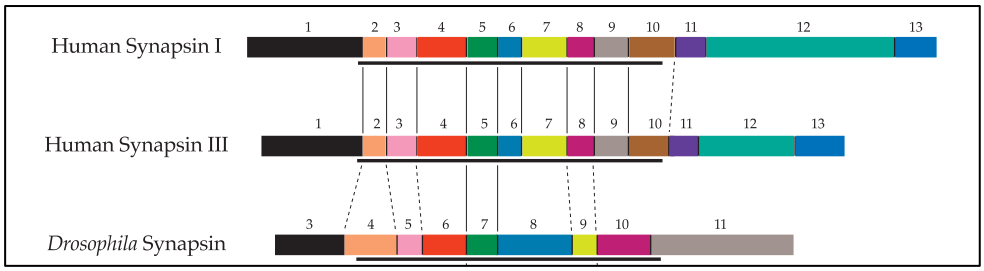


Figure 1.4. Synapsin intron-exon structure in human and *Drosophila* (adapted from Kao *et al.*, 1999). The Drosophila synapsin gene contains 12 exons and the contribution of these exons to the protein structure is very similar to the one seen in mammals.

1.4.4.1 Phosphorylation of Drosophila synapsins

Recently, seven novel phosphorylation sites in *Drosophila* synapsin were identified by mass spectrometry at Thr 86, Ser 87, Ser 464, Thr 466, Ser 538, Ser 961, and Tyr 982 (Figure 1.5) (Nuwal *et al.,* 2010). No phosphorylated peptides were found in this study for the conserved serine 6 in domain A (PKA motif 1) or for the other potential PKA phosphorylation site at serine 533 (PKA motif 2) that conforms to the canonical PKA target sequence RRxS.

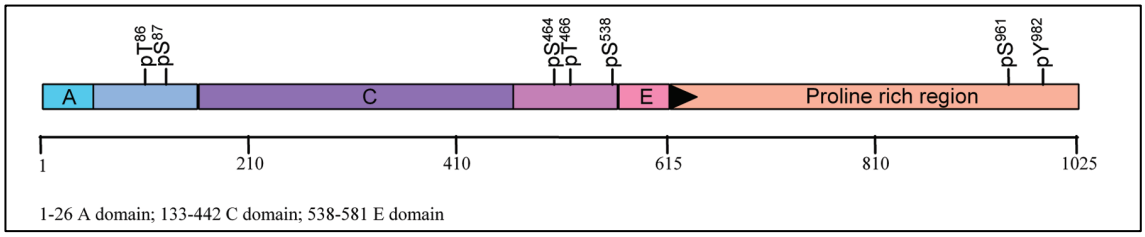


Figure 1.5. Identified phosphorylation sites in *Drosophila* synapsin (Nuwal *et al.,* 2010). Phosphorylations at the conserve serine 6 in domain A and serine 533, both consensus sequences for PKA phosphorylation, were not detected in this study.

In *Drosophila,* the conserved N-terminal phosphorylation consensus motif RRxS is encoded in the gene but it is modified (RRFS➔RGFS) in most mature mRNAs by pre-mRNA editing. The RNA modifying enzyme ADAR is responsible for an adenosine-to-

inosine conversion, which needs a special structure of the RNA (double stranded) that will form the editing region. By in-vitro phosphorylation experiments it was found that the N-terminal peptide containing the RRFS motif represents an excellent substrate for bovine PKA, whereas a peptide with the edited RGFS motif is not significantly phosphorylated by this enzyme (Figure 1.6) (Diegelmann *et al.,* 2006). Synapsins with S6A and S533A mutations at the PKA sites show associative learning defects (Michels *et al.,* 2011).

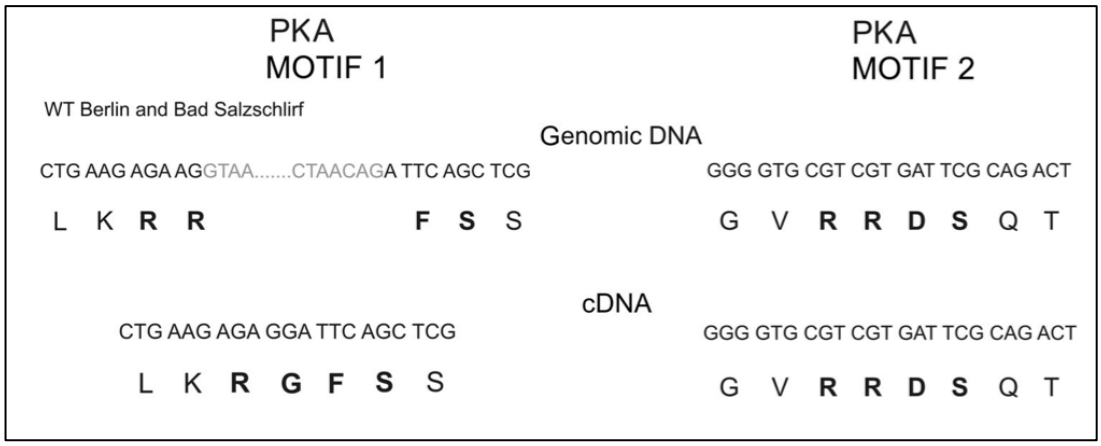


Figure 1.6. Comparison of the two consensus motifs between the genomic DNA and cDNA (Digelmann et al., 2006). Only motif 1 is subjected to modification by mRNA editing.

1.4.4.2. Synapsin null mutants

To study the function of synapsins in *Drosophila,* mutants were generated by P-transposon remobilization. Syn[97] null mutant was described as carrying a 1.4 kb deletion spanning parts of the regulatory sequence of the synapsin gene, its first exon, and half of its first intron (Godenschwege *et al.* 2004). The coding region is not affected in these mutants, but in Western blots and immunohistochemistry no synapsin can be detected by a monoclonal antibody that recognizes an epitope in the conserved domain C present in all known synapsin isoforms (mAb 3C11). In the Syn[79] null mutant, on the other hand, a deletion of ca.10 kb eliminates the first seven exons and thus no intact synapsin can be generated (Figure 1.7). An even larger deletion in the mutant Syn[28] eliminates exons 1-9 and in addition exons 2-5 of the TIMP gene (Godenschwege *et al.,* 2000).

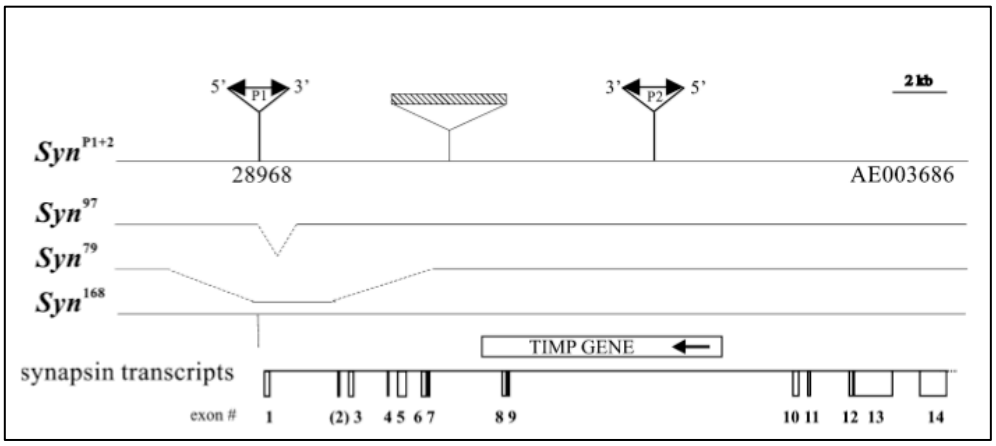


Figure 1.7. Genomic organization of *Drosophila* synapsin locus and mutant genes (Godenschwege *et al.,* 2004). The three synapsin mutants with the size of the deletion are shown in the figure.

The Syn[97] and Syn[79] null mutants are viable and fertile but several phenotypes affecting complex behavior have been described (Godenschwege *et al.,* 2004). The associative learning in Syn[97] larvae is reduced to more or less 50% of wild-type CS levels (Michels *et al.* 2005, 2011; Diegelmann *et al.,* 2013). Also, relatively low levels of learning can be observed in adults without synapsin (Knapek *et al.,* 2010). Furthermore, the reserve pool is diminished in Syn[97] null mutants while the recycling pool is not affected and they lack the ability to increase the quantal size upon intense depolarization (Akbergenova and Bykhovskaia, 2010). The phenotype showed by Syn[97] null mutants can be phenocopied by expressing RNAi-mediated synapsin knock-down (Michels *et al.,* 2011).

1.5 Synapse-associated protein of 47 kD (Sap47)

The monoclonal antibody nc46 from the Würzburg Hybridoma Library generated by Alois Hofbauer (Hofbauer, 1991; Hofbauer *et al.,* 2009), which binds to all synaptic terminals of the *Drosophila* nervous system, was the key to identify the synapse-associated protein of 47 KDa (Sap47). Sap47 is a novel conserved protein of unknown function identified by applying the expression cloning approach using the mAb nc46. Thus, it was possible to identify two alternative spliced transcripts that code for two predicted Sap47 isoforms differing only in their last amino acids (Reichmuth *et al.,* 1995). By the *Drosophila* genome project (flybase.org) 10 splice variants have been detected. Immunostained nervous tissue and muscle revealed that Sap47 is associated with the nerve terminal. In *Drosophila* brains, mAb nc46 strongly stains all neuropil except for

faint staining of a horizontal layer in the medulla and most of the lamina. In Western blots, the mAb nc46 gives a prominent signal for a protein of 47 kDa (Reichmuth *et al.,* 1995) and weaker signals near 62 and 83 kDa (Funk *et al.,* 2004). While Sap47 homologs are found in mammals and c.elegans, no obvious sequence similarity could be found with other known proteins. However, more sophisticated analysis revealed that Sap47 contains the BSD domain also seen in the transcription factors BTF2 and DOS2-like proteins (Doerks *et al.,* 2002). Using the P-element insertion approach, the Sap47^{156CS} null mutant was generated by jump-out mutagenesis. This mutant contains a deletion of 110 bp in the 3' UTR of the upstream adjacent gene *black pearl* (*blp*), 170 bp between *blp* and *Sap47* gene transcription start site, as well as the first exon of 1226 bp and 220 bp of the first intron of the *Sap47* gene. *Drosophila* Sap47^{156CS} null mutants are viable and fertile but show complex behavioral phenotypes (Funk *et al.,* 2004; Saumweber *et al.,* 2011).

1.6 Olfactory response in Drosophila

The olfactory response in *Drosophila* is initiated by activation of odorant receptors expressed in the olfactory sensory neurons (OSNs), which project into the antennal lobe (AL) and form synapses with glomerulus-specific projection neurons (PNs) that connect to the mushroom-bodies (MB) and the lateral protocerebrum. OSNs and PNs also activate multiglomerular excitatory and inhibitory local interneurons (LNs), which mediate lateral and intraglomerular inhibition in the AL (Vosshall and Stocker, 2007; Chou *et al.,* 2010). Recently, strong evidence was provided for a model in which *Drosophila* olfactory habituation, i.e. reduced olfactory avoidance caused by previous exposure to an odorant, arises through potentiation of inhibitory transmission between GABAergic local interneurons (LNs) and projections neurons (PNs) of the AL (Das *et al.,* 2011). In this study, the authors analyze the molecular basis of this potentiation and propose a mechanism that could lead to increased presynaptic GABA release after odorant exposure. In collaboration with this group, the role of synapsin in olfactory habituation of *Drosophila* was studied in the present thesis (Sadanandappa, Blanco *et al.,* 2013).

1.7 Gal4/UAS system in Drosophila melanogaster

The Gal4/UAS system consists of a bipartite system in which one transgenic line expresses Gal4 (transcriptional regulator of yeast) under the control of a cell-specific enhancer and the other line contains a transgene consisting of five optimized Gal4 binding sites (17-mer, four copies of which are found in the upstream activation sequence (UAS) of yeast), a TATA box, the DNA sequence to be expressed, and a terminator sequence. This system allows the ectopic expression of any RNA sequence (protein coding or non-coding) under the control of the UAS enhancer. Transgenic flies carrying this transgene are crossed to a Gal4-expressing line producing progeny in which the transgene is only expressed in a Gal4-dependent pattern (Figure 1.8). This system allows the transcription to be restricted to specific cells or tissues and makes possible the tissue-specific gain-of-function or loss-of-function experiments (Brand and Perrimon, 1993).

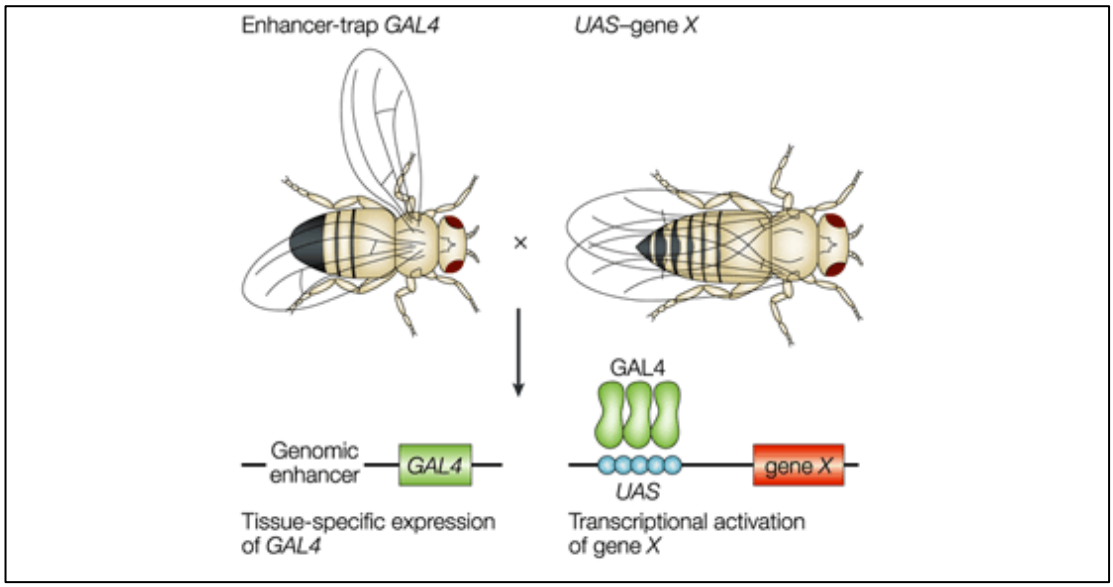


Figure 1.8. Gal4/UAS system. To activate the transcription, flies carrying the UAS-Gene X are crossed to flies expressing Gal4 (enhancer trap Gal4) (Brand and Perrimon, 1993).

1.8 Monoclonal antibodies

Antibodies are important tools for the analysis of neuronal circuits in the brain. The whole homogenate of *Drosophila* can be used as antigens in the hybridoma technique to generate monoclonal antibodies with anatomical and molecular specificity. The mAbs can be screened for any characteristics of choice or with the aim to discover new molecules. As a result, the hybridoma technique has become very useful. In the conventional hybridoma technology, a mouse is immunized with the antigen of interest.

After a high antibody titer has been reached, spleen cells of the immunized mouse are fused with myeloma cells to generate hybridomas that produce the mAbs of interest (Köhler and Milstein, 1975). Through such collections of mAb, it was possible to identify molecules from the ovary or oogenesis, products of segmentation and homeotic genes or genes involved in metamorphosis, and heat shock and nuclear proteins, among others. As an example, a mAb against *Drosophila* choline acetyltransferase was generated and used as a specific marker for cholinergic neurons (Crawford *et al.,* 1982; Buchner *et al.,* 1986). The first mAb collection against *Drosophila* brain with 148 mAbs was generated by Fujita and colleagues (Fujita et al., 1982, 1988). The Würzburg Hybridoma Library is a valuable resource as it contains more than 200 monoclonal antibodies, it has antibodies against antigens located in neuronal compartments like the cell body, the axon, or the synaptic neuropil, and antibodies that selectively stain individual cells or cell types in the retina or in the brain (Hofbauer, 1991). By either screening a cDNA expression library with the monoclonal antibodies or using the monoclonal antibody to affinity purify the antigen by immunoprecipitation or 2D-gels and subsequently apply mass spectrometry (MS) analysis, it was possible to indentify some proteins recognized by these monoclonal antibodies, like cysteine string protein (CSP), synapse-associated protein of 47 kDa (SAP47), Bruchpilot (BRP), Epidermal Growth Factor Receptor Substrate 15 (EPS15), Pigment Dispersing Factor (PDF) precursor (review Hofbauer, 2009). Nevertheless, the approach "from antibody to gene" has worked so far for complex mixtures of antigens with antibodies that recognize relatively abundant proteins like those found in all neuropil, or, as in the case of the PDF precursor, only if the cells that are stained by the mAb are known. It was noticed that mAbs cannot always detect every location of a protein and can bind to epitopes of unrelated molecules showing cross-reactivity. Furthermore, some molecules in the nervous system can only be detected by immunohistochemistry procedures but not by Western blots or are present in a concentration too low to be detected (Valentino *et al.,* 1985).

1.9 Goals of the thesis

The aim of the first part of the present thesis was to investigate the phosphorylation of synapsin at the conserved PKA (serine 6 in *Drosophila*) and at the newly identified serine 464 sites and the distribution of synapsin phosphorylated at these sites in the *Drosophila melanogaster* brain using phospho-specific antisera. The available data have lead to a hypothesis how the phosphorylation of synapsin might be involved in learning, memory and habituation. In view of this hypothesis, our aim is to find out which neurons might be involved.

In the second part we aimed to identify and structurally characterize interneurons of the *Drosophila* brain by monoclonal antibodies of the Würzburg Hybridoma Library. These antibodies only stain a few cells in the brain and due to the low amount of protein, no signal could be detected in Western blot. Therefore we wanted to compare their staining patterns with these of antibodies in which the antigen is known or with Gal4 lines with similar expression patterns.

2. Material

2.1. Fly strains

- Canton S — In lab
- w^{1118}; +;+ — In lab
- w^{-}; +; $Sap47^{156CS}$ — Funk, N.
- w^{-}; +; Syn^{97CS} — Funk, N.
- w^{-}; +; $Sap47^{156\ CS\text{-}NF}$, $Syn^{97\ CS\text{-}NF}$ — Nuwal, T.
- elav-Gal4;+;+ — Sigrist, S.
- w^{-}, Dicer; +; elav-Gal4 — Sigrist, S.
- w^{-}; amon-Gal4 (91D); + — Wegener, C.
- w^{-};+; amon-Gal4 (386Y) — Wegener, C.
- c155;; $tubGal80^{ts}$ — Bloomington
- w^{-}, elav Gal4; +; Syn^{97} — Godenschwege, T.
- y,w,UAS-mCD8GFP; UAS-mCD8GFP/CyO;+ (multiple inserts) — Bloomington
- w^{-};+; UAS *Sap* RNAi — Funk, N.
- w^{-}; +; UAS *Syn* cDNA PKA1 non-edited, PKA2 mutated, Syn^{97} — Virstyuk, O.
- w^{-}; +; UAS *Syn* cDNA PKA1 and PKA2 mutated, Syn^{97}/TM3 sb — Virstyuk, O.
- w^{-}; +; UAS *Syn* cDNA PKA1 non-edited, PKA2 WT, Syn^{97} — Chen, Y.
- w^{-};+; UAS *Syn* cDNA PKA1 mutated, PKA2 WT, Syn^{97}/TM3 sb — Husse, J.
- w;+; UAS *Syn* cDNA8 (edited) — Michels, B.

  (same construct but different insertion as in Syn $cDNA^{12}$ described in Löhr *et al.,* 2002)
- w^{-}; P {UAS-CaMKII-I.Ala}2; + — Bloomington
- w^{-}; P {UAS-CaMKII.T287A}3B3; + — Bloomington
- w^{-}; P {UAS-CaMKII.T287D}6B1; + — Bloomington
- w^{-}; P {UAS-CaMKII.T287D}10A; + — Bloomington
- P{w[+mC]=UAS-PKA-C1.W224R.F}5.3.2, y[1] w[*] — Bloomington
- y[1] w[*]; P{w[+mC]=UAS-PKA-C1.K75A}46.7 — Bloomington
- UAS PKA-C1 RNAi — VDRC

The following lines are from the Gal4 driver collection of Rubin Laboratory at Janelia farm:

- w^{1118}; +; P {GMR40E08-Gal4}attP2 — Bloomington
- w^{1118}; +; P {GMR42H01-Gal4}attP2 — Bloomington
- w^{1118}; +; P {GMR51C07-Gal4}attP2 — Bloomington

- w^{1118}; +; P {GMR64D09-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR67E08-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR72G03-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR74G10-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR75A02-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR75F06-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR78E11-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR49C04-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR49D04-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR14C06-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR59E08-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR83H07-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR58F01-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR70F10-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR80D06-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR12G09-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR33G11-Gal4}attP2 Bloomington
- w^{1118}; +; P {GMR70H05-Gal4}attP2 Bloomington

2.2. Buffers and reagents

2.2.1. DNA and RNA analysis

2.2.1.1. Primers

PRIMER NAME	PRIMER SEQUENCE
Syn 97-1F	5'-AGA AAA TTT GGC TTG CAT GG-3'
Syn 97-2R	5'-CGG GGT CTC AGT TTT GTT T-3'
Syn 97-3R	5'- CCT CTA CTT TTG GCT GCC TG-3'
pUAST ua	5'-CAT GTC CGT GGG GTT TGA AT-3'
exon4 rev	5'-GGC AGG CTA TTC GGA TCG-3'

Table 2.1- Primers sequences

2.2.1.2. Reagents for genomic DNA isolation

Homogenization buffer	10 mM Tris (pH 8.2) 1 mM EDTA 25 mM NaCl final volume up to 10 ml with ddH_2O add Proteinase K before use
dNTPs	dNTP Set 10mM (Gene Craft Germany)
Taq polymerase	5' Prime, Hamburg
6x Loading buffer	30% Glycerin 0.15% Bromophenol blue 0.15% Xylene cyanol 1x TAE
100% Ethanol	50 ml 100% Ethanol
70% Ethanol	35 ml 100% Ethanol 15 ml ddH_2O
10x buffer	5' Prime, Hamburg
DNA size standards	GeneRulerTM 100bp DNA Ladder GeneRulerTM 1kb DNA Ladder
10x TBE	151.4 ml of Tris 77.3 g of boric acid 23.3 g of EDTA final volume up to 2.5 l with ddH_2O
Agarose gel 2%	3 g Agarose 150 ml TBE buffer added 6.5 µl of Ethidium Bromide after the temperature reached 65°C.

Table 2.2- PCR reagents

2.2.2 Protein analysis

2.2.2.1 SDS-PAGE

Mini protean™ (BioRad®) electrophoresis (reagents for two mini gels)

Reagents	12%
30% Acrylamide	6 ml
1.88 M Tris pH 8.8	3.8 ml
10% SDS	150 µl
10% APS	150 µl
TEMED	6 µl
dH_2O	4.9 ml

Table 2.3- Reagents for resolving gel

Reagents	5%
30% Acrylamide	500 µl
0.635 M Tris pH 6.8	380 µl
10% SDS	300 µl
10% APS	30 µl
TEMED	3 µl
dH_2O	2.1 ml

Table 2.4- Reagents for stacking gel

10x SDS PAGE Running buffer	125 mM Tris 960 mM Glycine 0.5% SDS pH adjusted to 8.9 and final volume up to 1l with dH_2O
2x Laemmli buffer	125 mM Tris, pH 6.8 6% Glycerin 2% SDS 0.025% Bromophenol blue 5% 2-β-mercaptoethanol final volume up to 10 ml with dH_2O

NuPAGE® LDS Sample Buffer (4x)	Novex Mini XCell™ electrophoresis system Invitrogen, Carslbad, CA 92008 USA
NuPAGE® Sample Reducing Agent (10x)	Novex Mini XCell™ electrophoresis system Invitrogen, Carslbad, CA 92008 USA
NuPAGE® Antioxidant	Novex Mini XCell™ electrophoresis system Invitrogen, Carslbad, CA 92008 USA
NuPAGE® Novex 12% Bis-Tris ZOOM gel	Novex Mini XCell™ electrophoresis system Invitrogen, Carslbad, CA 92008 USA
20x MOPS SDS Running buffer	209.2 g MOPS 1 M 121.2 g Tris base 1 M 20 g SDS 69.3 mM 6 g EDTA free acid 20.5 mM final volume up to 1l with ddH_2O, pH 7.7
20x MES SDS Running buffer	195.2 g MES 1 M 121.3 g Tris base 1 M 20 g SDS 69.3 mM 6 g EDTA free acid 20.5 mM final volume up to 1l with ddH_2O, pH 7.3

Table 2.5- Reagents for SDS-PAGE

2.2.2.2 Western blot

1x Transfer buffer	25 mM Tris 150 mM Glycine 20% Methanol pH adjusted to 8.3 and volume made up to 2 l with dH_2O
10x TBST	100 mM Tris 1.5 M NaCl 0.5% Tween-20 pH adjusted to 7.6 and final volume made up 1 l with dH_2O
Blocking solution	1g Non-fat dry milk powder dissolved in 20 ml (per mini-blot) of 1x TBST, heated to 60°C and filtered.
Detection kit	ECL Western blotting detection reagents GE Healthcare UK Limited
X-Ray cassette	Hartenstein. Würzburg, Germany

X-Ray films	Contatyp ,ÖHRLEIN Handelsvertretung für Laborbedarf

Table 2.6- Items for Western blot

2.2.2.3 Silver staining (modified after Blum et al. for mass spectrometry)

Fixer	40% EtOH 10% HAc 50% H_2O Final volume set to 200 ml
Wash	30% EtOH 70% H_2O Final volume set to 200 ml
Sensitizing	0.02% $Na_2S_2O_3$ Final volume set to 200 ml
Silver nitrate	0.1% $AgNO_3$ Final volume set to 200 ml
Developer	3% Na_2CO_3 0.05% formalin Final volume set to 200 ml
Stopper	0.05 M EDTA Final volume set to 200 ml

Table 2.7- Reagents for Silver staining

2.2.2.4 Two dimensional SDS-PAGE

Sample homogenizing mix	Zoom® 2D Protein Solubilizer 1 or 2 1 M Tris Base 100x Protease Inhibitor Cocktail (Roche®) 2 M DTT ddH_2O
Sample loading/Strip rehydration mix	Zoom® 2D Protein Solubilizer 1 or 2 2 M DTT Ampholytes 3-10 (Serva) 1% Bromophenolblue Sample homogenate ddH_2O Final volume 165 µl
Equilibration solution (400 µl per strip)	4x NuPAGE™ LDS Sample Buffer

	10x NuPAGE™ Reducing Agent
Alkylation solution	1x NuPAGE™ LDS Sample Buffer 125 mM Iodoacetamide
Alkylating agent	DMA (N,N-Dimethylacrylamide)
Strip overlaying solution	0.5% agarose solution in SDS-PAGE running buffer
ZOOM® IPGRunner™ Mini-Cell System	Novex Mini XCell™ electrophoresis system Invitrogen, Carslbad, CA 92008 USA
ZOOM® IPGRunner™ Cassettes	Novex Mini XCell™ electrophoresis system Invitrogen, Carslbad, CA 92008 USA
ZOOM® IPGRunner™ Buffer Core	Novex Mini XCell™ electrophoresis system Invitrogen, Carslbad, CA 92008 USA
ZOOM® Dual Power	Novex Mini XCell™ electrophoresis system Invitrogen, Carslbad, CA 92008 USA
ZOOM® Strip pH 6-9L	Novex Mini XCell™ electrophoresis system Invitrogen, Carslbad, CA 92008 USA

Table 2.8- Items for 2D-PAGE electrophoresis

2.2.3 Immunochemistry

2.2.3.1 Immunoprecipitation

Homogenization buffer	25 mM Tris (pH 7.5) 150 mM NaCl 2 mM EDTA 2 mM EGTA 10% Glycerol 0.001% NP-40 pH 7.5 and final volume set to 500 ml
Phosphatase inhibitors	1 mM NaF 10 mM Na-pyrophosphate
Protease inhibitor cocktail	Complete Mini™, Roche® Diagnostics, Mannheim

Table 2.9- Reagents for immunoprecipitation

2.2.3.2 Covalent coupling of antibodies to Protein-G-Agarose for IP

Citrate buffer	4.7 g Citric acid 9.2 g Dinatriumhydrogen phosphate pH adjusted to 5
20 mM Dimethylpimelidate (DMP)	5.4 mg/ ml (0.2 M Triethalonamine)
PBST	PBS + 0.05% Tween 20 (pH 7.4)
Lysis buffer	25 mM Tris 150 mM NaCl 10% Glycerol 1% NP-40 1% Triton X-100 1 mM NaF 10 mM Na-pyrophosphate pH adjusted to 7.5
Washing buffer	50 mM Tris 100 mM NaCl 0.1% NP-40 0.1% Triton X-100 1 mM NaF 10 mM Na-pyrophosphate pH adjusted to 7.6

Table 2.10- Reagents for covalent coupling

2.2.4 Immunohistochemistry

2.2.4.1 Adult and larva brain whole mounts

Fixative	4% PFA (2 g dissolved initially in 25 ml dH_2O and heating up to 60ºC with constant stirring) 100 µl of 1 M NaOH was added and the solution turned clear on complete dissolution it was cooled to 20ºC 19 ml of (1/15) M Na_2HPO_4 and 5 ml of (1/15) M K_2HPO_4 were added pH adjusted to 7.4 by (1/15) K_2HPO_4 and final volume made to 50 ml Solution was chilled on ice before use
Drosophila Ringer	7.48 g NaCl

	0.35 g KCl 0.2 g $CaCl_2$ 0.105 g Na_2HPO_4 0.048 g KH_2PO_4 Final volume made up to 1 l with dH_2O
PBS 10x	14.8 g Na_2HPO_4 4.3 g KH_2PO_4 72 g NaCl pH adjusted to 7.4 and final volume made to 1 liter
Wash buffer	1x PBST (0.3% Triton X-100)
Blocking solution	5% Normal-Goat-Serum in 1x PBST

Table 2.11- Solutions for adult and larva whole mount stainings

2.2.4.2 Larval NMJ dissections

Ca^{2+} free saline	7.6 g NaCl 12.32 g Sucrose 0.37 g KCl 0.38 g $MgCl_2$ 1.3 g HEPES 0.19 g EDTA Final volume made up to 1 liter with dH_2O
Fixative	Described above in section 2.2.4.1
2x PEM buffer	20 ml PIPES (30.24 g in 100 ml dH_2O, pH 7.0) 20 ml EGTA (0.76 g in 100 ml dH_2O, pH 7.0) 20 ml $MgDO_4$ (0.24 g in 100 ml dH_2O) 40 ml dH_2O
Blocking solution	4.64 ml 1x PBS 100 mg 2% BSA 10 µl 0.2% Triton X-100 250 µl 5% Normal Horse/Goat Serum Final volume made up to 5 ml
Wash buffer	1x PBST (0.1% Triton X-100)

Table 2.12- Solutions for NMJ dissections

2.2.4.3 Immunostaining of cryosections

Fixative	Described above in section 2.2.4.1
Drosophila Ringer	Described above in section 2.2.4.1
Wash and Cryoprotectant	25% Saccharose (25 g in 85 ml of Ringer)
Embedding medium	16% Carboxymethyl Cellulose (1.6 g in 10 ml dH_20)
10x PBS	Described above in section 2.2.4.1
Microscope slide	Thermo Scientific, Menzel Gläser Superfrost® Plus

Table 2.13- Solutions for cryosections

2.2.5 Hybridoma cell culture

HT-medium	70 ml RPMI 1640 20 ml Medium 199 2 ml HT Supplement 50x 1 ml HCS 10 ml Fetal Calf Serum 1 ml Anti-Anti 100x 1 ml L-Glutamate all from Invitrogen, Carslbad, CA 92008 USA
HT-medium without FCS	70 ml RPMI 1640 20 ml Medium 199 10 ml HCS 2 ml HT Supplement 50x 1 ml Anti-Anti 100x 1 ml L-Glutamate all from Invitrogen, Carslbad, CA 92008 USA
0.2 µm filter	Whatman GE Healthcare
Staining solution	Trypan Blue (Sigma-Aldrich, Munich, Germany)

Table 2.14- Reagents for hybridoma cell culture

2.2.6 Chemicals

Chemicals were supplied by Applichem (Darmstadt), Calbiochem (Darmstadt), Roth (Karlsruhe), Merck (Darmstadt), Serva (Heildelberg) and Sigma (München).

2.3 Antibodies

2.3.1 Primary antibodies

Name	Species	Target protein	Dilution	Source
anti-Syn (3C11)	mouse	Synapsin	1:200	A. Hofbauer
nc46	mouse	Sap47	1:200	A. Hofbauer
8C3	mouse	Syntaxin	1:1000	DSHB*
na21	mouse	Protein X?	1:10	A. Hofbauer
ab49	mouse	CSP	1:200	A. Hofbauer
nc82	mouse	Bruchpilot	1:100	A. Hofbauer
P-tyr	mouse	Phosphotyrosin	1:1000	Millipore
anti-GFP	rabbit	GFP	1:1000	Life technologies
anti-P-Syn (S6)	rabbit	Phosphorylated peptide (S6)	1:200	Eurogentec
anti-P-Syn (S464)	rabbit	Phosphorylated peptide (S464)	1:1000	Eurogentec
anti-Syn (S6)	rabbit	Non-phosphorylated peptide (S6)	1:500	Eurogentec
anti-Syn (S464)	rabbit	Non-phosphorylated peptide (S464)	1:500	Eurogentec
nb168	mouse	PDF	1:20	A. Hofbauer
ab135	mouse	?	1:5-1:20	A.Hofbauer
ab158	mouse	?	1:5-1:20	A. Hofbauer
ca8	mouse	?	1:20	A. Hofbauer
fb20	mouse	?	1:5-1:20	A. Hofbauer
nb139	mouse	?	1:2	A. Hofbauer
ab47	mouse	?	1:5-1:20	A.Hofbauer
nb169	mouse	?	1:20	A. Hofbauer
nc24	mouse	?	1:5	A. Hofbauer
nc53	mouse	?	1:5	A. Hofbauer

Table 2.15- Primary antibodies. * Developmental Studies Hybridoma Bank

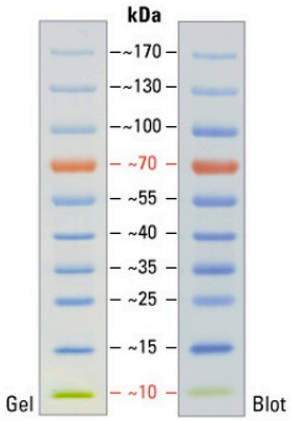
2.3.2 Secondary antibodies

NAME	SPECIES	DILUTION	APPLICATION	SOURCE
anti-rabbit-HRP	goat	1:3000	WB	Biorad
anti-mouse-HRP	goat	1:3000	WB	Biorad
anti-rabbit-Cy5	goat	1:400	IF	Jackson Immunoresearch
anti-mouse-Cy3	donkey	1:400	IF	Jackson Immunoresearch
anti-rabbit-Alexa488	goat	1:200	IF	Invitrogen
anti-mouse-Cy3	goat	1:1000	IF	Invitrogen
anti-rabbit-Cy3	goat	1:1000	IF	Invitrogen
anti-chicken- Alexa 488	goat	1:1000	IF	Jackson Immunoresearch

Table 2.16- Secondary antibodies

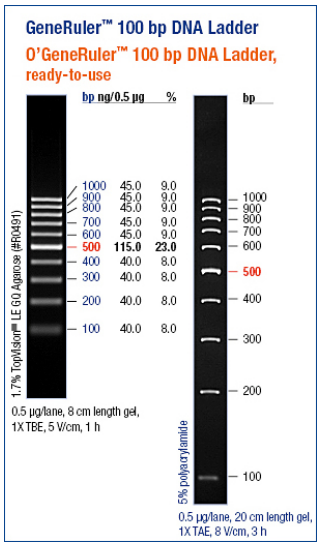
2.4 Ladders

2.4.1 PageRuler™Pre-stained Protein Ladder (Fermentas)



Images are from a 4-20% Tris-glycine gel (SDS-PAGE) and subsequent transfer to membrane.

2.4.2 GeneRuler™ 100bp DNA Ladder (Fermentas)



3. Methods

3.1 Handling Drosophila melanogaster

A detailed description of the use of *Drosophila* in biological research is given by Ralph J. Greenspan (Fly pushing). Here we briefly summarize the steps essential for the present work. Most commonly fruit flies are used for genetic experiments. Female flies will not mate for at least 4 hours after emerging from the pupa case. If the males and females are separated every 4 hours, a supply of virgin females is assured.

Male and female *Drosophila* may be distinguished from each other by examining the genital organs on the ventral posterior of the abdomen. Heavy bristles surround male fly genitalia. However, the females do not have these bristles.

Drosophila male has a black comb-like organ on the lateral areas of the 4th joint of the front leg. Sex combs cannot be seen without some means of magnification. Abdominal differences in both shape and colour are readily distinguished between males and females. The abdomen of the male is quite round on the end and is darker than the female abdomen. The abdomen of the females comes to a definite V-shape point in the rear. Males are generally smaller than *Drosophila* females.

Most *Drosophila* transfers and examinations are made while the flies are anesthetized by carbon dioxide.

- Composition of the fly medium:

Water	39 Liters
Yeast	675 Grams
Soy flour	390 Grams
Yellow cornmeal	2.85 Grams
Light malt extract (dehydrated)	1.8 Grams
Agar	225 Grams
Light corn syrup	3 Liters
Propionic acid	0.188 Liters

3.2 DNA analysis

The procedure for DNA analysis has been described in detail (Barany, 1991) and was followed with minor modifications.

3.2.1 Isolation and purification of genomic DNA

The requested number of flies was anesthetized and kept on ice. 10 flies per preparation were homogenized in 120 µl of homogenization buffer (100 mM NaCl, 100 mM Tris, 50 mM EDTA and 0.5% SDS) and ground with a rod (the rod was rinsed by adding 370 µl homogenization buffer). The homogenate was incubated at 68ºC for 30 minutes. The homogenate was incubated on ice for 30 minutes after addition of 75 µl of 8 M KAc. A centrifugation step at 14000 rpm for 10 minutes was done followed by a repetition of this step after transferring the supernatant to a fresh tube (550 µl). The DNA was precipitated with a 2.5 fold volume (1375 µl) of 100% ethanol for 10 minutes at room temperature. The sample was centrifuged for 15 minutes at 14000 rpm and the pellet was washed with 70% ethanol followed by another centrifugation for 15 minutes at 14000 rpm. The pellet was dried and dissolved in 25 µl dH_2O.

3.2.2 Polymerase chain reaction (PCR)

PCR is a method to amplify the DNA *in vitro* by using specific primers to obtain a high amount of a restricted, unique genomic region that is required for the analysis (Barlett and Stirling, 2003). Primers were designed using primer3 software available online at (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). CG concentration was set at 50%. Melting temperature of the primers was adjusted to be similar within an interval of 1-2ºC and primer length was usually restricted to 18-20 bases. Melting temperature was kept around 61ºC. Primers were prepared from the stocks (100 pmol/ µl) by re-suspending 2 µl of each primer in 18 µl ultrapure water (diluted 1:10 before use). Mastermix with premixed buffer, dNTPs and DNA polymerase was added upto 50% of the total PCR reaction volume.

Preparation of the Mastermix (standard)

1 µl DNA

0.25 µl Taq

2.5 µl buffer

0.5 µl dNTP

0.5 µl sense primer

0.5 µl antisense primer

19.75 µl of PCR H_2O (autoclaved water)

25 µl total volume

PCR program

Syn97 1F- Syn97 2R and Syn97 1F-Syn97 3R			pUAST ua exon4 rev		
95ºC	5:00		95ºC	5:00	
95ºC	0:45		95ºC	0:45	
62ºC	1:00	x30	58ºC/62ºC/62.2ºC	1:00	x30
68ºC	2:00		68ºC	2:00	
68ºC	5:00		68ºC	5:00	
4ºC	∞		4ºC	∞	

Table 3.1- PCR program

Usually a PCR with temperature gradient for the primers pUAST ua and exon4 rev was performed to determine the optimum annealing temperature. Afterwards, PCR 6x loading dye (4 µl in 25 µl sample) was added and stored at 4ºC.

3.2.3 PCR for sequencing

After running the 2% agarose gel (using the lowest possible concentration of agarose in the gel for the given size of DNA), the desired bands were visualized under UV light and excised with a clean blade. The PCR product was purified using the gel extraction kit (QIAquick gel extraction kit).

PCR for sequencing

10 ng/100 bp DNA

15 pmol primer

1 µl Termomix

4 µl Sequence buffer

upto 20 µl H_2O

The primer were prepared again from the stocks (100pmol/ µl) by re-suspending 2 µl of each primer in 18 µl ultrapure water before use (dilution of 1:10).

PCR program

pUAST down primer		
94ºC	1 min	
96ºC	10 sec	
52ºC	10 sec	x25
60ºC	4 min	
68ºC	5 min	
15ºC	∞	

Table 3.2- PCR program for sequencing

3.2.4 Purification of DNA from PCR for sequencing

After the PCR, 20 µl of the DNA sample was mixed with 50 µl 100% ethanol and 2 µl NaOAC (3 M, pH 4.8). The sample was kept for 10 minutes on ice and then centrifuged for 25 minutes at 14000 rpm. The pellet was washed with 250 µl of 70% ethanol and centrifuged for 5 minutes at 14000 rpm. The step was repeated under the same conditions. The resultant pellet was dried and 20 µl of HiDi Formamid was added.

3.3 Protein analysis

3.3.1 1D-SDS-PAGE analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a technique to separate proteins according to their electrophoretic mobility (Shapiro *et al.,* 1967; Laemmli, 1970). Proteins have identical charge per unit mass due to binding of SDS and this results in fractionation by individual size in SDS gel electrophoresis.

SDS is a powerful detergent, which has a very hydrophobic end (the lipid like dodecyl part) and highly charged part (the sulphate group). The dodecyl part interacts with hydrophobic amino acids in proteins. Since the 3D structure of most proteins depends on interactions between hydrophobic amino acids in their core, the detergent destroys 3D structures, transforming what were globular proteins into linear molecules now coated with negatively charged SDS groups. Therefore, after boiling proteins in SDS they become elongated with negative charges arraying down their length, so they will move towards a positive electrode. The reason why beta-mercaptoethanol is usually included in the sample buffer is to cleave disulphide bonds within or between molecules, allowing molecules to adopt an extended monomeric form. Since some proteins have few or no hydrophobic residues it is also not surprising that such molecules do not run on SDS page. Modifications such as phosphorylation and especially glycosylation can also cause proteins to run slower than expected. Finally, cross-linked proteins do not run as their molecular weight would predict, generally running slower and usually are analysed on higher percentage gels. However, a particular protein runs at a particular position on a particular percentage acrylamide gel in a reliable manner.

3.3.1.1 Non-pre-cast SDS-PAGE system

There are generally two gels, the 12% resolving gel and the 5% stacking gel. The stacking gel consists of very low acrylamide concentration and it is used to form the wells into which the proteins are loaded. The low acrylamide concentration also allows most proteins to be concentrated at the dye front, so that diluted protein samples can be compared to concentrated samples on the same gel. The higher the acrylamide concentration of the resolving gel, the slower the proteins migrate through the gel. In contrast, the lower percentage gels proteins migrate faster. Other chemicals such as

ammonium persulphate (APS), a free radical generator, and TEMED, a free radical stabilizer, initiate and facilitate polymerization of the gel.

Before loading the gel, the sample was denatured by adding 2X Laemmli buffer and heating to 90°C for 10 minutes. Bromophenol blue of the laemmli buffer serves as an indicator dye and one can observe the migration of protein samples as the dye front that runs ahead of the proteins. Another function of bromophenol blue is to make it easier to see the sample while loading it into the wells of the gel; glycerol in the Laemmli buffer increases the density of the sample so that it will fall to the bottom of the well, minimizing bubbling or loss of protein sample in the buffer. Dithiothreitol (DTT) is present in many formulations to help reduce any disulphide S-S bonds that could provide secondary/tertiary structure and/or dimer formation.

3.3.1.2 Pre-cast SDS-PAGE system (from Invitrogen)

When using ready-made gels provided by Invitrogen (NuPAGE® Novex 4-12% Bis-Tris ZOOM Gel, NuPAGE® Novex 10% Bis-Tris ZOOM Gel) the samples were mixed with NuPAGE® LDS Sample Buffer and NuPAGE® Reducing Agent according to the instructions by Invitrogen and heated at 70°C for 10 minutes.

1X SDS Running Buffer was prepared by adding 20 ml 20X NuPAGE® MES or MOPS SDS Running Buffer to 400 ml of deionised water. The Upper (inner) Buffer Chamber was filled with 200 ml 1X NuPAGE® SDS Running Buffer. For reduced samples, 200 ml 1X NuPAGE® SDS Running Buffer containing 500 µl NuPAGE® Antioxidant were used. The lower (outer) Buffer Chamber was filled with 200 ml 1X NuPAGE® SDS Running Buffer.

The running conditions were kept as provided by Invitrogen instructions: 120 V constant.

3.3.2 Western blotting

The western blot is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract (Towbin *et al.,* 1979). It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3D structure of the protein (native/non-denaturing conditions). The proteins are

transferred to a membrane (typically nitrocellulose or polyvinylidene difluoride), where they are detected using antibodies specific to the target protein(s).

3.3.2.1 Transfer

First, the proteins have to be accessible to the antibody for detection, so they are moved from within the gel onto a membrane made of nitrocellulose. The sandwich is assembled with a fibre pad, a filter paper on top, then the gel, the membrane is placed on top of the gel, another filter paper and finally again another fibre pad. The entire stack is placed in a buffer solution (1X Transfer buffer). Electroblotting uses electric current to pull proteins from the gel onto the PVDF or nitrocellulose membrane. I our experiments, the transfer was done using the Mini Trans-Blot® system from Bio-Rad. The membrane was placed between the gel and the anode, as the electric field will move the sample towards the anode since all proteins contain high negative charges as a result of SDS treatment.

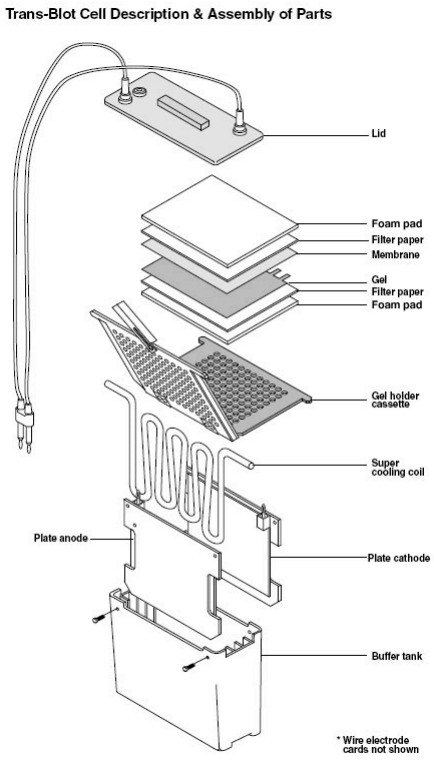


Figure 3.1- Wet blotting (Source: www.bio-rad.com)

The power supply was adjusted to a constant voltage of 110V for 1h. The uniformity and overall effectiveness of protein transfer from gel to membrane can be verified by a Coomassie or Ponceau S staining of the membrane.

3.3.2.2 Blocking

Blocking of the membrane is a step to prevent non-specific interactions between the membrane and the antibody. This is achieved by placing the membrane in a diluted solution of proteins, typically 5% non-fat dry milk in 1X TBST (washing buffer) either for 1h at room temperature or overnight at 4ºC depending on the quality of the primary antibody used for identification of the protein. The proteins in the diluted blocking solution attach to the membrane in all places, thus blocking the empty membrane surface and non-target proteins from interacting with the antibody later. When the antibody is added, it will remain attached after washing only at the high-affinity binding sites of the specific target protein. This reduces background signals and detection of unspecific proteins in the Western blot, leading to clearer results, and eliminates false positives.

3.3.2.3 Detection

Antibodies are used to detect and visualize specific proteins. They are generated when a host specie is exposed to the protein of interest. Normally, the antibody production is part of the immune response, whereas here they are harvested and used as sensitive and specific tools for the detection of the proteins of interest.

After blocking, a diluted solution of primary antibody in 1X TBST was incubated with the membrane for 2 hours at room temperature under gentle agitation. After the incubation, the membrane was washed with 1X TBST 3 times for 5 minutes to remove unbound primary antibody. Next, the membrane was exposed to a secondary antibody, directed at a species-specific portion of the primary antibody. The secondary antibody is usually linked to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. Each primary antibody is usually bound by more than one secondary antibody.

The membrane was incubated for 1h at room temperature with the secondary antibody. A horseradish peroxidase (HRP) linked secondary antibody was used. After the incubation, the membrane is washed again 3 times 10 minutes each in 1X TBST as before to remove all unbound antibodies, since this reduces the background noise. The chemiluminescent solutions in the ECL Kit (GE Amersham) were first mixed in the ratio 1:1 and then poured over the membrane for an incubation of 1 minute. During this incubation, the luminogenic substrates in this mixture are cleaved by the HRP and

release energy in the form of light. The light produced by this chemiluminescence reaction is in proportion to the amount of the protein detected by the primary antibody. A light-sensitive X-ray film is placed against the membrane in a dark room and the location and intensity of the signal on the film correlates with the size and amount of the specific protein. The exposed X-Ray film was then developed and fixed in an automatic developing machine (AGFA CP-1000).

3.3.2.4 Alkaline phosphatase treatment

The protocol for the alkaline phosphatase treatment explained in Schubert PhD thesis (Schubert, 2010) was followed with minor modifications. The desire number of heads was homogenized in the alkaline phosphatase homogenization buffer (25mM Tris, 150mM NaCl, 10% Glycerol, 0.1% NP-40). The samples were divided in four different treatments: a) treatment with 10% Enzyme Units of the total volume and 1X Shrimp Alkaline Phosphatase (SAP) buffer (Fermentas) heated to 37ºC during 15 minutes; b) treatment with 10% Enzyme Units of the total volume and 1X Shrimp Alkaline phosphatase (SAP) buffer incubated on ice during 15 minutes; c) treatment with 1X Shrimp Alkaline Phosphatase (SAP) buffer heated to 37ºC during 15 minutes; and d) treatment with 1X Shrimp Alkaline Phosphatase (SAP) buffer incubated on ice during 15 minutes. The reaction was stopped heating the samples at 65ºC during 15 minutes. The wild-type control flies were treated only with Laemmli buffer. A SDS-PAGE followed by a Western blot was performed.

3.3.3 2D-SDS-PAGE analysis

2D-PAGE is used to separate mixtures of proteins according to their isoelectric point and their size. The polyacrylamide gel provides a supporting matrix through which proteins can migrate. The gel strips for isoelectric focusing have a pH gradient along their length.

A complex protein mixture is loaded into the sample well and the IPG strip is inserted. After rehydration of the strip a high voltage is applied across the gel. The proteins migrate through the gel until they reach their isoelectric point (the point at which due to the local pH their charge is the zero). This technique is called isoelectric focussing.

The gel strip is then soaked in a denaturing solution containing the detergent sodium dodecyl sulphate (SDS), which causes the proteins to unfold and become negatively charged. The strip is placed horizontally into a large pocket of a normal SDS gel and in the electric field the proteins all move towards the anode as described above, thus separating the proteins according to their size.

The required numbers of flies were collected, frozen and their heads were separated. The heads were homogenized in 100 µl of the Sample Homogenizing Mix (ZOOM® 2D Protein Solubilizer 1) and 1X protease inhibitors (10x Complete-Mini, Roche®). The homogenate was then centrifuged twice for 15 minutes with 13.000 rpm at 4ºC. 1 µl of 99% N,N.Dimethylacrylamide (DMA, Sigma) was added to the post-nuclear supernatant and incubated on a rotatory shaker at room temperature for 15 minutes to alkylate the proteins. Thereafter 2 µl of 1 M DTT were added to quench any excess of DMA. The required amount of supernatant (25 µl, equivalent to 25 fly heads) was then used to prepare the final rehydration mix and sample. The supernatant was mixed with the Sample loading solution (137 µl ZOOM® 2D Protein solubilizer 1/2, 2 µl 1 M DTT, 2µl Ampholytes (Servalyte 6-9 pH ampholyte, Serva), 1% Bromophenol blue). 165 µl of this sample were loaded into the sample loading well (ZOOM® IPG Runner™ Cassette). The ZOOM® Strip (pH 6-10) was inserted into the cassette. The acidic end (+) of the IPG strip was inserted into the channel while holding the strip with forceps at the basic end (-) taking care that there were no bubbles inside with the printed side facing down and the gel facing up. Up to six strips could be accommodated by the cassette. The wells were sealed using the sealing tape provided. The loaded and sealed ZOOM® IPGRunner® Cassette was left at 18ºC overnight.

The next day, the sealing tape and the Sample Loading Devices were removed from the cassette. Two electrode wicks were placed over the exposed ends of the strips on each end of the cassette. 600 µl of not deionized plain water were added to the anode wick and 600 µl of 3% DDT were added to the cathode wick. The cassette was placed against the ZOOM® IPG Runner™ Core and slid into the Mini-Cell Chamber of the IPG Runner® and locked. The outer chamber was filled with 600 ml of deionized water without pouring any liquid into the inner chamber. The chamber was closed with the lid

and the electrode cords were connected to the power supply (ZOOM® Dual Power by Invitrogen). The proteins were focused by five voltage steps (Table 3.3) providing 1835.45 Vh.

Broad Range ZOOM® strips

Step	Voltage (V)	Time (min)	Total Volthours (Vh)
1	200	20	66.7
2	450	15	112.5
3	750	15	187.5
4	750-2000	45	468.75
5	2000	30	1000

Table 3.3- Voltage conditions for isoelectric focusing

Thereafter the strips were taken out of the cassette and incubated in the equilibration (1X NuPAGE® LDS Sample buffer, 1X NuPAGE Reducing Agent) and alkylation solutions (1X NuPAGE® LDS Sample buffer, Iodacetamide) respectively for 15 minutes each with shaking. The strips were placed into the IPG well of the 4-12% Bis-Tris NuPAGE™ 2DE gel. The strips were overlaid with 400 µl of 0.5% agarose solution prepared in the running buffer (1X MOPS). Air bubbles were avoided. Once the agarose was solidified, 5 µl of the protein marker were loaded into the marker well. The chamber was filled with 1X MOP/MES running buffer and 500 µl of NuPAGE® Antioxidant were added to the inner buffer chamber. The second dimension of electrophoresis (SDS-PAGE) was performed at 100-200 V.

After electrophoresis, the second dimension gel was subjected to either staining (see 3.3.4 Blum Silver Staining) or Western blot (see 3.3.2. Western Blotting) according to the need.

3.3.4 Blum Silver Staining

Silver staining is used for sensitive detection of proteins separated by 1D and 2D SDS PAGE. The method is sensitive and mass spectrometry compatible.

Silver binds to the amino acid side chains, primarily the sulfhydryl and carboxyl groups of proteins, and are then reduced to free metallic silver. As little as one nanogram of protein can be visualized with silver stain. Based on recently published literature (Mortz *et al.*, 2001), identification of silver-stained proteins from electrophoretic gels is feasible by enzymatic digestion in conjunction with highly sensitive and accurate mass spectrometry.

According to the modified Blum silver staining protocol for mass spectrometry after electrophoresis, the gel was removed from the cassette and placed into a box containing 40% EtOH, 10% HAc and 50% H_2O fixing solution for 1 hour or overnight on a rotatory shaker. The fixation restricts protein movement from the gel matrix and removes interfering ions and detergent from the gel. Thereafter the gel was washed with washing solution (30% EtOH, 70% H_2O) 2 times for 20 minutes each and afterwards once with milliQ H_2O for 20 minutes. Sensitization solution (0.02% $Na_2S_2O_3$) was added for 1 minute (exact) to increase the sensitivity and the contrast of the staining and, again, the gel was washed with milli-Q H_2O 3 times for 20 seconds each (exact). The gel was incubated with cold (4ºC) silver nitrate solution (0.1% $AgNO_3$) and was placed on a shaker for 20 minutes at 4ºC. After the incubation, the gel was washed again with milli-Q H_2O 3 times for 20 seconds each (exact) and was transferred from the old box to a new one. The gel was washed briefly with milli-Q H_2O for 1 minute (exact) and right afterwards the proteins could be visualized with the developing solution (3% Na2CO3, 0.005% formalin). First, a little bit of the developing solution was added and when it changed to a yellow solution, it was discarded and the rest of the developer was added. The reaction was stopped with the stopper solution (0.05M EDTA) when the intensity of the bands were strong enough. The gel can be stored in the same stopper solution at 4ºC for 2-3 days or in a preserving solution (1% Acetic acid) until further analysis of the proteins bands.

3.4 Immunochemistry procedures

3.4.1 Immunoprecipitation

Immunoprecipitation (IP) is a method that uses the antigen-antibody reaction principle to isolate a protein that reacts specifically with an antibody from a mixture of proteins so that its quantity or physical characteristics can be examined (Bonifacino *et al.,* 2001). Historically, the solid-phase support for immunoprecipitation used by the majority of scientists has been highly porous agarose beads coated with Protein A or G which bind to the antibody, which in turn binds to its target antigen. The immunoprecipitation experiment performed here contained the following steps: 1 ml of the primary antibody and 50 µl of Protein G-agarose beads (Roche Diagnostics, Mannheim) were incubated together overnight to make sure that the primary antibody is bound to the beads. The tubes with the antibody/beads mixture are centrifuged 5-10 minutes at 13000 rpm and 4ºC. The supernatant with unbound antibody was discarded. 100 heads were homogenized in 120 µl homogenization buffer. The homogenate was centrifuged for 20 minutes at 13000 rpm and 4ºC. The supernatant was transferred to a new eppendorf tube and the pellet was discarded. 40 µl of the lysate was mixed with 30 µl of 1X Laemmli buffer and kept at -20ºC as input control. The rest of the lysate was incubated with the beads overnight. The antibody-beads complex were mixed with 50 µl of the homogenate and another 300 µl of the homogenization buffer. This mixture was incubated from 2 hours to overnight under gentle rotation. The bead/immune complex was spun down at 13000 rpm for 3 minutes. The supernatant was saved as another control (40 µl of the sample and 30 µl of Laemmli buffer) and the pellet was washed 3 times with 100 µl homogenization buffer with the help of a syringe and centrifuged again at 13000 rpm. Since only the target protein and its interaction partners bind to the antibody and are precipitated, other proteins that do not bind are washed away. Components of the bound immune complex (antigen, interaction partners, and antibody) were eluted from the beads by adding 80 µl of 1X Laemmli sample buffer and heating the sample briefly to 70ºC to dissolve all hydrophobic interactions between the antigen-antibody and to break the disulphide bonds holding together polypeptide subunits of the proteins or maintaining their tertiary structure. Immunoprecipitated proteins were further analysed by SDS-PAGE followed by staining and/or immunoblotting to analyse various characteristics

of the involved proteins such as their molecular weights, their interaction partners or their enzymatic activity.

3.4.2 Covalent coupling of antibodies to Protein-G-Agarose for Immunoprecipitation

3.4.2.1 Preparation of Protein-G-Agarose

The first step was calculating the total amount of beads. For a single IP, 50 µl of Protein-G- Agarose beads is enough. The beads were spun down at 3500 rpm and the supernatant was removed. The pellet was washed 3 times with 400 µl Citrate buffer at pH 5 and spun down at 3500 rpm. The supernatant was removed and the antibody was added and incubated overnight at 4°C on a rotatory shaker. Thereafter, the same amount of citrate buffer as the antibody was added and incubated for 40 minutes at room temperature and gently shaked. After the incubation, the sample was centrifuged and the supernatant was discarded. The pellet was washed 3 times with citrate buffer and twice with 0.2 M triethanolamine (TEA) pH 8.2.

3.4.2.2 Coupling procedure

500 µl of 0.2 M triethanolamine pH 8.2 containing 20 mM dimethylpimelidate (DMP) was added to the sample and incubated for 40 minutes at room temperature and gentle shaking. The reaction was stopped by spinning down and removing the supernatant and adding 500 µl of 50 mM Tris pH 7.5. The sample was incubated for 15 minutes at room temperature and gentle shaking. Thereafter, three washes with 500 µl PBS and 0.05% Tween-20 at pH 7.4 were done followed by 3 washes in 100 µl 0.1 M citrate buffer pH 3.0 for 2 minutes at room temperature and gentle shaking. Finally, the beads were washed once with 500 µl of PBS and 0.05% Tween-20 at pH 7.4 and another one with the preferred buffer (homogenization buffer). After the covalent coupling the beads were used for immunoprecipitation as described above.

3.5 Immunohistochemistry procedures

Immunostaining is a general term in biochemistry that applies to any use of antibody-based methods to detect a specific protein(s) in a sample. The term immunostaining was originally used to refer to the immunochemical staining of tissue sections. Now, however, immunostaining encompasses a broad range of techniques used in histology, cell biology, and molecular biology that use antibody-based staining methods.

3.5.1 Adult brain whole mounts

The procedure for antibody staining of adult whole mount brains of *Drosophila* has been described in detail (Wu and Luo, 2007) and was followed with minor modifications. Adult flies were anesthetized with CO_2, collected over a glass plate and kept on ice. The flies were placed onto a silgel (Wacker Chemie AG) dish with dorsal side down and fixed with a pin through its abdomen or thorax. The brain preparation was done in Ringer's solution at room temperature and the brains were fixed for 1 hour in 4% paraformaldehyde (PFA). Thereafter, the brains were washed three times in 1X PBST (0.3% Triton X-100) for 10 minutes each. Afterwards, the brains were blocked in PBST containing 0.3% Triton X-100 and 5% normal goat serum for 2 hours at room temperature or overnight at 4°C. The brains were incubated two nights at 4°C with the primary antibody diluted in the blocking solution with 5% normal goat serum and 1X PBST (0.3% Triton X-100). The next day, the brains were washed six times in 1X PBST (0.3% Triton X-100) for 10 minutes each at room temperature. Thereafter, the brains were incubated overnight at 4°C with the secondary antibodies diluted in blocking solution (1X PBST with 0.3% Triton X-100 and 5% Normal Goat Serum). After incubation with the secondary antibodies, the brains were washed six times in 1X PBST (0.3% Triton X-100) for 20 minutes at room temperature. After antibody staining, whole the brains were mounted in Vectashield™ (Vetor Labs) for fluorescence microscopy. The preparations were examined using a confocal laser-scanning microscope Olympus IX81. The confocal stacks were analyzed using the Fiji (Schindelin *et al.,* 2012; Schmid *et al.,* 2010) based on ImageJ (Collins, 2007) and Adobe Photoshop CS5.

3.5.2 Larva brain whole mounts

The brains were dissected in PBS solution, fixed in 4% paraformaldehyde and blocked for 90 minutes at room temperature in PBS with 5% normal goat serum and 0.3% Triton X-100 followed by two nights incubation at 4°C with the primary antibodies diluted in PBS with 5% Normal Goat Serum and 0.3% Triton X-100. The next day, the brains were washed six times in 1X PBST (0.3% Triton X-100) for 10 minutes each at room temperature. Thereafter, the brains were incubated overnight at 4°C with the secondary antibodies diluted in blocking solution (1X PBST with 0.3% Triton X-100 and 5% Normal Goat Serum). After incubation, the brains were washed six times in 1X PBST (0.3% Triton X-100) for 20 minutes at room temperature. Larval brains were imaged

using a confocal laser scanning microscope Olympus IX81. The confocal stacks were analyzed using the Fiji (Schindelin *et al.,* 2012; Schmid *et al.,* 2010) based on ImageJ (Collins, 2007) and Adobe Photoshop CS5.

3.5.3 Larva NMJ dissections

Third instar larvae were collected in pre-chilled Ca^{2+}-free saline and kept on ice for 10 minutes. The dormant larvae were transferred onto a silgel (Wacker Chemie AG) surface of a dissection dish containing pre-chilled Ca^{2+}-free saline. The larva was observed under the microscope and placed on its dorsal surface (two major longitudinal trachea are visible on the ventral side). One needle was fixed at the posterior tip and the second one at the head. A small cut was made in the cuticle near the middle of the ventral side. Then the cuticle was cut longitudinally on either side along the middle cut to the posterior and anterior. The cuticle was then cut open and pinned on either side to expose the viscera. All unnecessary body parts were removed leaving only the CNS and the body wall musculature. The Ca^{2+}-free saline was the discarded and the dissected larva was incubated in the fixative (4% PFA) for 90 minutes at 4ºC. Thereafter, the fixative was discarded and the dissected larva was washed with 1X PEM buffer twice at room temperature for 15 minutes each. Then the PEM buffer was discarded and the larva was incubated in blocking solution for 1 hour at room temperature and gentle shaking followed by the addition of the primary antibody. The primary antibody was incubated overnight at 4ºC. The next day, the primary antibody was discarded and the larva was washed six times in 1X PBST for 30 minutes each. Thereafter, the larva was incubated in (fluorescently labelled) secondary antibody diluted in 1X PBST overnight at 4ºC. After the incubation the larva was washed six times for 30 minutes on a shaker with 1X PBST. Finally, the larvae were mounted on a glass slide overlaid with Vectashield™ and covered with a coverslip. The edges of the coverslip were sealed onto the glass with transparent nailpolish. After drying for 30 minutes, the slides can be stored at 4ºC.

3.5.4 Immunostaining of Cryosections

The procedure for immunostaining of cryosections has been recently described (Halder *et al.,* 2011). Five adult, large female CS flies were anesthetized by CO_2 and placed on a glass Petri dish kept on ice bath. The flies were glued by their dorsal thoraces to a thin fibre stick using nail polish, which was allowed to dry for 10 minutes. Fibre sticks with immobilized flies were dipped in EtOH for 3 seconds and jerked to shed off hairs

and bristles, and excess EtOH was soaked away with a tissue paper. The flies immobilized on the fibre stick were then dipped in freshly prepared ice cold 4% PFA solution and the stick is clamped to a holder so that the flies are horizontally oriented with their ventral side facing upwards and the setup is placed on an ice bath and focussed under a bright field stereomicroscope. The proboscis and ventral air sacs were removed from each fly head with fine tweezers. The flies with removed proboscis (on fibre) were incubated in 4% PFA solution (in small glass tubes) for 3 hours at 4ºC to fix the tissues. Thereafter, they were incubated in 25% Saccharose solution at 4ºC overnight. The next morning, the heads of the flies (on the fibre) were cut off under a bright field stereomicroscope and were embedded in a small drop of 16% carboxymethylcellulose (CMC) placed on a holder (metal bolt). After adjusting the head to the correct orientation in the CMC drop, the holder was slowly dipped into liquid N_2, to gradually freeze it from bottom to top. The holder with the frozen head embedded in CMC was then attached to a cryotome and 15 µm thick cryosections were cut at -26ºC. Ribbons of sections were collected on a subbed microscope slide (Thermo Scientific-Menzel-Gläser Superfrost® Plus) one beside the other, briefly thawed, refrozen and then air dried. The sections were encircled with Roti-liquid Barrier Marker (Roth) and blocked with 200 µl of 1X PBST + 0.1% Triton X-100 + 2.5% NGS pH 7.4 for 30 minutes at room temperature in a humid chamber. Thereafter the sections were incubated with primary antibody (diluted in 1X PBST+1.5% NGS) at room temperature for 2 hours in a humid chamber. Next, the excess primary antibody was drained off and the slide was washed once briefly and twice for 10 minutes each with 1X PBST. Secondary antibody (α-mouse or α-rabbit Alexa 488 or Cy3) was added to the sections (5 µl/ml in 1X PBST) and incubated at 37ºC for 2 hours. The slides were washed 3 times for 10 minutes with 1X PBST and once with 1X PBS. One drop of Vectashield™ was added to the sections before covering them with a coverslip and observing them under a fluorescence microscope to check if the staining was successful. The slides were sealed with nail polish and stored at 4ºC.

3.6 Hybridoma cell culture

Hybridoma clones were generated as described earlier (Hofbauer, 1991; Hofbauer *et al.,* 2009). The preparation can be done using HT-medium or mixing 50% of HT-medium and 50% of HT-medium without FCS. The medium was mixed in a beaker and filtered sterile. If the addition of antibiotics was necessary, 1 ml of the

antibiotic was added. One day before thawing the cells, a 24-well plate was prepared (per well: 0,5 ml HT-Medium, one drop FCS and one drop HY). The solution without cells was kept in the incubator overnight. A small sample was stored as a control to check if the sample suffered any contamination. Before adding the medium, the incubator was warmed-up and enriched with CO_2 (7%). The cells were defrosted at 37ºC and transferred immediately in a 10 ml blue cap with 9.5 ml of medium to wash the excess of glycerol that was used during the freezing step. The sample was centrifuged 1000 rpm/min (55%) for 5 minutes. The supernatant was discarded and the pellet was dissolved in 1ml HT-Medium and added to the wells (half of the well was filled up). The next day, 1 ml of medium was added per well. After the washing step, the cells were resuspended in small bottles adding 5 ml of medium and 1 ml was kept in the wells. The cells which grew well were transferred to large bottles (40-50 ml final volume). When the medium turned yellow, it was removed and checked on Western blot.

3.7 Behavioral assays

3.7.1 Short-term olfactory habituation

The protocol was adapted from the previously described protocols for measuring olfactory responses of flies in a Y-maze and for inducing short-term habituation to odorants (Das *et al.,* 2011, Sudhakaran *et al.,* 2012). 4-day old flies were starved overnight on a moist filter paper (around 25-30 flies per vial). These flies were subjected to a pretest to measure the naïve response to 10^{-3} dilution of ethyl butyrate (EB) or 5% CO_2. For EB exposure, flies were transferred to a 125 ml glass bottle in which a 1.5 ml Eppendorf tube containing 5% EB (diluted in paraffin oil) with a perforated lid was suspended. Flies in an empty vial covered with cheesecloth were placed in a CO_2 incubator for inducing Short-term olfactory habituation (STH) to CO_2. After 30 minutes of odorant exposure, the flies responses were tested to 10^{-3} EB or to 5% CO_2. A response index (RI) was calculated for naïve and odor-exposed flies, based on the fraction of flies in the odorant compartment minus the fraction of flies in the air compartment divided by the total number of flies that participated in the experiment. The RI values are presented as the percentage response of the naïve response of the flies.

3.8 Antisera generation

New antisera against phosphorylated synapsin were tested. Those antisera were made by Eurogentec (www.eurogentec.com) and small aliquots of antisera and glycerol (1:1) were frozen. To generate these antisera, Eurogentec used crude serum from a rabbit immunized with the phosphorylated peptide and, at first, antibodies against phosphorylated epitope and against other epitopes of the peptide were captured on a column with the phosphorylated peptide. Both types of antibodies were eluted and loaded on a matrix coupled with the non-modified peptide, such that the antibodies again the other epitopes were captured and only the desired immunoglobulins specific for the modification were in the flow-through. The captured antisera were eluted and saved. The antisera were named anti-PSyn (S6) and anti-PSyn (S464) for the phosphorylated epitopes of the peptides and correspondingly anti-Syn (S6) and anti-Syn (S464) for the other epitopes of the peptide, respectively. Anti-Syn denotes the monoclonal antibody 3C11. The peptides used for the immunization were:

-For P-Syn (S6): NH2-MKRRFSPSGDLSSC-CONH2 (non-edited)
-For P-Syn (S464): NH2-VSSRAESPPTDEGV-CONH2

4. Results

4.1 Studies of synapsin phosphorylation

4.1.1 Specificity of the antisera

Name (synonymous)	epitope/peptide	Specie
α-Syn (3C11, Synorf1)	LFFMEVCGL in the C domain	mouse
α-Syn(S6)	NH2-MKRRFSSGDLSSC-CONH2	rabbit
α-Syn(S464)	NH2-VSSRAESPTDEGV-CONH2	rabbit
α- PSyn(S6)	NH2-MKRRFS^{p}SGDLSSC-CONH2	rabbit
α- PSyn(S464)	NH2-VSSRAES^{p}PTDEGV-CONH2	rabbit

Table 4.1- Synapsin antibodies used in this study

Synapsin plays a role in the fine regulation of neurotransmitter release. When synapsins are phosphorylated, neurotransmitter release is facilitated (see Introduction). The monoclonal synapsin antibody α-Syn (3C11) (Hofbauer *et al.,* 2009) detects all synapsin isoforms regardless of their phosphorylation status. The following antibodies were generated with the aim to study synapsin phosphorylation at serine 6 (S6) (PKA/CaMKII consensus motif) and serine 464 (S464) (CaMKII consensus motif) in *Drosophila melanogaster* brain (Table 4.1, see Methods). First, the specificity of the antisera was studied by Western blots analysis. Wild-type flies, Syn^{97} or Syn^{79} null mutants, Sap47^{156CS} null mutants and double mutants were used for that purpose. Of the three small synapsin isoforms recognized by the monoclonal antibody α-Syn (3C11) at 70, 74, and 80 kDa and the two large isoforms at around 143 kDa (Godenschwege *et al.,* 2004) often only two fused signals between 70 and 80 kDa are visible in the control blots using short exposure times. Likewise, only the most abundant 47 kDa isoform of Sap47 (Funk *et al.,* 2004) is used as loading control. In order to avoid signals in the synapsin blots from the larger Sap47 isoforms, the blots were cut horizontally near 60 kDa and the upper part was developed for synapsin detection and the lower part for Sap47 detection (nc46). An increase in synapsin expression possibly combined with enhanced phosphorylation in the Sap47^{156CS} null mutants was previously shown (Funk, unpublished; Nuwal, 2010). It is usually seen as an additional (fused) band around 85 kDa. The antisera Syn(S6) and

Syn(S464) that should be specific for the non-phosphorylated peptides containing S6 and S464, respectively, were tested in the Western blots shown in Figures 4.1 and 4.2.

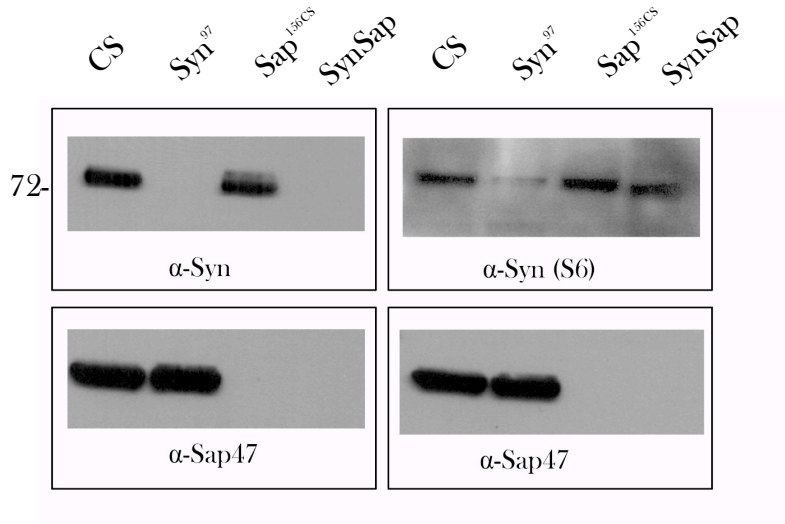


Figure 4.1. Specificity of α-Syn(S6) antiserum. Western blot of head homogenate using a 12% precast gel for detection of non-phosphorylated synapsin. In the right part of the blot, synapsin was detected with α-Syn(S6) antiserum (diluted 1:1000); the positive control with α-Syn (mAb 3C11) (diluted 1:200) is shown in the left part of the blot. The loading control with anti-Sap47 (mAb nc46) (diluted 1:200) is shown in the bottom part of both blots. CS: Canton S wild type flies; Syn^{97}: synapsin mutant Syn^{97}; Sap^{156CS}: Sap mutant $Sap47^{156CS}$ and SynSap: double mutants $Syn^{97}/Sap47^{156CS}$ (2 heads per lane).

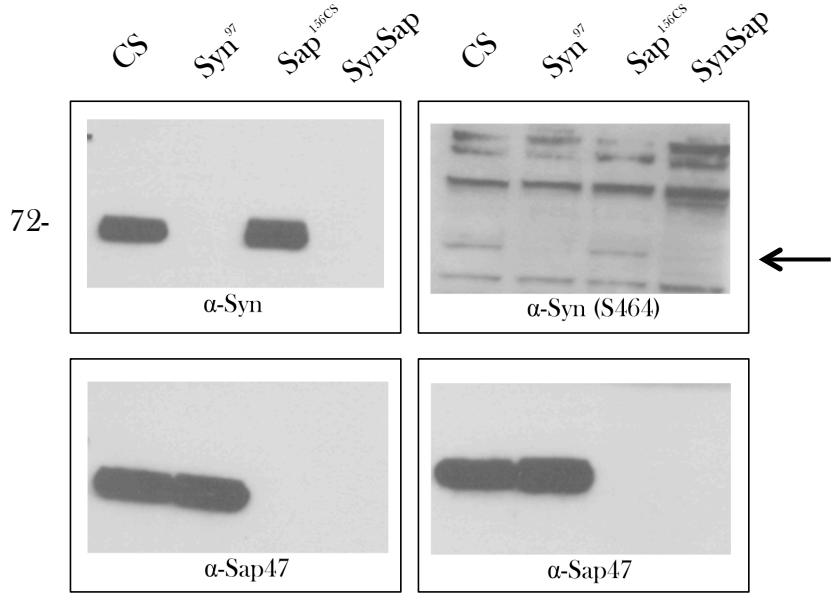


Figure 4.2. Specificity of α-Syn(S464) antiserum. Western blot of head homogenate using a 12% precast gel for detection of non-phosphorylated synapsin. In the right part of the blot, synapsin was detected with α-Syn(S464) antiserum (diluted 1:1000); the positive control with α-Syn (mAb 3C11) (diluted 1:200) is shown in the left part of the blot. The loading control with anti-Sap47 (mAb nc46) (diluted 1:200) is shown in the bottom part of both blots. CS: Canton S wild type flies; Syn^{97}: synapsin mutant Syn^{97}; Sap^{156CS}: Sap mutant $Sap47^{156CS}$ and SynSap: double mutants $Syn^{97}/Sap47^{156CS}$ (2 heads per lane).

The control blots with α-Syn (3C11) and α-Sap47 (nc46) demonstrate the specificity of these two antibodies (no signals in the respective mutants). However, no clear difference between wild-type and Syn^{97} null mutants was found for α-Syn(S6) antiserum (Figure 4.1, right blot), indicating that the antiserum may not recognize synapsin. Unexpectedly, it cross-reacts with a protein of about the same apparent molecular weight that is not influenced by the Syn^{97} or the $Sap47^{156CS}$ null mutants. The α-Syn (S464) antiserum against the non-phosphorylated peptide with S464 (Figure 4.2, right blot) contains a synapsin-specific fraction (arrow) but in addition cross-reacts with various proteins unrelated to synapsin, as revealed by comparison to the blot developed with mAb α-Syn (3C11). Following these experiments, the antisera PSyn(S6) and PSyn(S464) that should be specific for the phosphorylated peptides containing S6 and S464, respectively, were tested in the Western blots shown in Figures 4.3 and 4.4.

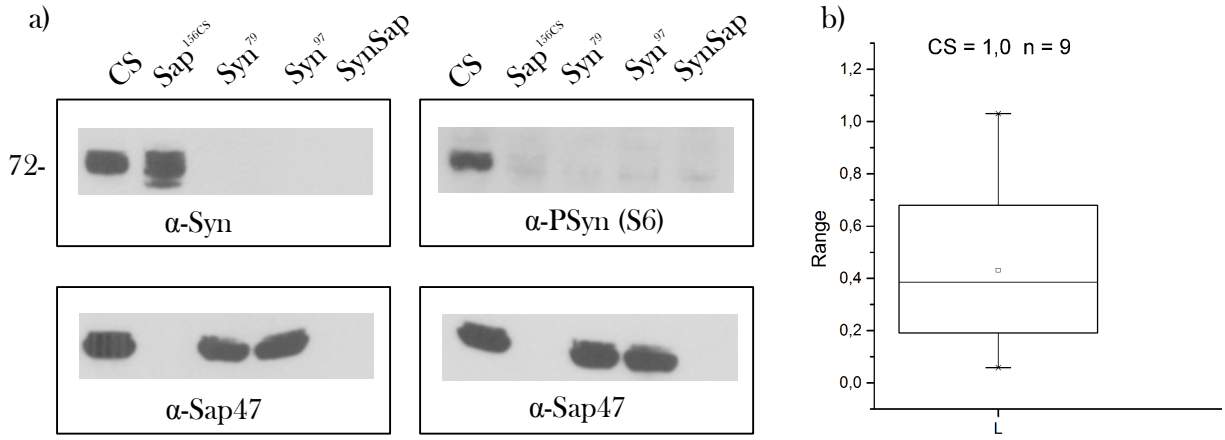


Figure 4.3. Specificity of α-PSyn(S6). a) Western blot of head homogenate using a 12% precast gel for detection of synapsin phosphorylated at S6 (site 1). In the right part of the blot, synapsin was developed with PSyn(S6) antiserum (diluted 1:500); the positive control with anti-Syn (mAb 3C11) (diluted 1:200) is shown in the left part of the blot. The loading control with anti-Sap47 (mAb nc46) (diluted 1:200) is shown in the bottom part of the blot. CS: Canton S wild type flies; Syn^{79}: synapsin mutant Syn^{79}; Syn^{97}: synapsin mutant Syn^{97}; Sap^{156CS}: Sap mutant $Sap47^{156CS}$ and SynSap: double mutants (2 heads per lane). **b)** Normalized Sap^{156CS} ratio PSyn(S6)/3C11 (*p*-value <0.01, calculated using Student's *t*-test with Bonferoni correction. Error bars indicate standard deviations. n=9).

In the Western blot of Figure 4.3 developed with α-Syn (3C11) (left part of the blot) the (fused) 74 and 80 kDa bands for synapsin can be detected. The 70 kDa band as well as the extra band near 85 kDa in the $Sap47^{156CS}$ null mutant mentioned above can be

recognized. For the PSyn(S6) antiserum, a (fused) double band at the position expected for synapsin can be detected in wild-type flies (CS) and the fact that it is absent in Syn^{97} null mutants and strongly reduced in Sap^{156CS} null mutants indicates that this antibody could be specific for synapsin phosphorylated at S6 and that although the total amount of synapsin is increased, the amount of S6-phosphorylated isoforms is reduced in $Sap47^{156CS}$ null mutants (Figure 4.3 b)

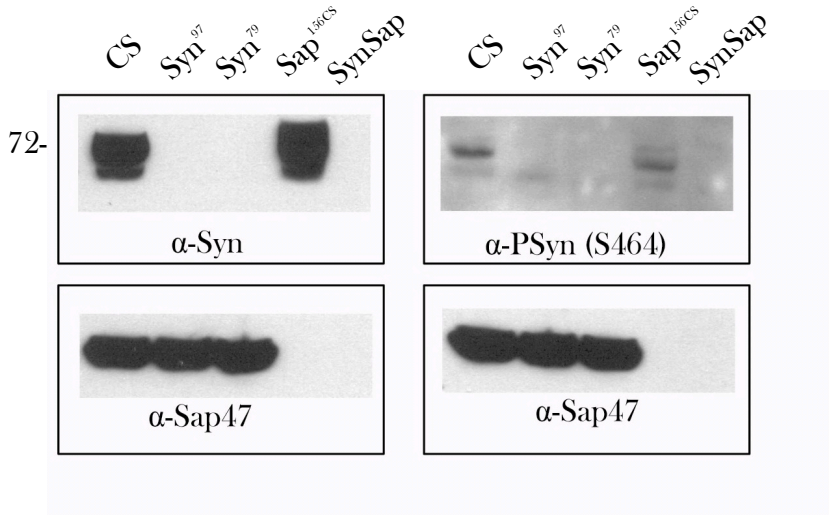


Figure 4.4. Specificity of α-PSyn(S464). Western blot of head homogenate using a 12% precast gel for detection of synapsin phosphorylated at S464 (site 2). In the right part of the blot, synapsin was developed with PSyn(S464) antiserum (diluted 1:1000); the positive control with anti-Syn (mAb 3C11) (diluted 1:200) is shown in the left part of the blot. The loading control with anti-Sap47 (mAb nc46) (diluted 1:200) is shown in the bottom part of the blot. CS: Canton S wild type flies; Syn^{97}: synapsin mutant Syn^{97}; Syn^{79}: synapsin mutant Syn^{79}; Sap^{156CS}: Sap mutant $Sap47^{156CS}$ and SynSap: double mutants (2 heads per lane).

In the Western blot of Figure 4.4 (left half of the blot) the fused three bands for synapsin are detected in CS flies with mAb 3C11 (anti-Syn). The fused extra band in $Sap47^{156CS}$ null mutants is clearly shifted to a higher molecular weight. The shifted band for synapsin is unique to $Sap47^{156CS}$ null mutants, as it has been reproduced in the independent mutant allele $Sap47^{208}$ (data not shown). This shift could be due to posttranslational modifications (see Discussion). For PSyn(S464) (right half of the blot) a single specific band can be detected in wild-type flies but this band was not found for either of the two synapsin mutants. This leads to the tentative conclusion that this antibody may be specific for synapsin phosphorylated at S464 (see Discussion). The blot also indicates that the abundance of the isoform phosphorylated at S464 is not strongly reduced in the $Sap47^{156CS}$ null mutants.

Once the specificity of the antibodies was checked using 1D-PAGE, 2D gel electrophoresis was performed in order to determine if the phosphorylated synapsin isoforms could be identified. Unfortunately, the 2D blot developed with α-PSyn(S464) was inconclusive (not shown).

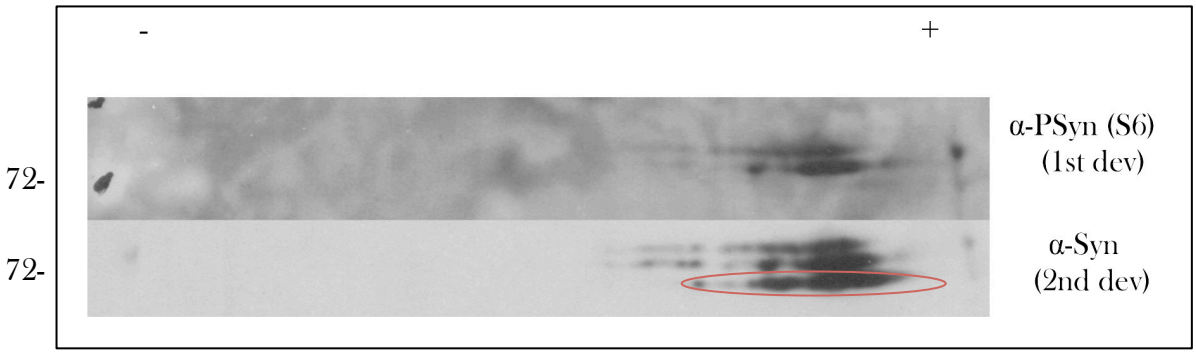


Figure 4.5. Comparison between PSyn(S6) antiserum and Synapsin antibody using 2D-PAGE gels. Western blot of head homogenate using a 4-12% Bis-Tris IPG 2D-PAGE precast gel for the detection of synapsin phosphorylated at S6. The upper part of the diagram shows the 2D blot membrane developed with α-PSyn(S6) (1:500) antiserum and the lower part the membrane developed again with the positive control α-Syn (mAb 3C11) (diluted 1:200). Canton S wild type flies.

The Western blot of the 2D-PAGE for detection of S6-phosphorylated synapsin isoforms was developed two times, first with α-PSyn(S6) antiserum and after washing of the membrane developed again with α-Syn (3C11). By comparing the two developments, some missing spots can be observed in the first development (red encircled in the second development), supporting the view that the α-PSyn(S6) antiserum could be specific for S6-phosphorylated synapsin. The lowest band observed for α-Syn development represents synapsin isoforms not phosphorylated at S6 (Figure 4.5), whereas most or all isoforms of the upper two bands seem to be phosphorylated at serine 6. The large number of spots differing in their isoelectric point suggests that *Drosophila* synapsins may be phosphorylated differentially at multiple sites (see Discussion). The question of antiserum specificity and isoform characterization was further investigated using transgenic animals.

4.1.2 Transgenic synapsin mutants

Short name	Construct (see Methods and Michels *et al.,* 2011)
S6A-S533A	UAS- *Syn* cDNA PKA1 and PKA2 sites mutated
non-edited, S533A	UAS- *Syn* cDNA PKA1 site non-edited, PKA2 site mutated
non-edited	UAS- *Syn* cDNA PKA1 site non-edited, PKA2 site wild-type
edited	UAS- *Syn* cDNA PKA1 site edited, "rescue construct"

Table 4.2- Transgenic lines containing modified synapsin cDNA constructs under UAS control in Syn^{97} background.

The Gerber group (Michels *et al.,* 2005; 2011; Diegelmann *et al.,* 2013) has generated the following transgenic flies containing mutated synapsin cDNA constructs under the control of the yeast enhancer UAS that can be activated by the yeast transcription factor Gal4 (Table 4.2). To obtain animals expressing only this defined synapsin isoform, flies with the mutated cDNA construct were crossed to the line elav-Gal4;;Syn^{97}, a pan-neural Gal4 driver line, also in Syn^{97} null mutant background (stocks kindly provided by the Gerber group). These four transgenic lines were tested with the phospho-antisera.

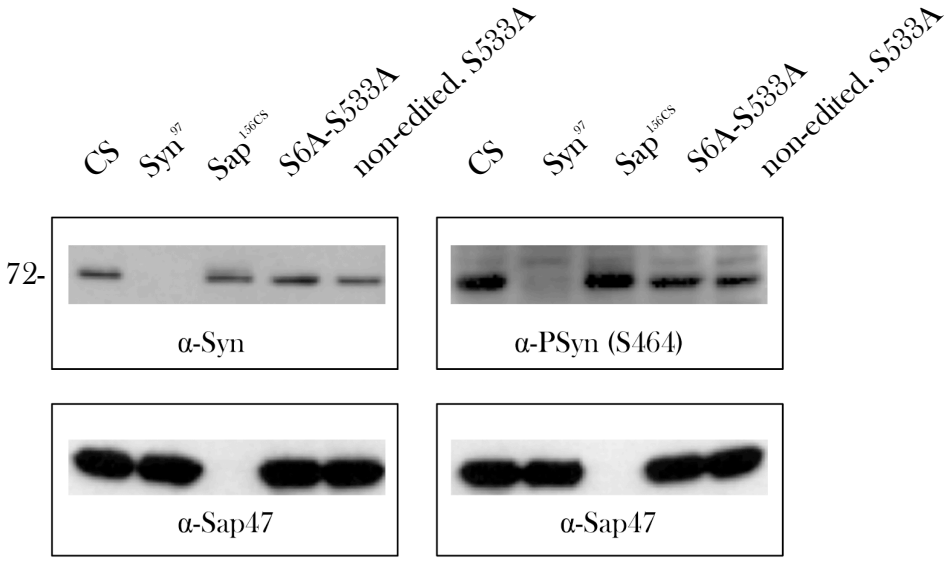


Figure 4.6. Transgenic flies in Syn^{97} background expressing pan-neurally (elav-Gal4) a synapsin cDNA with S6A and S533A mutations or the non-edited form detected with PSyn(S464) antiserum and Synapsin antibody. Western blot of head homogenate using a 12% precast gel for detection of synapsin phosphorylated at S464. The positive control with anti-Syn (mAb 3C11) (diluted 1:200) is shown in the left part of the blot, the right part of the blot was developed with PSyn(S464) antiserum (diluted 1:500). The loading control with anti-Sap47 (mAb nc46) (diluted 1:200) is

shown in the bottom part of the blot. In the mutated cDNAs, the conserved N-terminal PKA/CaMKII recognition site RRFS, which is edited in most mRNAs to RGFS (consensus for recognition of CaMKII but not PKA), is modified. Mutated cDNAs include S6A, S533A and the non-edited cDNA encoding RRFS. CS: Canton S wild type flies; Syn^{97}: synapsin mutant Syn^{97}; Sap^{156CS}: Sap mutant $Sap47^{156CS}$; S6A-S533A: UAS-Syn cDNA edited PKA site 1 (S6) and PKA site 2 (S533) mutated and non-edited, S533A: UAS- Syn cDNA PKA site 1 (S6) non-edited and PKA site 2 (S533) mutated (2 heads per lane).

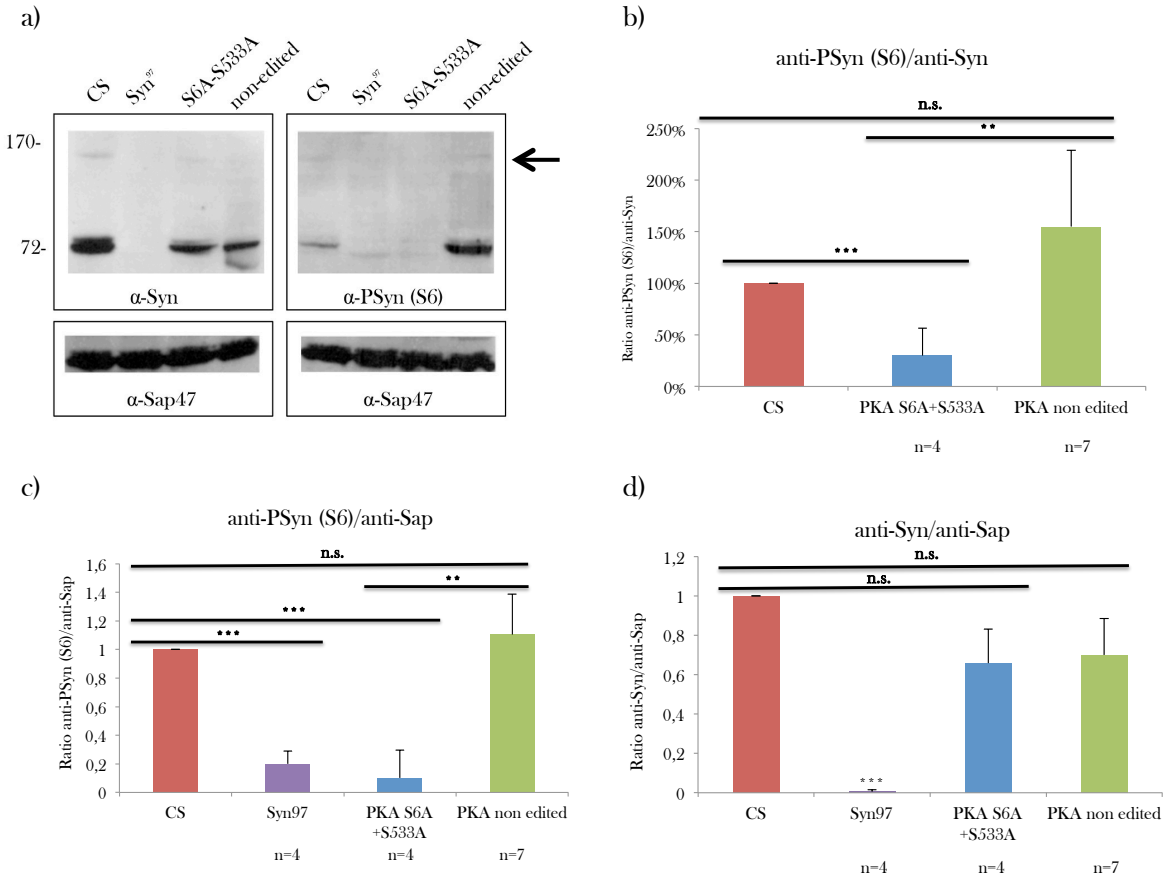


Figure 4.7. Transgenic flies in Syn^{97} background expressing pan-neurally (elav-Gal4) a synapsin cDNA with S6A and S533A mutations or the non-edited form detected with PSyn(S6) antiserum and Synapsin antibody. a) Western blot of head homogenate using a 12% precast gel for detection of synapsin phosphorylated at S6. The loading control with anti-Sap47 (mAb nc46) (diluted 1:200) is shown in the bottom part of the blot. The positive control with anti-Syn (mAb 3C11) (diluted 1:200) is shown in the upper left part of the blot, the upper right part of the blot was developed with PSyn(S6) antiserum (diluted 1:200). Transgenic lines are defined in Table 4.2 (2 heads per lane). **b)** Quantification of the relative phosphorylation as revealed by the signal ratio of PSyn(S6) antiserum (1:200) and anti-Syn antibody (1:200) normalized to CS. **c)** Quantification of the synapsin phosphorylation at S6 using PSyn(S6) antiserum (1:200) relative to the loading control (Sap47 signal), normalized to CS. The amount of phosphosynapsin at S6 site is reduced in the transgenic flies S6A-S533A and in the synapsin mutants, Syn^{97}. **d)** Quantification of total synapsin relative to total Sap47 using anti-Syn and anti-Sap antibodies (1:200), normalized to CS. The amount of synapsin is reduced only in synapsin mutants. (*P<0.05, **P<0.01, ***P<0.001; mean ± s.d, from four to seven independent experiments, one-way

ANOVA, n.s. not significant. Error bars indicate standard deviations). Notice that the signal for Syn^{97} null mutants in the PSyn(S6) quantification is due to the background of the blot.

As seen in the Western blots above (Figure 4.6), no qualitative difference could be observed between α-Syn and α-PSyn(S464) synapsin detection in the CS or synapsin transgenic flies. Under the culture conditions used (25°C) the expression level of the transgenes are similar to that of the wild-type gene. On the other hand, the antiserum against phosphorylated serine 6 recognizes small and large isoforms in wild-type, but not in the null mutant Syn^{97} nor in transgenic flies expressing a cDNA with S6A mutation in Syn^{97} background (Figure 4.7). The reduced anti-PSyn(S6)/anti-Syn ratio for S6A mutants in the quantification demonstrates again the specificity of the PSyn(S6) antiserum. The transgenic flies expressing a cDNA with S6A mutation show no significant synapsin phosphorylation at serine 6, highly significantly less than CS or the transgenic flies with the non-edited cDNA ($P<0.01$, one-way ANOVA). The higher level of S6 phosphorylation in flies expressing the non-edited synapsin ($P<0.05$, one-way ANOVA) may be due to enhanced phosphorylation by PKA (Figure 4.7 b).

Wild-type flies and the transgenics containing a Gal4 driver and a UAS construct do not share the same genetic background. We therefore decided to compare S6 phosphorylation in the mutant transgenic flies and flies expressing the edited wild-type synapsin cDNA (rescue construct UAS-Syn^{8},Syn^{97}) under UAS control using the same pan-neural Gal4 driver (elav Gal4). Again, the signal ratio of α-PSyn(S6) and α-Syn is significantly larger for the flies expressing the non-edited synapsin compared to those with the edited wild-type rescue contruct (Figure 4.8 a and quantification in 4.8 b).

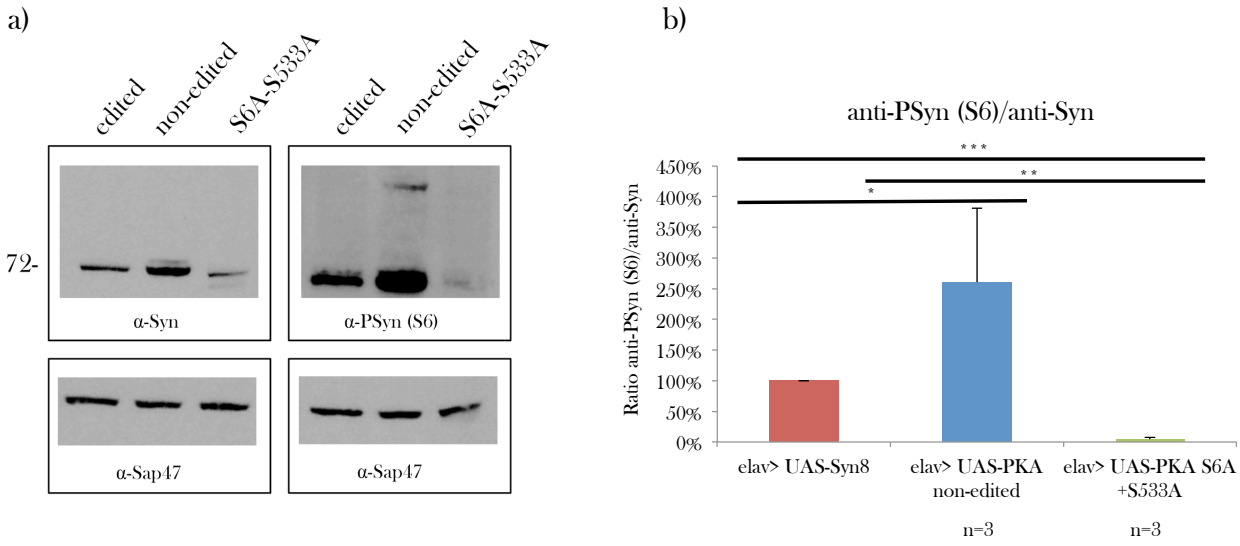


Figure 4.8. Comparison of the phosphorylation status of the synapsin isoform expressed from the edited wild-type cDNA rescue construct and from mutated isoforms, all in Syn^{97} background. a) Western blot of head homogenate using a 12% precast gel for detection of synapsin phosphorylated at S6. The loading control with anti-Sap47 (mAb nc46) (diluted 1:200) is shown in the bottom part of the blot. The positive control with anti-Syn (mAb 3C11) (diluted 1:200) is shown in the left part of the blot, the right part of the blot was developed with PSyn(S6) antiserum (diluted 1:200). The transgenic lines are defined in Table 4.2 (2 heads per lane). **b)** Quantification of the relative phosphorylation as revealed by the signal ratio of PSyn(S6) antiserum (1:200) and anti-Syn antibody (1:200) normalized to CS. (*P<0.05, **P<0.01, ***P<0.001; mean ± s.d, from three independent experiments, one-way ANOVA. Error bars indicate standard deviations).

Since the PSyn(S464) antiserum does not show any qualitative differences compared to the α-Syn antibody (Figure 4.6), we decided to focus the study and analyze differences only with the PSyn(S6) antiserum. The Western blot comparing the edited wild-type synapsin isoform expressed from the rescue construct and the isoforms expressed from the cDNA with S6A and S533A mutation or from the non-edited cDNA (Figures 4.7 and 4.8) demonstrates again the specificity of the PSyn(S6) antiserum. A signal at the expected position is detected by this antiserum in the synapsin rescue line but it is absent in the transgenic flies with the S6A mutated isoform. The non-edited form apparently is hyperphosphorylated as indicated by the higher α-PSyn(S6)/α-Syn signal ratio compared to the rescue isoform (Figure 4.8).

4.1.3 The role of PKA

Genotype	Type of allele	Reference	Source
P{w[+mC]=UAS-PKA-C1.W224R.F}5.3.2, y[1] w[*]	Expresses a non-functional PKA-C1 protein (X chromosome)	Kiger et al., 1999	Bloomington 35556
y[1] w[*]; P{w[+mC]=UAS-PKA-C1.K75A}46.7	Expresses PKA-C1 with no catalytic activity (3rd chromosome)	Kiger and O'Shea, 2011	Bloomington 35559
UAS PKA-C1 RNAi	RNAi against PKA- C1 CG4379 (2nd chromosome)	-	VDRC – 101524 KK

Table 4.3- Transgenic lines containing modified PKA-C1 under UAS control.

To investigate the influence of PKA (Protein Kinase A) on synapsin phosphorylation, we decided to examine differences in synapsin phosphorylation at S6 position in transgenic fly lines expressing PKA-C1 RNAi, PKA-C1 with no catalytic activity or PKA-C1 non-functional (Table 4.3).

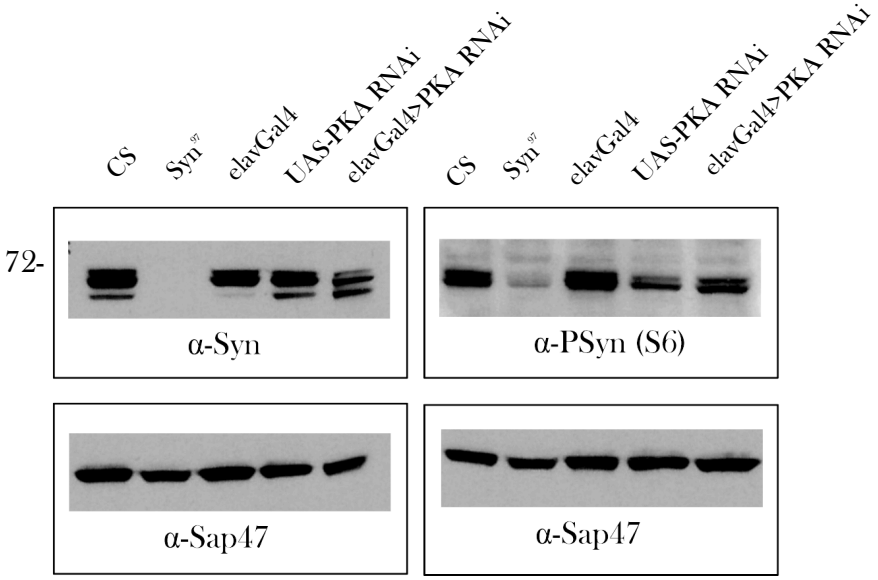


Figure 4.9. Synapsin phosphorylation at S6 in transgenic flies expressing panneurally a PKA-C1 RNAi construct. Western blot of head homogenate using a 12% precast gel for detection of phosphorylated synapsin at S6 (site 1). The right part of the blots was developed with PSyn(S6) (diluted 1:200) antiserum; the positive control with anti-Syn (mAb 3C11) (diluted 1:200) is shown in the left part of the blot. The loading control with anti-Sap47 (mAb nc46) (diluted 1:200) is shown in the bottom part of the blot. CS: Canton S wild type flies; Syn^{97}: synapsin mutant Syn^{97}; elavGal4: elav Gal4 (X) and UAS-PKA RNAi: UAS -PKA C1 RNAi (II) (2 heads per lane).

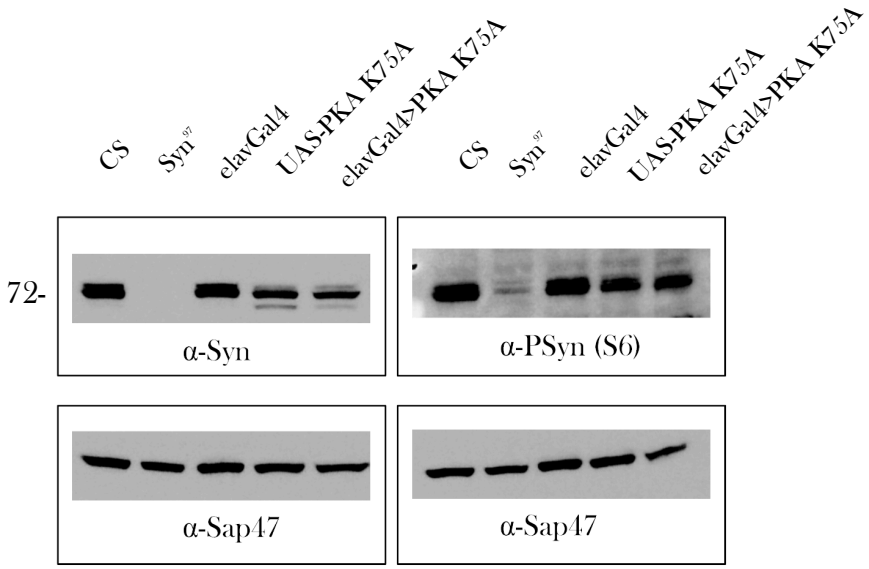


Figure 4.10. Synapsin phosphorylation at S6 in transgenic flies expressing panneurally PKA C1 with no catalytic activity. Western blot of head homogenate using a 12% precast gel for detection of phosphorylated synapsin at S6 (site 1). The right part of the blots was developed with PSyn(S6) (diluted 1:200) antiserum; the positive control with anti-Syn (mAb 3C11) (diluted 1:200) is shown in the left part of the blot. The loading control with anti-Sap47 (mAb nc46) (diluted 1:200) is shown in the bottom part of the blot. CS: Canton S wild type flies; Syn^{97}: synapsin mutant Syn^{97}; elavGal4: elav Gal4 (X) and UAS-PKA K75A: UAS -PKA C1 K75A (III) (2 heads per lane).

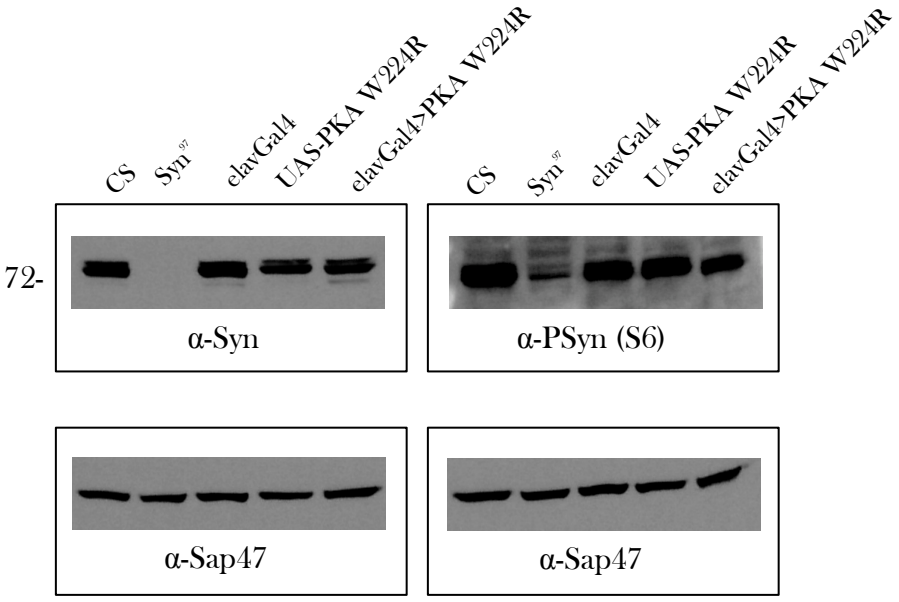


Figure 4.11. Synapsin phosphorylation at S6 in transgenic flies expressing panneurally PKA C1 non-functional. Western blot of head homogenate using a 12% precast gel for detection of phosphorylated synapsin at S6 (site 1). The right part of the blots was developed with PSyn(S6) (diluted 1:200) antiserum; the positive controls with anti-Syn (mAb 3C11) (diluted 1:200) is shown in the left part of the blot. The loading control with anti-Sap47 (mAb nc46) (diluted 1:200) is shown in the bottom part of the blot. CS: Canton S wild type flies; Syn^{97}: synapsin mutant Syn^{97}; elavGal4: elav Gal4 (X) and UAS-PKA W224R: UAS -PKA C1 W224R (X) (2 heads per lane).

The Western blots (Figure 4.9, 4.10 and 4.11) illustrate the variability of the detection of the 70 kDa isoform by the α-Syn antibody both between different fly lines and between experiments (see Discussion). Expressing the PKA-C1 RNAi construct (Figure 4.9) seems to reduce synapsin phosphorylation both in the UAS PKA-C1 RNAi parental line (leaky expression) and in F1 after the cross with elav Gal4 (pan-neural expression). Panneural overexpression of a PKA-C1 subunit with no catalytic activity or PKA-C1 non-functional apparently does not significantly alter the phosphorylation of synapsin at S6. The fact that the PKA consensus motif at S6 (RRFS) is edited in most synapsin mRNAs (Diegelmann *et al.,* 2006) could perhaps explain the similarity between the wild-type or the parents and the transgenic progeny (see Discussion).

4.1.4 The role of CaMKII

The amino acid sequence of *Drosophila* synapsin PKA site 1 (S6) (irrespective of editing) and site 2 (S533) both conform to the consensus target sequence for vertebrate CaMKII (RxxS). In addition, however the S464 site also conforms to this sequence. Therefore, the influence of transgenic expression of the CaMKII inhibitory peptide ala on the Western blot signals of the phospho-specific antisera of PSyn(S6) (site 1) or PSyn(S464) (site 2) was tested. As described in the introduction, the UAS-CaMKII Ala expresses a peptide analog of the autoinhibitory domain of CaMKII with T287A mutation. In the UAS-CaMKII T287A expressing transgenic flies, CaMKII activity is reduced because of a substitution of an alanine in place of threonine at position 287 that corresponds to the target domain for autosphosphorylation required for CaMKII activation (Griffith *et al.,* 1993).

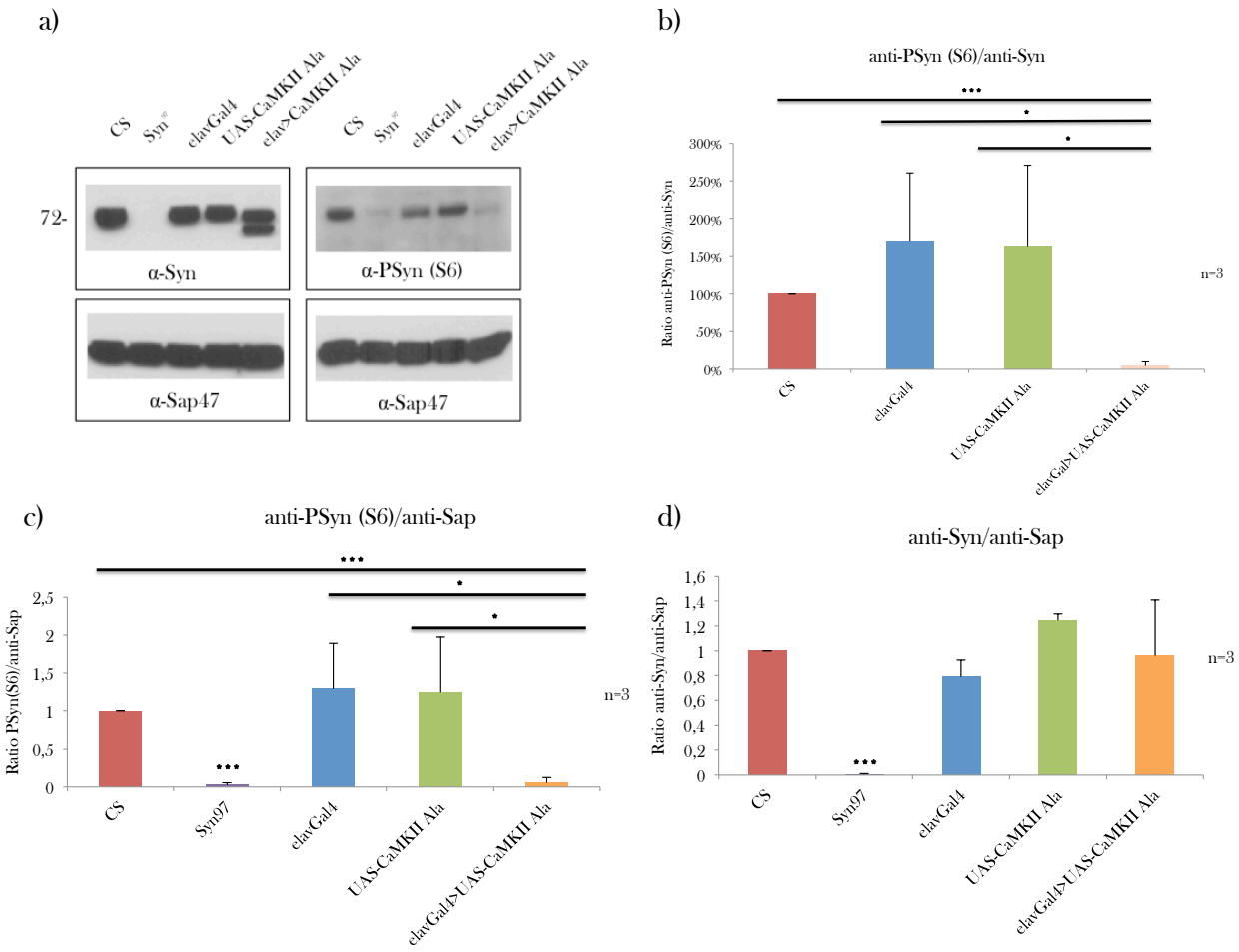


Figure 4.12. Evidence for CaMKII-dependent synapsin phosphorylation at the S6 site using UAS-CaMKII-Ala, an inhibitory peptide for CaMKII. a) Western blot of head homogenate using a 12% precast gel for detection of phosphorylated synapsin at S6 (site 1). The right part of the blot was developed with PSyn(S6) (diluted 1:200) antiserum; the positive control with anti-Syn (mAb 3C11) (diluted 1:200) is shown in the left part of the blot. The loading control with anti-Sap47 (mAb nc46) (diluted 1:200) is shown in the bottom part of the blot. CS: Canton S wild type flies; Syn^{97}: synapsin mutant Syn^{97}; elavGal4: elav Gal4 (X) and UAS-CaMKII Ala: inhibitory peptide for CaMKII under UAS control expression (2 heads per lane). **b)** Quantification of the relative phosphorylation as revealed by the ratio between PSyn(S6) antiserum (1:200) and anti-Syn antibody, normalized to CS. **c)** Quantification of the synapsin phosphorylation at S6 relative to the loading control using PSyn(S6) antiserum and anti-Sap antibody (1:200). The amount of phosphosynapsin at S6 site is reduced in the transgenic elavGal4>UAS-CaMKII Ala and synapsin mutants. **d)** Quantification of total synapsin relative to Sap47 using anti-Syn and anti-Sap antibodies (1:200). The amount of synapsin is reduced only in synapsin mutants. (*P<0.05, **P<0.01, ***P<0.001; mean ± s.d, from three independent experiments, one-way ANOVA. Error bars indicate standard deviations).

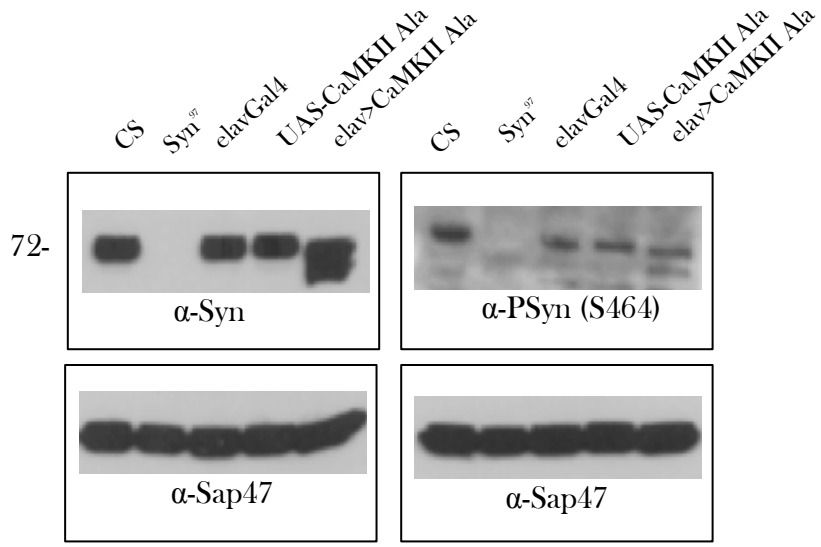


Figure 4.13. No evidence for CaMKII-dependent synapsin phosphorylation at the S464 site using UAS-CaMKII-Ala, an inhibitory peptide for CaMKII. Western blot of head homogenate using a 12% precast gel for detection of phosphorylated synapsin at S464. The right part of the blot was developed with PSyn(S464) (diluted 1:500) antiserum; the positive control with anti-Syn (mAb 3C11) (diluted 1:200) is shown in the left part of the blot. The loading control with anti-Sap47 (mAb nc46) (diluted 1:200) is shown in the bottom part of the blot. CS: Canton S wild type flies; Syn^{97}: synapsin mutant Syn^{97}; elavGal4: elav Gal4 (X) and UAS-CaMKII Ala: inhibitory peptide for CaMKII under UAS control expression (2 heads per lane).

The expression of the ala inhibitory peptide for CaMKII significantly increases the amount of the 70 kDa synapsin isoform (Figures 4.12-4.13). In addition, it reduces the phosphorylation at S6 (Figure 4.12) but not at S464 (Figure 4.13). The transgenic flies expressing an inhibitory peptide for CaMKII show more than 80% downregulated synapsin phosphorylation ($P<0.05$, one-way ANOVA, n=3). On the other hand, the parents of this progeny do not show any reduction in synapsin phosphorylation at S6 compared to wild-type (Figure 4.12 b). Accordingly, CaMKII could phosphorylate serine in position 6 (see Discussion).

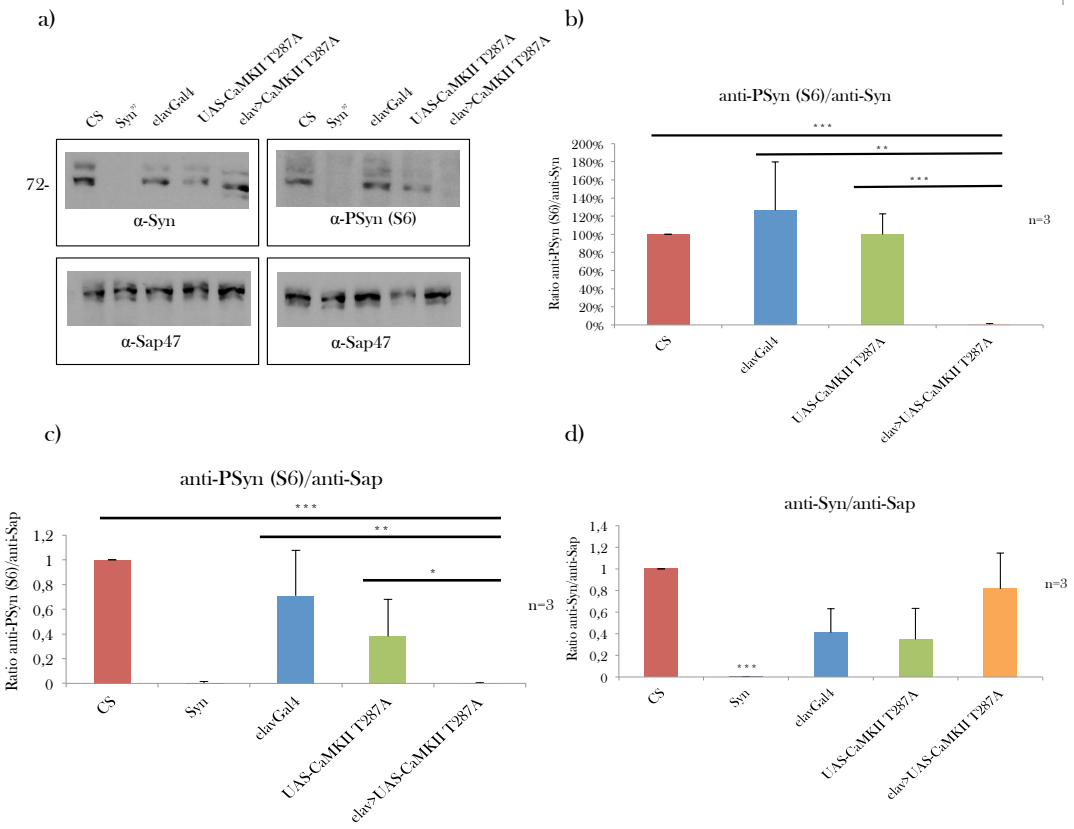


Figure 4.14. Further evidence for CaMKII-dependent synapsin phosphorylation at the S6 site using UAS-CaMKII-T287A construct. a) Western blot of head homogenate using a 12% non-precast gel for detection of phosphorylated synapsin at S6. The right part of the blot was developed with PSyn(S6) (diluted 1:200) antiserum; the positive control with anti-Syn (mAb 3C11) (diluted 1:200) is shown in the left part of the blot. The loading control with anti-Sap47 (mAb nc46) (diluted 1:200) is shown in the bottom part of the blot. CS: Canton S wild type flies; Syn^{97}: synapsin mutant Syn^{97}; elavGal4: elav Gal4 (X) and UAS-CaMKII T287A: mutation on CaMKII at the position T287 under UAS control expression (2 heads per lane). **b)** Quantification of the relative phosphorylation as revealed by the ratio between PSyn(S6) antiserum (1:200) and anti-Syn antibody (1:200), normalized to CS. **c)** Quantification of the synapsin phosphorylation at S6 relative to the loading control using PSyn(S6) antiserum and anti-Sap antibody (1:200). The amount of phosphosynapsin at S6 site is reduced in the transgenic elavGal4>UAS-CaMKII T287A (F1) and synapsin mutants. **d)** Quantification of total synapsin relative to Sap47 using anti-Syn and anti-Sap antibodies (1:200). The amount of synapsin is reduced only in synapsin mutants. (*P<0.05, **P<0.01, ***P<0.001; mean ± s.d, from three independent independent experiments, one-way ANOVA. Error bars indicate standard deviations).

The transgenic flies over-expressing a mutated form of CaMKII containing a change of threonine for alanine at position 287 show more than 80% downregulated synapsin phosphorylation at S6 ($P<0.05$, one-way ANOVA, n=3) (Figure 4.14). CaMKII inhibitory peptide and CaMKII mutated at the autophosphorylation site (T287A) have the same effect in synapsin phosphorylation at S6. This supports the view that CaMKII might be involved in synapsin phosphorylation at S6 position (see Discussion).

4.1.5. Age studies

As it will be discussed later, the increase in PKA-dependent signaling was shown to be involved in age-related memory impairment in *Drosophila melanogaster* (Yamazaki *et al.*, 2010). We decided to study if this observation might correlate with an increase in synapsin phosphorylation during aging. Western blots of young wild-type flies (between 1-4 days old) and old wild-type flies (between 22 and 31 days old) from the same vial were tested for differences in phosphorylation. For this study, different experimental conditions were used in order to establish a reproducible protocol. In some experiments young flies as well as old flies were frozen at -80°C to preserve their phosphorylation status. Other experiments with flies from different vials using CO_2 instead of freezing or separating males and females were also performed. The flies were transferred from the old vial to the new vial simultaneously every second day.

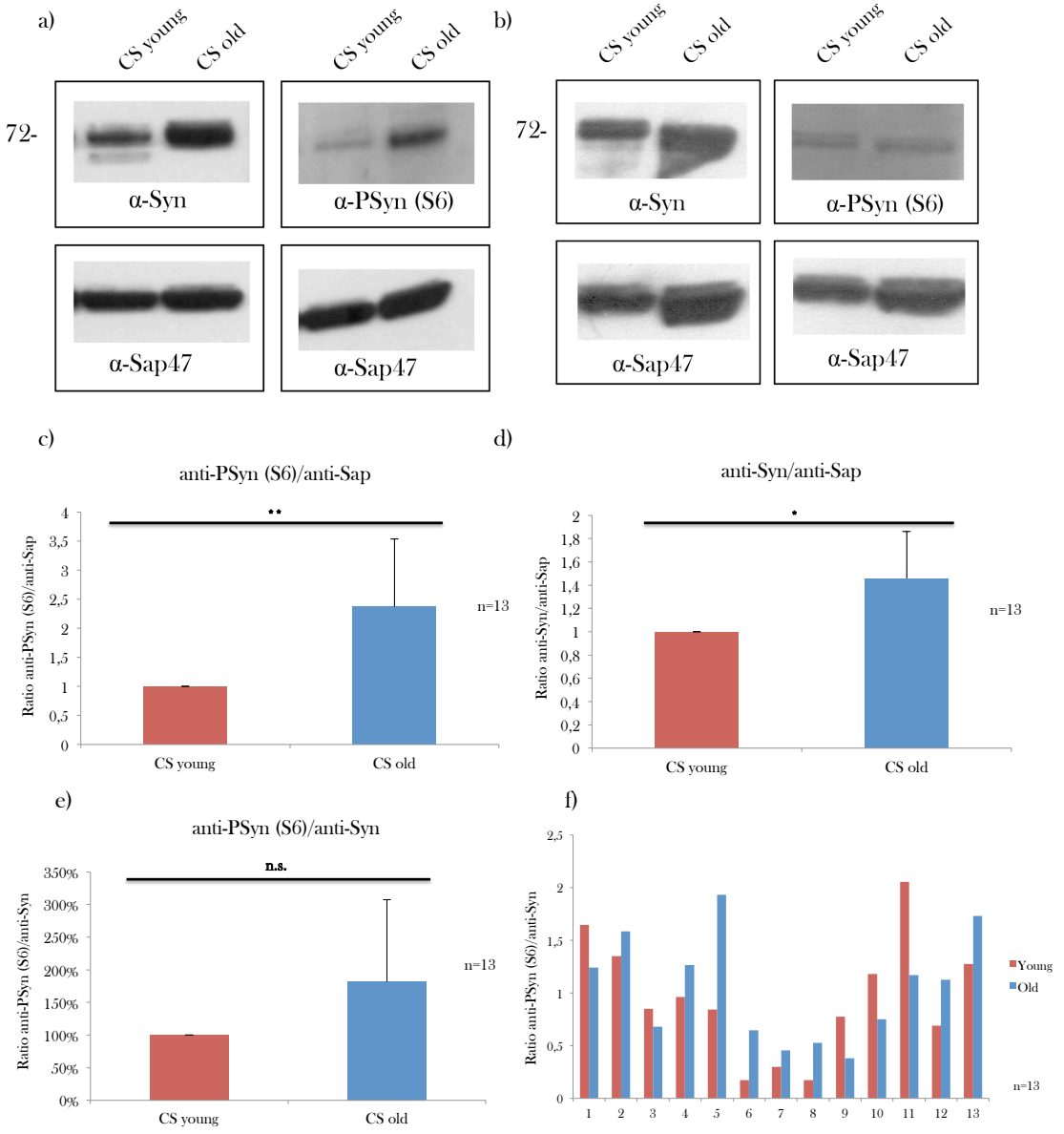


Figure 4.15. Test for differences in phosphorylation in young (1-4 days old) and old (22-31 days old) flies at the S6 site. a, b) Differences in synapsin phosophorylation at S6 in old flies in two different Western blots of head homogenate using a 12% precast gel for detection of phosphorylated synapsin at S6. The right part of the blots was developed with PSyn(S6) (diluted 1:200) antiserum; the positive controls with anti-Syn (mAb 3C11) (diluted 1:200) is shown in the left part of the blots. The loading control with anti-Sap47 (diluted 1:200) is shown in the bottom part of the blot. CS: Canton S wild type flies (2 heads per lane). **c)** Quantification of the synapsin phosphorylation at S6 using PSyn(S6) antiserum (1:200). The amount of phosphosynapsin at S6 site is increased in the old flies. **d)** Quantification of total synapsin using anti-Syn antibody (1:200). The amount of synapsin is also increased in old flies. **e)** Quantification of the phosphorylation as revealed by the signal ratio between PSyn(S6) antiserum (1:200) and anti-Syn antibody (1:200). **f)** Phosphorylation signal ratios between PSyn(S6) and anti-Syn in old (22-31 days old) and young (1-4 days old) flies in thirteen different experiments averaged in (e). The phosphorylation between young and old flies is highly variable between different experiments. (*P<0.05, **P<0.01, ***P<0.001; mean ± s.d, from thirteen independent experiments, one-way ANOVA. n.s. not significant. Error bars indicate standard deviations).

The Western blots and the quantification (Figure 4.15) showed a significant increase in the phosphorylation at S6 position in old compared to young flies. However, when we look at the signal from the anti-Syn antibody, which recognizes all synapsin isoforms independent of their phosphorylation, as well as the protein Sap47 used as a loading control, we also see a significant increase. But when analyzing the ratio between phosphosynapsin and total synapsin, the increase in phosphorylation in old flies is not significant. The phosphorylation is very variable between the different independent experiments.

4.1.6 Behavioral analysis

[Most of these results have been published in the manuscript "Sadanandappa, **Blanco Redondo** *et al.*, Synapsin function in GABA-ergic interneurons is required for short-term olfactory habituation. The Journal of Neuroscience, 16 October 2013, 33 (42): 16576-16585; doi:10.1523/JNEUROSCI.3142-13.2013]

It is known that synapsin null mutants (Syn^{97}) are viable and fertile, but several phenotypes affecting complex behavior have been described (Godenschwege *et al.,* 2004). The Y-maze (see Methods) is a very sensitive method to test the olfactory behavior in adult flies. With the aim of studying the role of synapsin in short-term olfactory habituation (STH), the next experiments were performed.

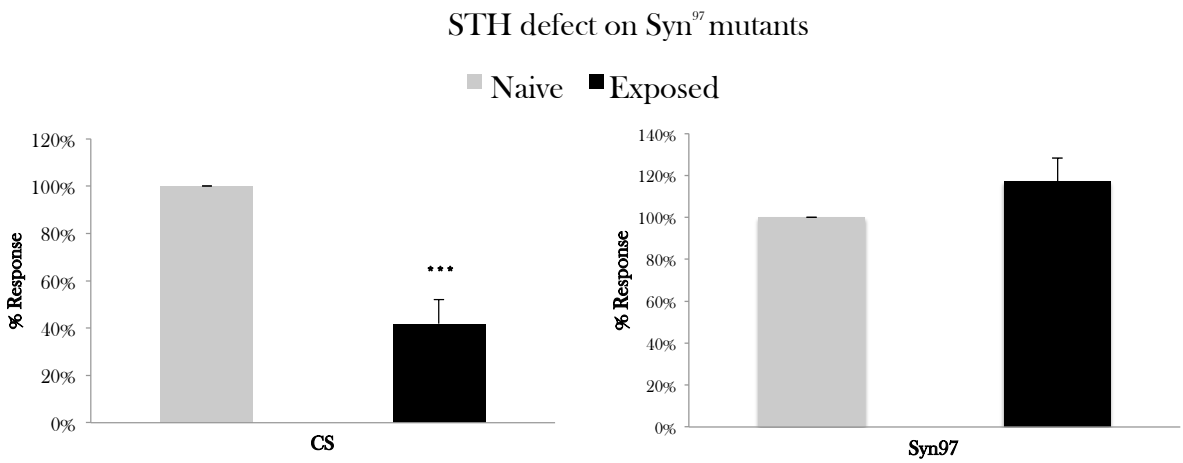
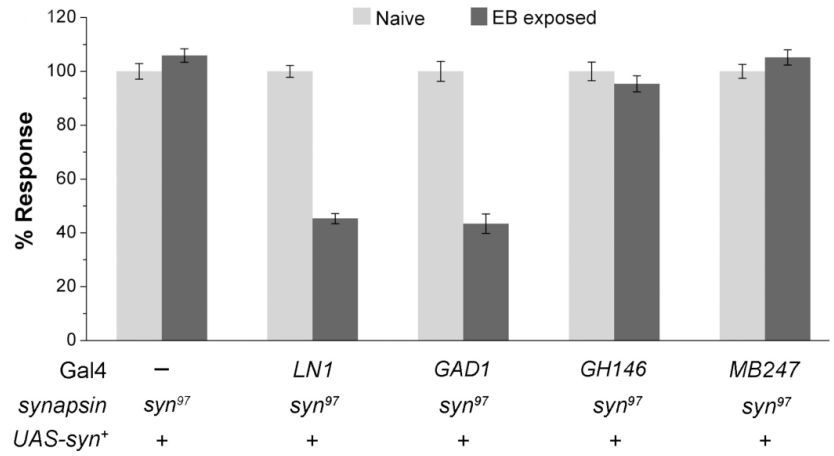


Figure 4.16. Synapsin null mutants (syn^{97}) are defective for STH. Histogram showing the efficiency of STH for CS and *syn*^{97} flies to EB. Light grey bars correspond to the naïve response to a 10^{-3} dilution of EB and the dark grey bars depict the corresponding behavioral response after 30 minutes of 5% EB exposure. *** represents *p*-value <0.001, calculated using Student's *t*-test with Bonferoni correction. Bars indicate mean ± SEM. N is 3 to 6 sets of flies for each bar.

In wild-type *Drosophila,* 30 minutes of odorant exposure (5% EB or 15% CO_2) results in the odorant specific decrement of olfactory avoidance in the Y-maze that recovers within an hour (Figure 4.16) (Das *et al.,* 2011). This phenomenon of short-term olfactory habituation (STH) is lost in Syn^{97} mutants, indicating that synapsin is necessary for the establishment of this form of non-associative memory.

Drosophila synapsin is phosphorylated at least at seven sites identified by mass spectrometry and verified by phosphatase treatment (Nuwal *et al.,* 2010). As we have seen, two additional sites, motif 1 in the conserved "A" domain and motif 2 near the "E" domain, conform to both the PKA (RRxS) and the CaMKII (RxxS) consensus motifs (Klagges *et al.,* 1996; Diegelmann *et al.,* 2006). We tested whether these two potential phosphorylation sites are essential for synapsin function in STH, using a transgenic synapsin construct mutated at these predicted kinase-phosphorylation sites.

a)



b)

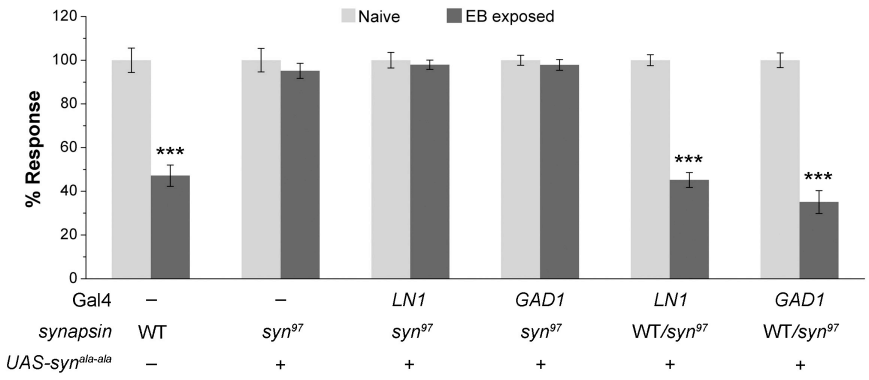


Figure 4.17. Synapsin phosphorylation sites S6 and/or S533 is essential for STH formation. a) Transgenic expression of synapsin in LN1 or GAD1 subsets of neurons rescues the *syn*^{97} STH defect. **b)** Rescue expression of synapsin with mutated motifs 1 and 2 (S6A, S533A) (serine to alanine) in LN1 GABA-ergic olfactory local interneurons (LN1) or most GABA-ergic neurons (GAD1) fails to rescue the *syn*^{97} defect for STH. Olfactory avoidance in a Y-maze before and after odorant exposure are represented as light grey and dark grey bars, respectively, along with mean ± SEM. N for each bar is 8 – 11 sets. *** represents *p*-value <0.001, calculated using Student's *t*-test with Bonferoni correction. Bars indicate mean ± SEM. (Experiment done by Madhumala K. Sadanandappa).

This phosphorylation of synapsin appears to be necessary for its role in LN1 or GAD1 expressing GABAergic neurons during STH. Thus, while expression of a wild-type synapsin transgene construct in these cells rescues the Syn^{97} defect in STH (Figure 4.17 a), expression of the UAS-syn$^{ala\text{-}ala}$ construct (previously named also as UAS-Syn cDNA PKA S6A+S533A) in either local LN1 GABAergic interneurons (LN1-Gal4> UAS-syn$^{ala\text{-}ala}$,syn^{97}/syn^{97}) or in GABAergic neurons (GAD1-Gal4> UAS-syn$^{ala\text{-}ala}$,syn^{97}/syn^{97}) has no effect on the syn^{97} defects in STH to EB or CO_2 (Figure 4.17 b), although mutant and wild-type transgenes are expressed at comparable levels . Intact STH in heterozygotes

indicates that the effect seen in the transgenic homozygotes flies is not a dominant negative effect due to the toxicity of the mutation.

The PKA target consensus RRxS also conforms to the CaMKII target consensus RxxS, which is particularly relevant since mammalian synapsin is a known substrate for CaMKII, although at different site (Kao *et al.,* 1999; Hilfiker *et al.,* 2005). We therefore first tested whether *Drosophila* synapsin could be a substrate for CaMKII (as seen in Figure 4.12 and 4.13) and then tested whether CaMKII activity is necessary for behavioral habituation.

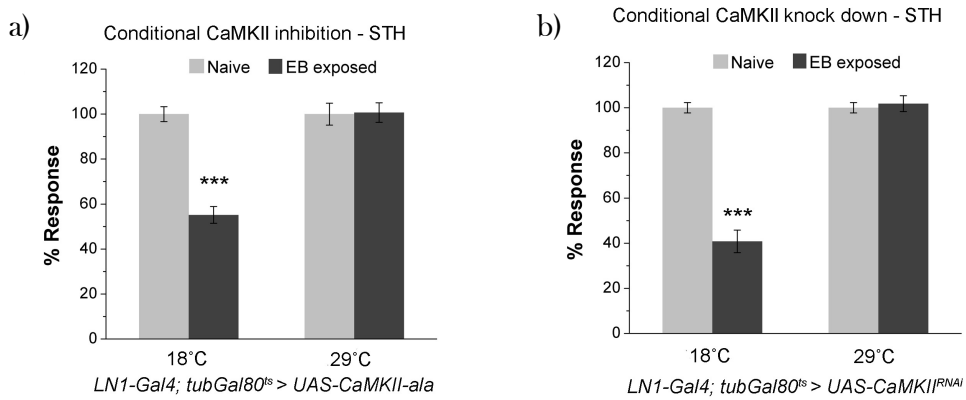


Figure 4.18. CaMKII dependent phosphorylation in LN1 neurons is necessary for STH. Under conditions of **a)** over-expression of CaMKII-inhibitory ala peptide (LN1-Gal4; tub-Gal80^{ts}> UAS-CaMKII-ala) and **b)** knock down of CaMKII (LN1-Gal4; tub-Gal80^{ts}> UAS-CaMKII^{RNAi}) in LN1 neurons of the antennal lobe, flies fail to show STH at 29˚C, whereas flies maintained at the permissive temperature (18˚C) displayed normal STH. Light grey and dark grey bars correspond to naïve and post-odor exposure responses repectively, measured for 10^{-3} dilution of EB. *** represents p-value <0.001, calculated using Student's t-test with Bonferoni correction. Bars indicate mean ± SEM. (Experiment done by Madhumala K. Sadanandappa).

Using the tub-Gal80^{ts} conditional expression system, we expressed the CaMKII inhibitory-peptide in the LN1 subset of local interneurons (LN1-Gal4; tub-Gal80^{ts}> UAS-CaMKII-ala). When raised, maintained and tested at a temperature permissive for Gal80^{ts} function (18˚C), flies displayed normal STH. However, when the 18˚C - raised adult flies were shifted to 29˚C for four days to induce the CaMKII inhibitor-peptide in LN1 neurons, flies showed significantly reduced or absent STH compared to controls (Figure 4.18 a). The conclusion that CaMKII is necessary in LN1 neurons for STH is further supported by the observation that RNAi-mediated knockdown of CaMKII in LN1 cells

has the same effect as the CaMKII inhibitory peptide expression. Thus, LN1-Gal4; tub-Gal80^{ts}> UAS-CaMKII^{RNAi} flies reared at 18˚C and shifted to 29˚C for 4 days, showed no detectable STH (Figure 4.18 b).

4.1.7 Distribution of synapsin phosphorylated at S6 site in the Drosophila brain

In order to determine where in the *Drosophila* brain synapsin isoforms phopshorylated at S6 are found, whole-mount preparations or cryostat sections of paraformaldehyde-fixed brains were stained using mAb 3C11 (anti-Syn) in combination with PSyn(S6) antiserum.

4.1.7.1 Wild-type flies

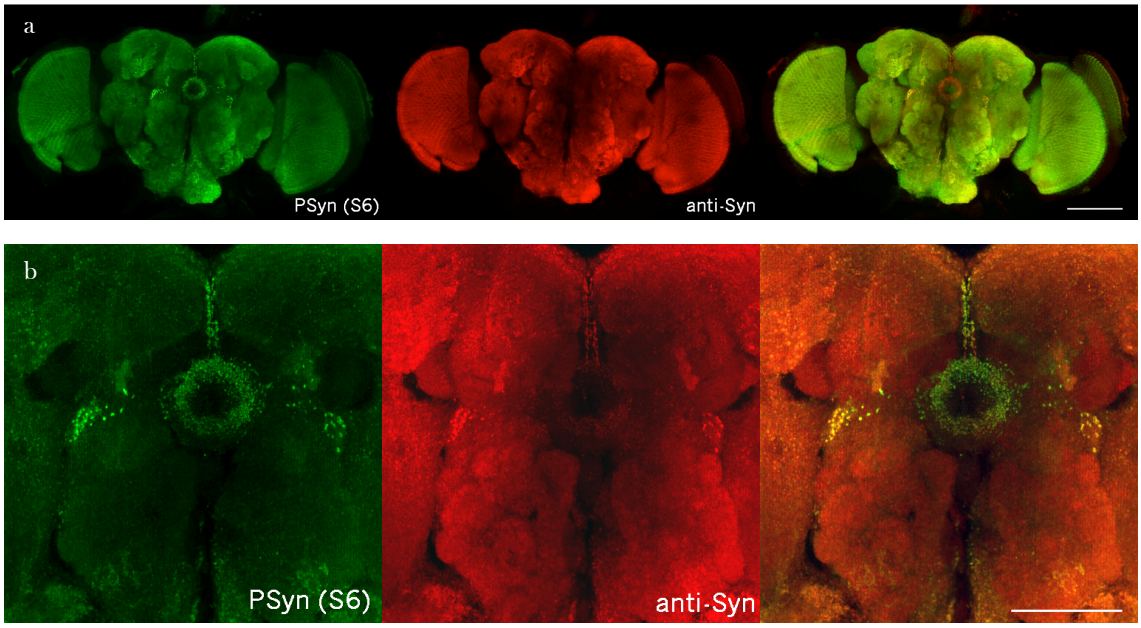


Figure 4.19. Z-projections of confocal stacks of a whole-mount adult wild-type *Drosophila* brains (frontal view) stained with anti-PSyn(S6) antiserum and anti-Syn (mAb 3C11) antibody. a) The overview reveals enhanced PSyn(S6) (green) staining in the ellipsoid body, whereas anti-Syn (red) stains the entire neuropil of the *Drosophila* brain (20x objective). **b)** Detail of anti-PSyn(S6) and anti-Syn staining at higher magnification (40x objective). PSyn(S6) is present at a high concentration in a subset of the ring neurons of the central complex (anti-PSyn(S6) 1:500; anti-Syn 1:20). Scale bar: 100 µm.

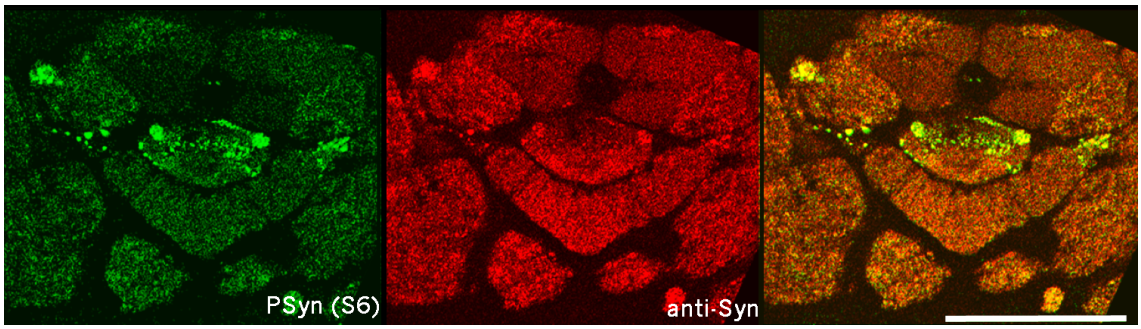


Figure 4.20. Horizontal cryostat section of a wild-type adult *Drosophila* brain shows the specific staining for anti-PSyn(S6) in the ellipsoid body. Sections were stained with mouse monoclonal antibody 3C11 (anti-Syn) (red) (diluted 1:50) and rabbit anti-PSyn(S6) antiserum (green) (diluted 1:300). Scale bar: 100 µm.

Synapsin isoforms phosphorylated at S6 are prominent in the ellipsoid body of *Drosophila* brain (Figures 4.19 and 4.20), but the functional meaning of the presence of synapsin isoforms phosphorylated at S6 prominently in the ellipsoid body of *Drosophila* brain remains to be determined.

4.1.7.2 Synapsin mutants

To test the specificity of the antiserum, stainings using Syn^{97} and Syn^{79} mutant flies were done.

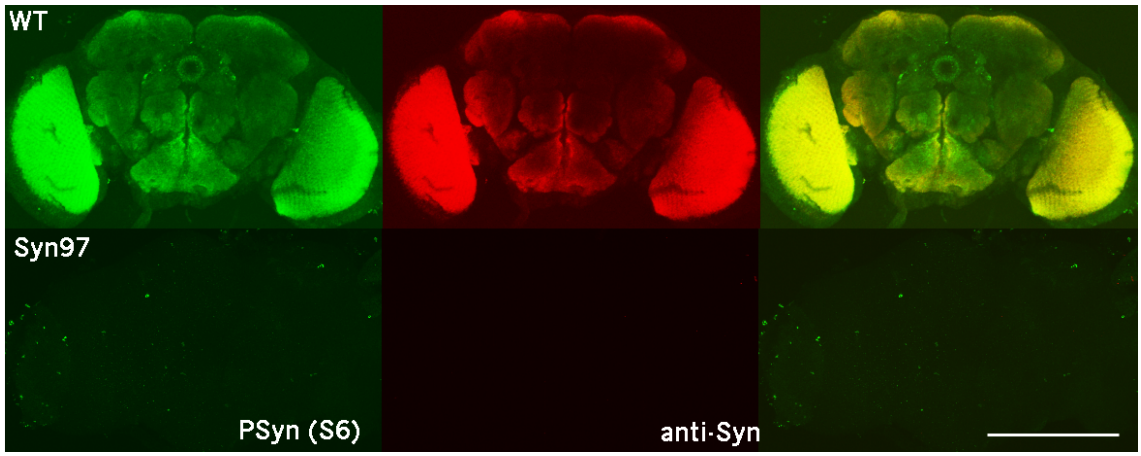


Figure 4.21. Z-projections of confocal stacks of whole-mount adult (synapsin knockout) Syn^{97} and wild-type *Drosophila* brain stained with PSyn(S6) antiserum and anti-Syn (mAb 3C11) antibody. The figure demonstrates the specificity of PSyn(S6) antiserum. An overview of anti-PSyn(S6) (green) and anti-Syn (red) detection in *Drosophila* brain (20x objective) (anti-PSyn(S6) 1:500; anti-Syn 1:20). Scale bar: 100 µm.

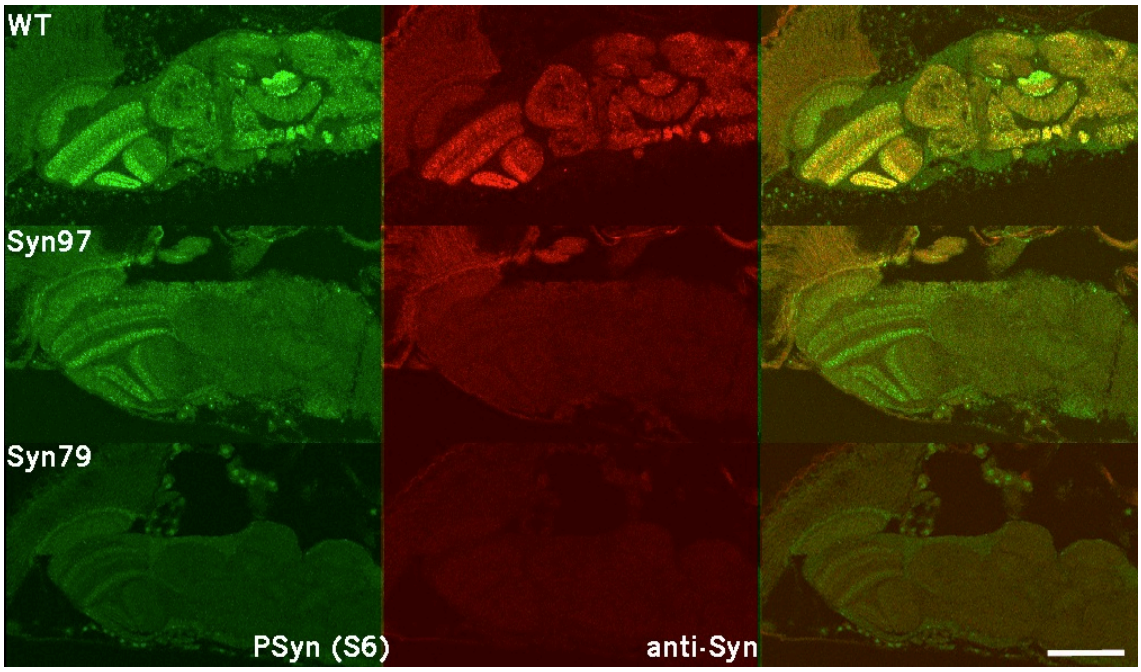


Figure 4.22. Horizontal cryostat sections of wild-type, Syn^{97} and Syn^{79} adult *Drosophila* brain. The staining demonstrates the specificity of PSyn(S6) antiserum for synapsin. Sections were stained with mouse monoclonal antibody 3C11 (anti-Syn) (red) (diluted 1:50) and rabbit anti-PSyn(S6) antiserum (green) (diluted 1:300). Scale bar: 100 μm.

In the Syn^{97} null mutant (red-eyed, outcrossed to CS) (Figure 4.22) weak staining in the optic lobe can be observed. This may be due to a cross-reaction of the antibody with other phosphoproteins or to autofluorescence of the eye pigment diffusing into the optic neuropil. The absence of staining in the eye and optic lobe of the white-eyed w;;Syn^{97} null mutant (Figure 4.22 bottom) indicates that the Syn^{97} null mutant staining was not specific for synapsin but due to the eye pigment in Syn^{97} null mutant. The whole mount staining also confirms the specificity of the antibody and the non-specificity of the staining in the eye and optic lobe in cryosections of Syn^{97} null mutant (Figure 4.21).

4.1.7.3 Synapsin transgenic flies

Transgenic flies in Syn^{97} background expressing pan-neurally (elav Gal4) cDNA with S6A and S533A mutations were used to prove the specificity of the staining.

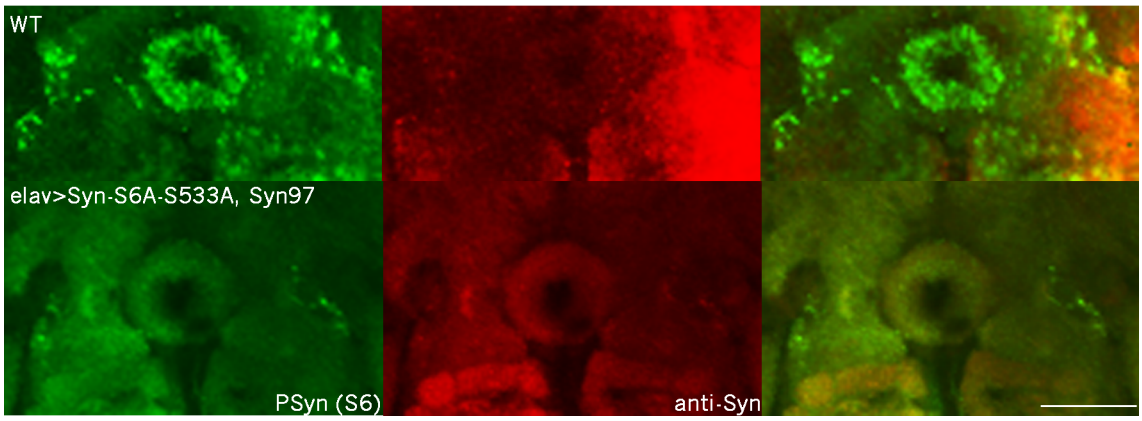


Figure 4.23. Confocal stacks of whole-mount of a transgenic fly in Syn^{97} background expressing pan-neurally (elav-Gal4) cDNA with S6A and S533A mutations compared to wild-type. The PSyn(S6) antiserum is specific for synapsin phosphorylated at S6. Detail of the central complex stained with anti-PSyn(S6) (green) and anti-Syn (red) in *Drosophila* brain (anti-PSyn(S6) 1:500; anti-Syn 1:20). Scale bar: 50 μm.

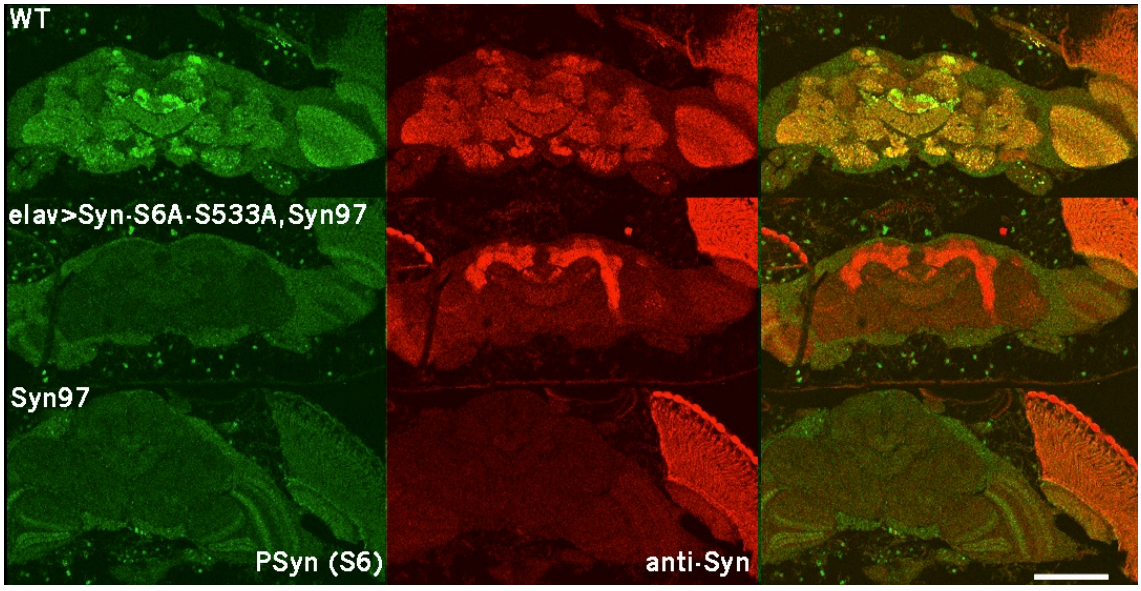


Figure 4.24. Horizontal cryosection of a wild-type, a transgenic fly in Syn^{97} background expressing pan-neurally (elav-Gal4) a cDNA with S6A and S533A mutations, and a Syn^{97} mutant. PSyn(S6) staining is absent in the subset of the ring neurons of the central complex in the transgenic fly with S6 mutated as well as in the Syn^{97} mutants. Sections were stained with mouse monoclonal antibody 3C11 (anti-Syn) (red) (diluted 1:50) and rabbit anti-PSyn(S6) antiserum (green) (diluted 1:300), Alexa 488 labelled goat anti-rabbit and Cy3 labelled goat anti-rmouse as a secondary antibodies. Scale bar: 100 μm.

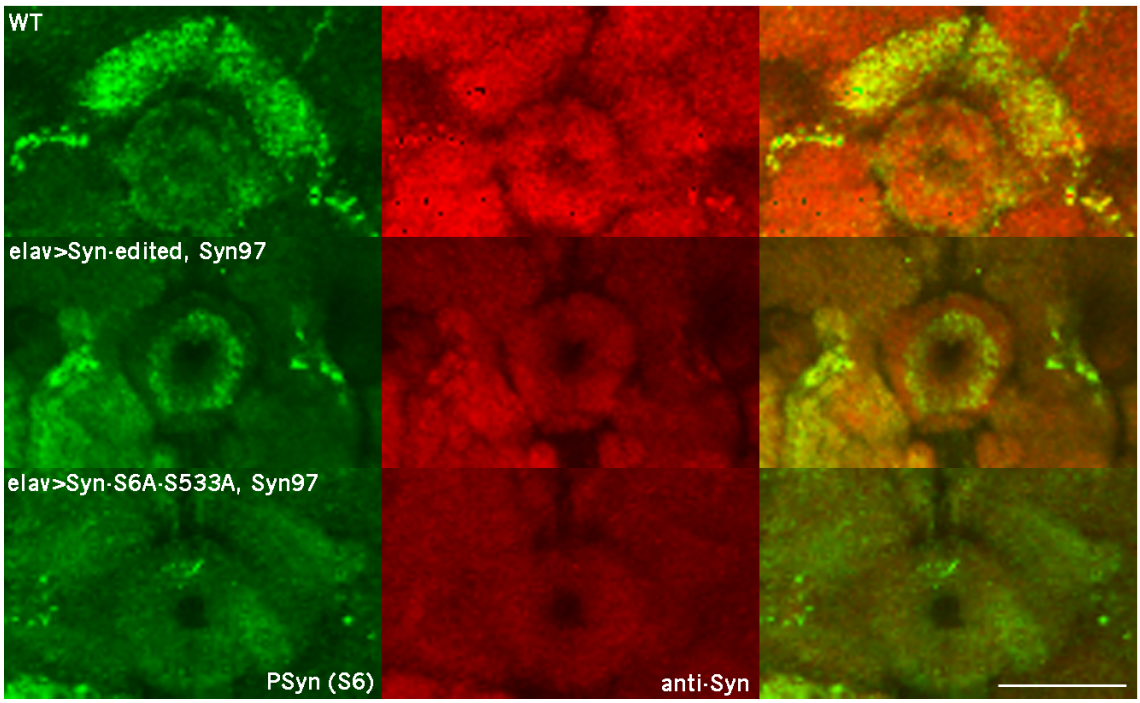


Figure 4.25. Confocal stacks of whole-mount of a transgenic fly in Syn^{97} background expressing pan-neurally (elav-Gal4) cDNA with S6A and S533A mutations compared to a transgenic fly in Syn^{97} background expressing pan-neurally (elav-Gal4) cDNA with the synapsin rescue, and a wild-type fly. The staining in the ellipsoid body of the transgenic fly expressing cDNA with the synapsin rescue is comparable to the wild-type staining, whereas the transgenic fly expressing cDNA with S6A mutation shows a reduced staining. Detail of the central complex stained with anti-PSyn(S6) (green) and anti-Syn (red) in *Drosophila* brain (anti-PSyn(S6) 1:500; anti-Syn 1:20). Scale bar: 50 µm.

Transgenic flies in Syn^{97} background expressing pan-neurally (elav Gal4) a cDNA with S6A and S533A mutations (Figure 4.23, 4.24, and 4.25) were used to prove the specificity of the immunohistochemical staining, similar to the results obtained in Western blots (Figures 4.7 and 4.8). The wild-type fly of Figures 4.23, 4.24 and 4.25 as well as the transgenic synapsin rescue of Figure 4.25 show staining in the ellipsoid body, but this staining is absent in the transgenic fly as well as in the Syn^{97} null mutant. With these results we can assume the specificity of our antiserum anti-PSyn(S6) also in immunohistochemical applications for synapsin phosphorylation at S6.

4.1.7.4 Ring neurons specific (c232-Gal4 line)

The staining showed by PSyn(S6) in a subset of ring neurons seems to be very specific. Nevertheless, the function played by synapsin in this part of the central brain remains unknown. With the aim to identify the cells which strongly express synapsin phosphorylated at S6, preliminary stainings using Gal4 lines that label some subsets of the

ring neurons were performed. The expression of Gal4 in the c232-Gal4 line is limited to a few cell types. In the adult brain, expression is found in the ring neurons of the ellipsoid body, specifically in the R3 and R4d morphological subtypes that arborized in the distal part of the ellipsoid body (Hanesch *et al.,* 1989; Renn *et al.,* 1999; Yang *et al.,* 2000; Neuser *et al.,* 2008; Seeling and Jayaraman, 2013).

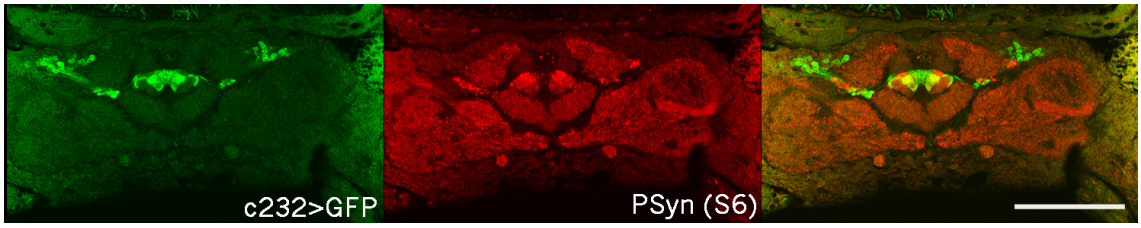


Figure 4.26. Horizontal cryosection of a fly expressing GFP in a subset of ring neurons (c232-Gal4) and stained with PSyn(S6). A partial co-localization is observed comparing the c232-Gal4 line expressing GFP and the PSyn(S6) staining. Sections were stained with rabbit anti-PSyn(S6) antiserum (diluted 1:300) and mouse monoclonal anti-GFP (diluted 1:1000) as primary antibodies, and Alexa 488 labelled goat anti-mouse and Cy3 labelled goat anti-rabbit as a secondary antibodies. Scale bar: 100 µm.

Comparing the GFP expression of this line with the staining of PSyn(S6) (Figure 4.26) a partial but not total co-localization could be observed. Further experiments using some other Gal4 lines which specifically label some subsets of the ring neurons have to be done in order to identify which neurons are labeled by PSyn(S6) antiserum.

4.1.8 Life expectancy

It is known that Syn^{97} null mutants are viable and fertile (Godenschwege *et al.,* 2004) but nothing was known about their life expectancy before. We decided to investigate whether Syn^{97} and also $Sap47^{156CS}$ null mutants have shorter life expectancy than wild-type flies. For that purpose, 4 sets with three vials of 10 flies each of wild-type, Syn^{97} null mutants, $Sap47^{156CS}$ null mutants and Syn/Sap double mutants were tested during three months. The food of each group was changed every second or third day avoiding the use of CO_2.

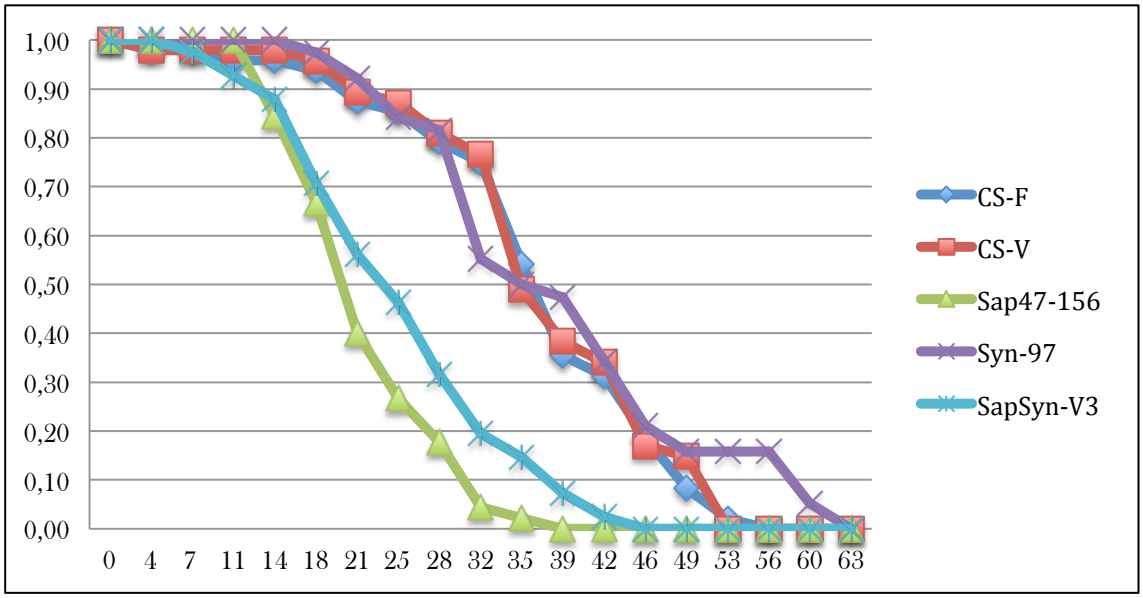
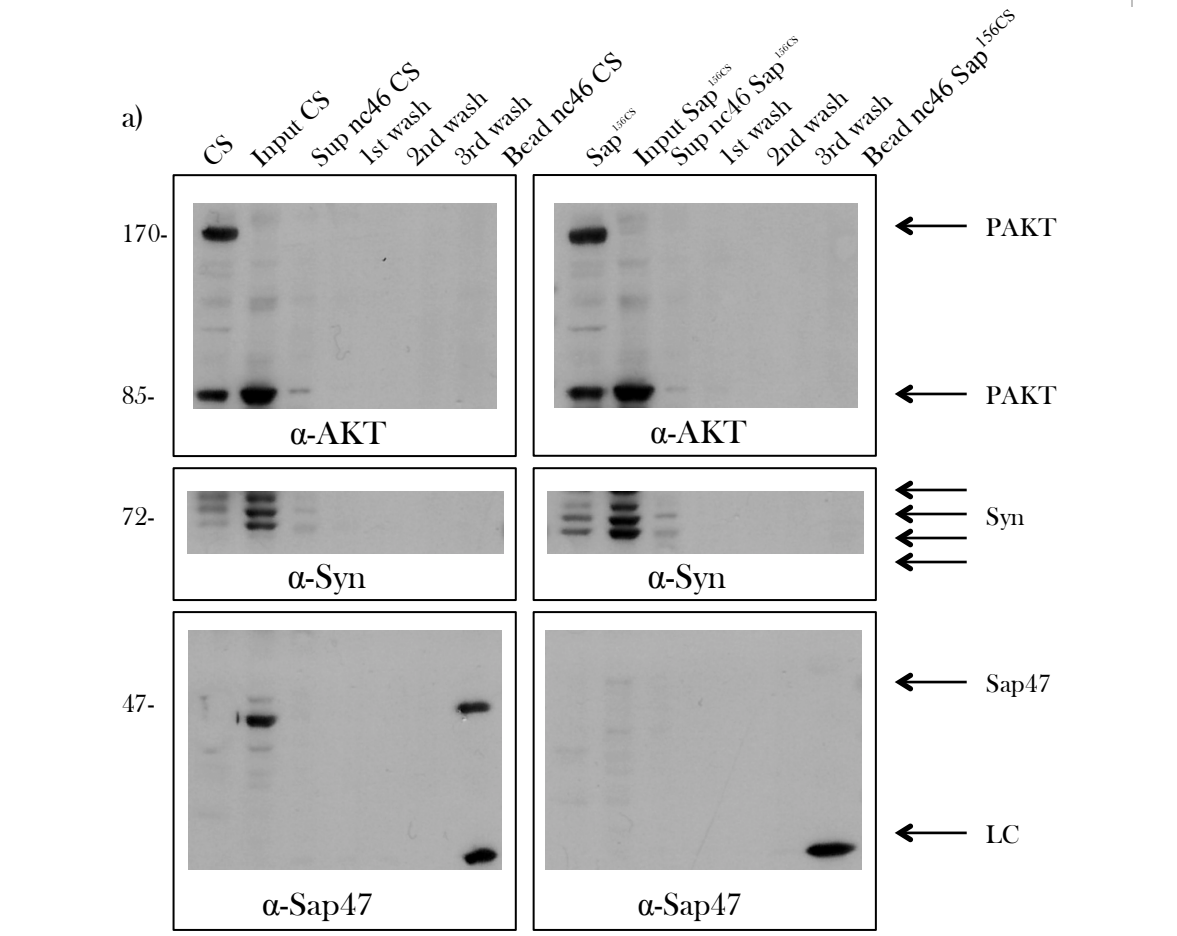


Figure 4.27. Lifespan is not reduced in Syn^{97} null mutants. CS-F and CS-V: Canton S wild type strains used for cantonizing Syn^{97} stocks (CSNF) or double mutant; Syn-97: synapsin mutant Syn^{97}; Sap47-156: SAP mutant Sap47^{156CS} and SapSyn-V3: syn-sap double mutants.

Surprisingly, the Sap47^{156CS} but not Syn^{97} null mutants show a shorter lifespan than the wild-type flies (Figure 4.27). Whether the difference of the double mutants and Sap47^{156CS} null mutants is significant remains unclear due to the small number of animals tested. Between wild-type flies and Syn^{97} null mutants, no differences can be observed meaning that the Syn^{97} mutation does not have any effect on the life expectancy under our conditions.

4.1.9 Interaction between Synapsin and Sap47

As seen before, the Sap47^{156CS} null mutant flies show an extra band for synapsin when it is detected with anti-Syn antibody in Western blots. Therefore, we decided to perform a co-immunoprecipitation (Co-IP) using Synapsin and Sap47 antibodies checking for interactions between these two proteins. We also wanted to investigate the interaction between phospo-AKT and Sap47 in the brain of *Drosophila* since Yao and colleagues (2013) demonstrated that Syap1, also named BSD domain-containing signal tranducer and AKT interactor (BSTA) (the homolog to Sap47 in mammals) promotes the phosphorylation of AKT in various human and murine cells due to a formation of a complex. A silver staining was performed with the aim to detect differences between synapsin and Sap47 protein expression and identify Sap47 interaction partners.


a)
CS
Input CS
Sup nc46 CS
1st wash
2nd wash
3rd wash
Bead nc46 CS
Sap 156CS
Input Sap 156CS
Sup nc46 Sap 156CS
1st wash
2nd wash
3rd wash
Bead nc46 Sap 156CS
170-
85-
72-
47-
α-AKT
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PAKT
PAKT
Syn
Sap47
LC

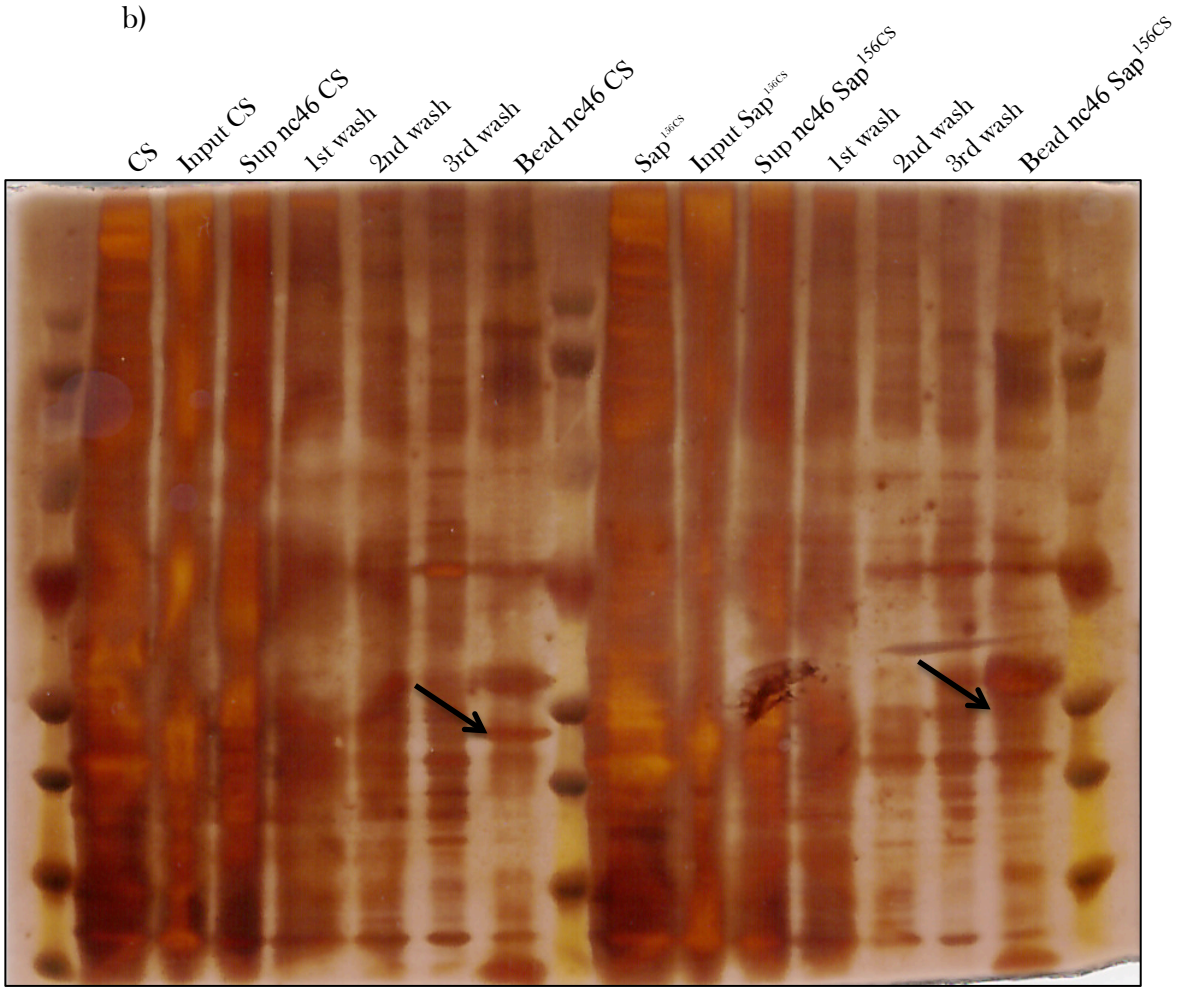
b)
CS
Input CS
Sup nc46 CS
1st wash
2nd wash
3rd wash
Bead nc46 CS
Sap 156CS
Input Sap 156CS
Sup nc46 Sap 156CS
1st wash
2nd wash
3rd wash
Bead nc46 Sap 156CS

Figure 4.28. Co-IP experiment using head homogenates of wild-type and Sap^{156CS} null mutant flies with anti-Sap47 as precipitating antibody coupled to protein G-agarose. a) Western blots of a 4-12% precast gel were developed with anti-phosphoAKT antibody (diluted 1:3000, upper part of the blot), anti-Syn antibody (3C11 diluted 1:200, middle part of the blot) and anti-Sap47 antibody (nc46 diluted 1:200, lower part of the blot). **CS, Sap47^{156CS}:** 2 fresh heads of Canton S wild-type or Sap^{156CS} null mutants homogenized in 2 µl Laemmli buffer; **Input:** Input controls (≈8 head equivalents, see Methods); **Sup nc46:** Supernatant controls (≈8 head equivalent). **Washes:** 40 µl each of the three wash solutions; **Bead:** Immunoprecipitated proteins (≈20 head equivalents). Most of the anti-Sap47 signal in CS fresh head lane is missing due to a blotting artifact (air bubble). The light chain of the precipitating antibody (LC) is detected by the light chain-specific secondary antibody. **b)** Gel identical to the gel in (a) subjected to silver staining after half blotting. The arrows point to the enriched Sap47 protein (left) that is missing in the Sap^{156CS} null mutant (right).

An immunoprecipitation using mAb nc46 (anti-Sap47) and protein G-coupled agarose beads from head homogenates of Canton S wild-type and Sap47^{156CS} null mutant flies was performed with the aim to detect by silver staining some bands in the Canton S flies that are not present in the Sap^{156CS} mutants or vice versa. In case proteins can be found and purified that co-immunoprecipitate with Sap47 or whose binding to synapsin is modified by Sap47, such potential interaction partners of Sap47 or synapsin could be identified by mass spectrometry experiments in order to obtain further information on the function of the two synaptic vesicle-associated proteins synapsin and Sap47. As can be clearly seen, Sap47 and phospho-AKT do not interact with each other in the Co-IP under the present conditions (Figure 4.28 a). The extra anti-Syn signal in the Sap^{156CS} null mutant lanes described above is visible. The anti-AKT signal near 170 kDa only in the fresh head lanes could represent a nuclear complex not dissolved by the sample buffer. For the silver staining (Figure 4.28 b) no differences in any band can be found between Sap47^{156CS} null mutants and wild-type flies under our conditions except for the presence of the enriched Sap47 protein in wild-type flies (left arrow) and its absence in the Sap47^{156CS} null mutants (right arrow). Some more experiments under changed conditions are needed to clearly detect bands which are only present in wild-type flies but not Sap47^{156CS} null mutants.

4.1.10 Variability of synapsin protein

As seen above, protein amounts of each synapsin isoform change between different blots. Therefore we were interested if synapsin isoform patterns could also vary among different wild-type strains. For that reason, a Western blot was performed using five different wild-type strains of *Drosophila melanogaster* kindly provided by the group of Charlotte Förster.

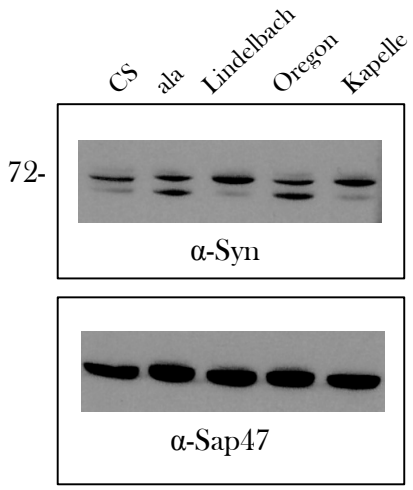

Figure 4.29. Variability of synapsin isoforms between different wild-type species. Western blot of head homogenate using a 12% precast gel for detection of synapsin. The blot was developed with anti-Syn (mAb 3C11) (diluted 1:200). The loading control with anti-Sap47 (mAb nc46) (diluted 1:200) is shown in the bottom part of the blot. CS: Canton S wild type flies; ala: ala wild-type flies; Lindelbach: Lindelbach wild-type flies; Oregon: Oregon wild-type flies and Kapelle: Kapelle wild-type flies (2 heads per lane).

The Western blot (Figure 4.29) confirms the high variability existing between different wild-type strains. It is thus not surprising to see also this variability in flies containing transgenic constructs or stocks with different genetic background as seen above. In some strains, the lowest band, which could be dephosphorylated synapsin (see Discussion), is more intense than for some other strains. Further experiments need to be done to clarify if this is due to a phosphorylation-dephosphorylation event or some other events.

4.1.11 Alkaline Phosphatase treatment

It is known that synapsin is phosphorylated at different positions (Nuwal *et al.,* 2010). The alkaline phosphatase treatment allows the removal of the phosphate groups from the protein, and then only non-phosphorylated protein should remain and be detected on the blot. We investigated which bands shown on the blots appear to be modified by phosphorylation using an alkaline phosphatase enzyme (see Methods).

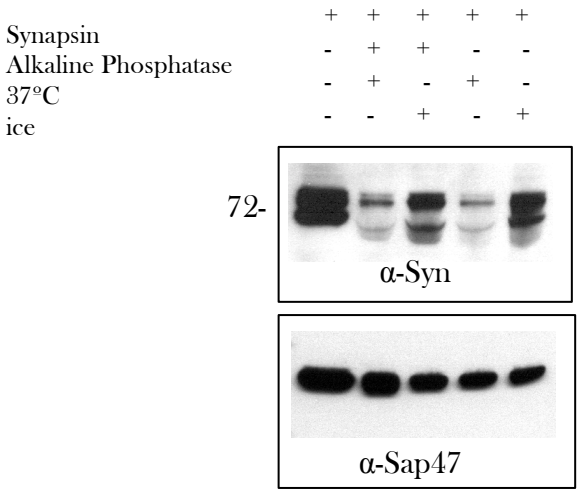

Figure 4.30. Alkaline phosphatase treatment. Western blot of head homogenate using a 12% precast gel for detection of synapsin. The blot was developed with anti-Syn (mAb 3C11) (diluted 1:200). The loading control with anti-Sap47 (mAb nc46) (diluted 1:200) is shown in the bottom part of the blot (2 heads per lane).

Wild-type fly head homogenates were treated with or without alkaline phosphatase at 37ºC or 0ºC and detected with anti-Syn antibody in a Western blot (Figure 4.30) to document any changes in the synapsin isoforms that could be related to phosphorylation. It can be seen that the incubation of the samples at high temperature shows a significant change in the isoform pattern irrespective of the presence or absence of the phosphatase enzyme. The Sap47 control signals are not significantly modified by the different treatments. Further experiments using different phosphatases have to be done in order to determine which phosphatases dephosphorylate synapsin.

4.2 Characterization of monoclonal antibodies from the Würzburg hybridoma library against Drosophila brain

[Most of these results have been published in the manuscript "**Blanco Redondo B**, Bunz M, Halder P, Sadanandappa MK, Mühlbauer B, *et al.* (2013) Identification and Structural Characterization of Interneurons of the *Drosophila* Brain by Monoclonal Antibodies of the Würzburg Hybridoma Library. PLoS ONE 8(9): e75420. doi:10.1371/journal.pone.0075420]

4.2.1 Antibody description

The Würzburg Hybridoma Library contains about 200 mAbs (Hofbauer, 1991), which selectively stain specific structures in the brain of *Drosophila melanogaster.* Unfortunately, most of these mAbs are still uncharacterized. Frontal z-projections of confocal stacks of the staining patterns of ten mAbs are described below. Each staining pattern is characteristic of the particular antibody and was obtained at least three times.

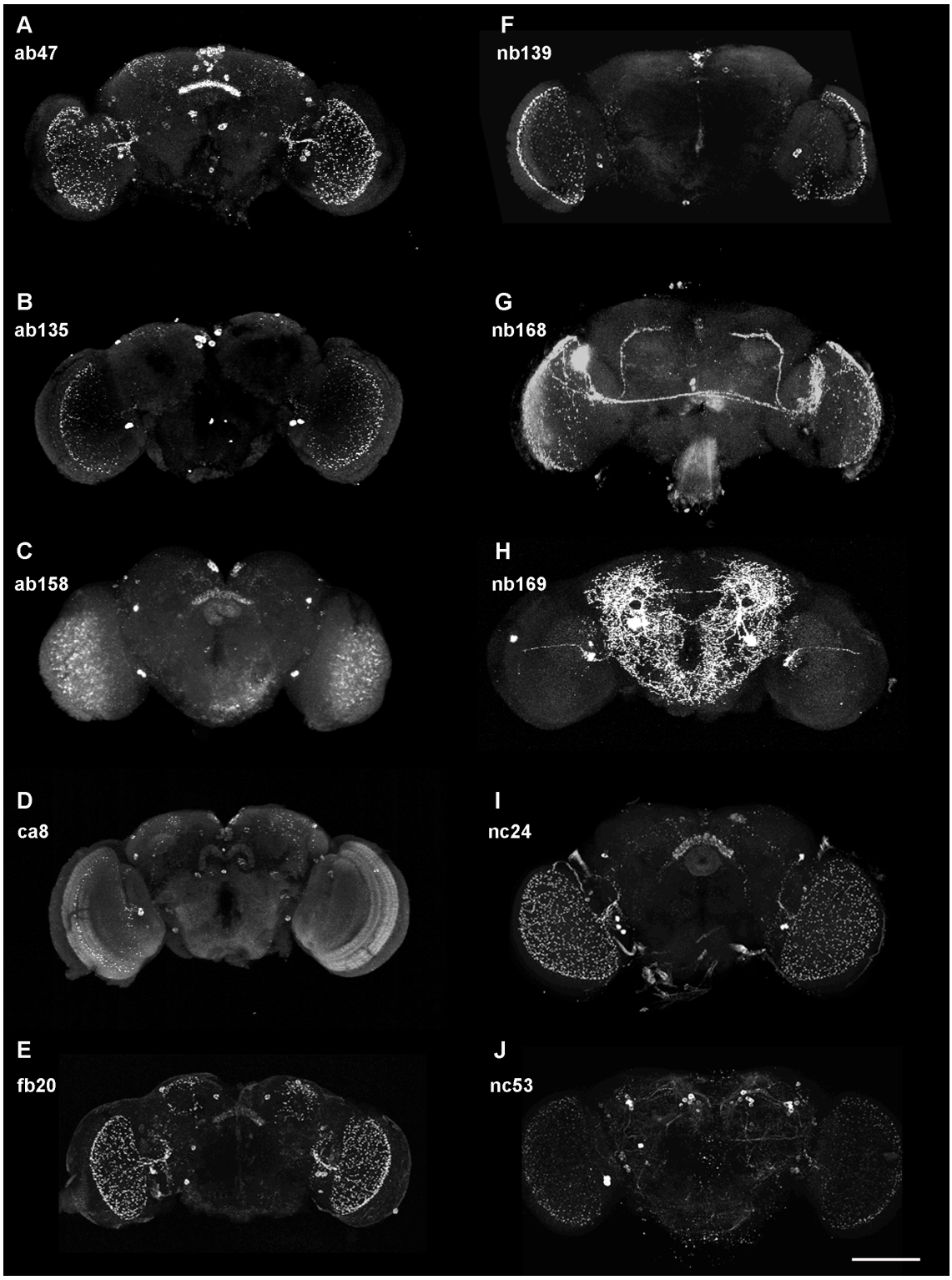

Figure 4.31. Staining pattern of ten different monoclonal antibodies. Z-projections of confocal stacks of whole-mount adult *Drosophila* brains (frontal view) stained with ten different monoclonal antibodies of the Würzburg Hybridoma Library. Note in (H) that the bright spot at the left rim of the projection is due to a staining artefact from outside the brain. Scale bar 100 µm. (Stainings in A,B,D,E, and H performed by Barbara Mühlbauer and in F,I, and J performed by Melanie Bunz).

Figure 4.31 shows maximum intensity z-projections of wild-type brain wholemount preparations stained with 10 different monoclonal antibodies of the Würzburg Hybridoma Library. Five of the antibodies (ab47, ab135, ab158, ca8, nb139) label cell bodies in the pars intercerebralis, four (ab47, ab158, fb20, nc24) highlight layers of the fan-shaped body, one (ca8) binds to the protocerebral bridge, and all except nb169 detect fine arborizations in tangential layers of the medulla. Only three of these antibodies showed staining in larva brain (Figure 4.32).

Figure 4.32. Z-projections of confocal stacks of larval nervous system stained with three of the monoclonal antibodies shown in Figure 4.31. The other seven antibodies produced no reliable staining in larvae. Scale bar 100 μm.

4.2.2 Co-localization of nb168 and PDF

The similarity of mAb nb168 staining to the known distribution of pigment dispersing hormone (PDH) (Helfrich-Förster *et al.,* 2007; Lohr *et al.,* 1993) is obvious. We decided to double-stain the brains with mAb nb168 and anti-PDH antiserum checking for co-localization.

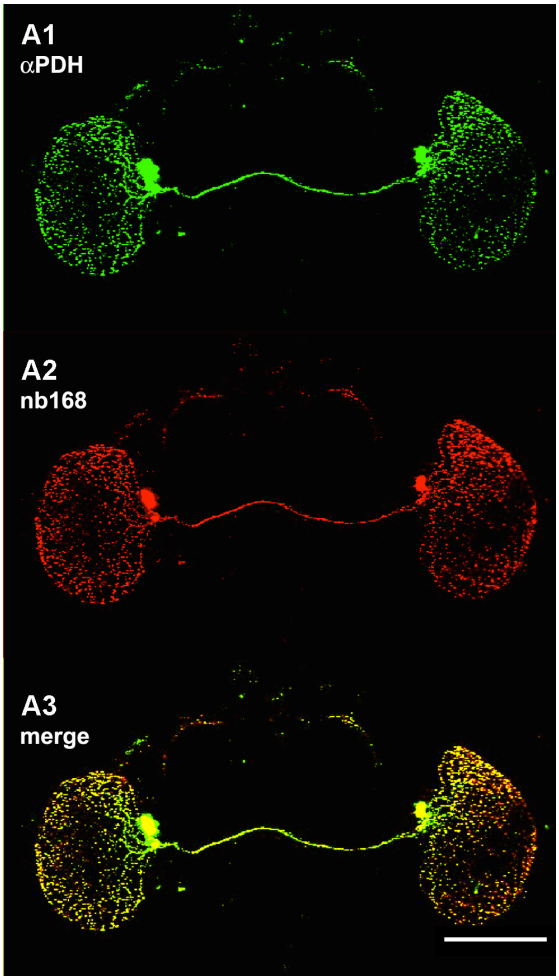

Figure 4.33. Z-projections of confocal stack of whole-mount brains double-stained with mAb nb168 and anti-PDH antiserum. The perfect co-localization in A3 indicates that nb168 could recognize *Drosophila* PDF or its precursor protein. Scale bar 100 µm.

Double stainings of whole mount brains with mAb nb168 and an antiserum against PDH reveal that the same neurons are labeled by the two antibodies (Figure 4.33). It was shown by Alois Hofbauer that the mutation of the *Pdf* gene eliminates mAb nb168 staining and thus demonstrates that mAb nb168 very likely recognizes PDF (Pigment Dispersing Factor) or its precursor protein.

4.2.3 nb169 antibody

The staining pattern of mAb nb169 in the adult brain is of particular interest. It includes one very large cell body in each brain hemisphere and a diffuse network of arborisations, in addition to a small number of cell bodies in the dorso-lateral protocerebrum and the visual system (Figure 4.31). The distribution of the corresponding antigen was investigated in more detail.

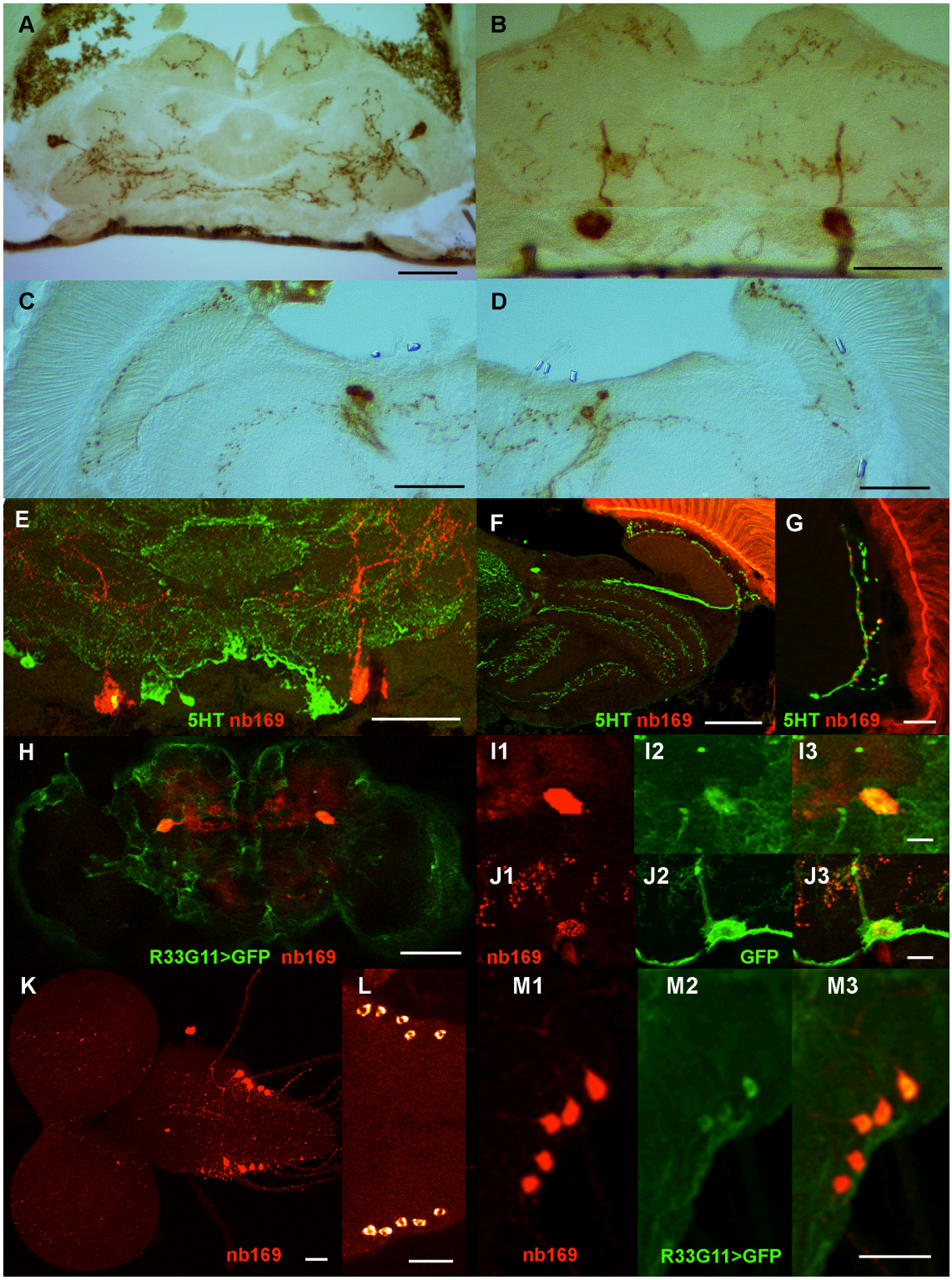

Figure 4.34. Staining details for mAb nb169. (A-D) The horizontal cryo-sections stained with mAb nb169 and DAB development show the cell bodies in the lateral protocerebrum at the dorso-ventral level of the fan-shaped and ellipsoid bodies (A), the giant neurons in the caudal cellular cortex with their main axons (in an adjacent section) at the level of the noduli (B), and the cell bodies innervating the accessory medulla, a horizontal layer of the medulla, and a fiber extending to and branching with varicosities in the lamina cortex (C, D). **(E-G)** Horizontal cryo-sections double-stained with nb169 (red) and anti-serotonin antiserum (green) demonstrate that serotonergic neurons and cells that contain the

nb169 antigen are distinct but that their neurites in the lamina cortex are closely apposed. **(H-J)** GFP (green) expressed under the control of the Janelia Gal4 line 45964 co-localizes with the nb169 antigen (red) in frontal confocal optical section (H, I1-3) and in horizontal cryo-section (J1-3). **(K-M)** In the larval nervous system the nb169 antigen (red) is localized in 10 cells (K, L) of which at least 8 also express GFP (green) under the control of 45964-Gal4 (M, enlargement of K). Scale bars in A-F, H, K-M: 50 µm; in G, I, J: 10 µm. (A-D provided by Alois Hofbauer)

Horizontal diamino benzidine (DAB)-stained frozen sections reveal two immuno-positive cell bodies of the lateral protocerebrum with some of their arborizations at the dorso-ventral level of the fan-shaped body (Figure 4.34 A), the two very large cell bodies with their prominent neurite (in an adjacent section) in the posterior protocerebrum at the level of the noduli (Figure 4.34 B), as well as the labeling of cell bodies in the visual system with fine arborizations and varicosities in the accessory medulla, in a tangential layer of the medulla and, conspicuously, in the cell body layer of the lamina (Figure 4.34 C, D). These features were partly reminiscent of stainings using antisera against serotonin (Buchner *et al.,* 1988). Thus, we wanted to determine if the cells identified by mAb nb169 contained serotonin (5HT). Fluorescent double staining with anti-5HT serum and nb169 (Figure 4.34 E, F) clearly demonstrate that mAb nb169 identifies a set of cells similar to, but distinct from, serotonergic neurons in the *Drosophila* brain. The similarity is particularly striking in the visual system where the two antibodies stain distinct neurons of nearly identical axon course and pattern of arborizations in the cellular cortex of the lamina (compare Figure 4.34 C with F, G). (Note that the fluorescence of the retina is unspecific). The serotonergic fibers and varicosities in this region have previously been investigated in detail in various insects (Nässel *et al.,* 1983; Nässel *et al.* 1985, Nässel, 1991) and are assumed to serve a paracrine function (Nässel *et al.,* 1984). In the larval ventral ganglion, mAb nb169 selectively labels five neurons on each side (Figure 4.32 and 4.34 K-M). Screening patterns of GFP expression driven by subsets of the Janelia Gal4 collection (Jenett *et al.,* 2012) for similarities with our mAb stainings, we discovered two lines (R33G11 and R49C04) that drove GFP expression in two large cell bodies in the caudal protocerebral cellular cortex. Double staining of brains from such flies with mAb nb169 and anti-GFP antiserum revealed that both antibodies label the same two perikarya (Figure 4.35 A1-3 for line R49C04, Figure 4.34 H, enlarged in I1-3 (whole-mount), and 4J1-3 (cryo-section) for line R33G11), while GFP expression was also prominent in various other structures (Figures 4.35 A1 and 4.34 H, J2). The co-localization of the

nb169 antigen and GFP driven by line R33G11 is also seen in the larval ventral nerve cord (Figure 4.34 M1-3).

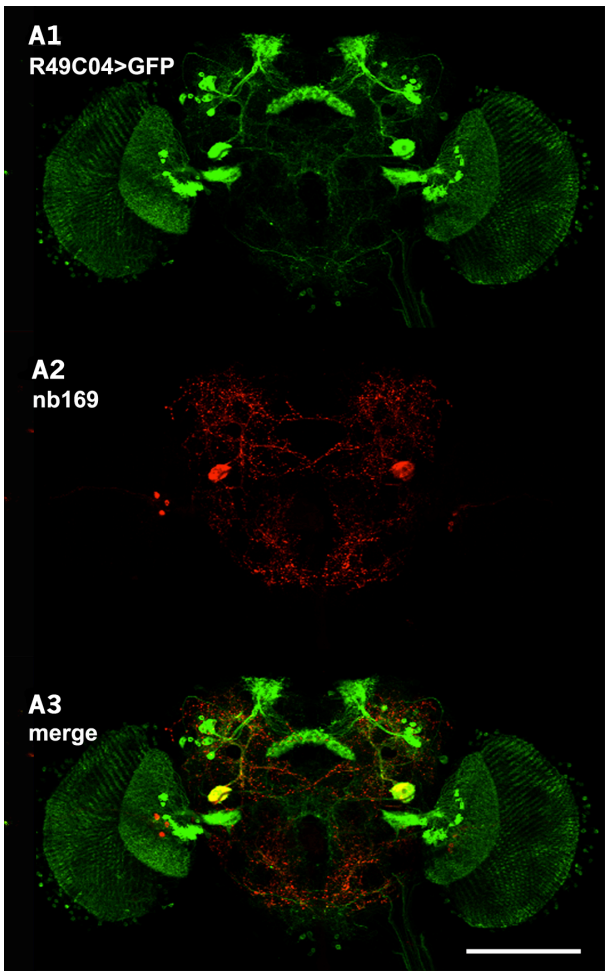

Figure 4.35. Z-projections of confocal stack of whole-mount brains double-stained with our mAb nb169 and anti-GFP. The co-localization is restricted to the two very large cells stained by nb169 in the posterior protocerebrum. Scale bar 100 µm.

4.2.4 Comparison between Janelia farm Gal4 lines and the monoclonal antibodies

Recently, the number of lines expressing Gal4 in subsets of *Drosophila* brain neurons has been significantly increased by a high-throughput approach employing fragments of regulatory sequences of genes expressed in the nervous system (Jenett *et al.,* 2012). We screened subsets of the Janelia Gal4 lines looking for similarities in the expression pattern with our monoclonal antibodies. The lines presented in the next table were selected and tested for co-localization.

mAb/subclone	Dilution	Isotype	Gal4-line or antibody [Bloomington stock #]	co-localization + = complete (+) = in subset
ab47/7	(1:5-1:20)	IgM κ	R49D04 [38679]	(+)
"	(1:5-1:20)	"	R51C07 [38773]	-
"	(1:5-1:20)	"	R67E08 [39445]	-
"	(1:5-1:20)	"	R57A02 [39878]	(+)
"	(1:5-1:20)	"	R75F06 [39901]	-
"	(1:5-1:20)	"	R78E11 [40001]	-
"	(1:5-1:20)	"	R40E08 [41238]	-
"	(1:5-1:20)	"	R42H01 [48150]	-
"	(1:5-1:20)	"	R12G09 [48525]	-
"	(1:5-1:20)	"	R49C05 [50415]	(+)
ab135/4	(1:20)	IgM	386Y (amon-Gal4)	-
ab158	(1:20)	IgM	386Y (amon-Gal4)	(+)
ca8/3/3	(1:20)	IgM κ	386Y (amon-Gal4)	(+)
″	(1:20)	″	R59E08 [39219]	-
″	(1:20)	″	R83H07 [40371]	-
"	(:20)	"	R14C06 [48604]	-
fb20/1	(1:5-1:20)	IgM κ	R49D04 [38679]	-
″	(1:5-1:20)	″	R51C07 [38773]	(+)
″	(1:5-1:20)	″	R67E08 [39445]	(+)
"	(1:5-1:20)	"	R57A02 [39878]	-
"	(1:5-1:20)	"	R75F06 [39901]	-
"	(1:5-1:20)	"	R78E11 [40001]	-
"	(1:5-1:20)	"	R40E08 [41238]	-
"	(1:5-1:20)	"	R42H01 [48150]	-
"	(1:5-1:20)	"	R12G09 [48525]	-
"	(1:5-1:20)	"	R49C05 [50415]	-
nb139	(1:2)	n.d.	anti-PDH	-
nb168	(1:20)	IgG	anti-PDH	+
nb169/2	(1:20)	IgG1	386Y (amon-Gal4)	(+)
″	(1:20)	″	anti-PDH	-
″	(1:20)	″	anti-5HT	-
"	(1:20)	"	R33G11 [45964]	(+)
"	(1:20)	"	R49C05 [50415]	(+)
nc24	(1:5)	IgM	anti-PDH	-
″	(1:5)	″	anti-MIP	-
"	(1:5)	"	R49D04 [38679]	(+)
"	(1:5)	"	R51C07 [38773]	-
"	(1:5)	"	R67E08 [39445]	-
"	(1:5)	"	R57A02 [39878]	(+)
"	(1:5)	"	R75F06 [39901]	-
"	(1:5)	"	R78E11 [40001]	-
"	(1:5)	"	R40E08 [41238]	(+)
"	(1:5)	"	R12G09 [48525]	-
"	(1:5)	"	R49C05 [50415]	(+)
nc53	(1:5)	IgM	anti-PDH	-
″	(1:5)	″	anti-NPF	-
″	(1:5)	″	anti-PER	-

Table 4.4. List of mAb, antibodies against known antigens and Gal4 lines used. List of mAbs compared with GFP (detected by anti-GFP (1:1000) driven by Gal4-lines) or stainings with antibodies against known antigens (pigment dispersing hormone, PDH (1:1500); myoinhibitory peptide, MIP (1:1000); neuropeptide F, NPF (1:300); period protein, PER (1:2000); serotonin, 5HT (1:400)).

The Janelia Gal4 lines showed on the list were tested for co-localization with the ten monoclonal antibodies from Figure 4.34. Some examples of positive co-localization of stainings are shown in the figure below.

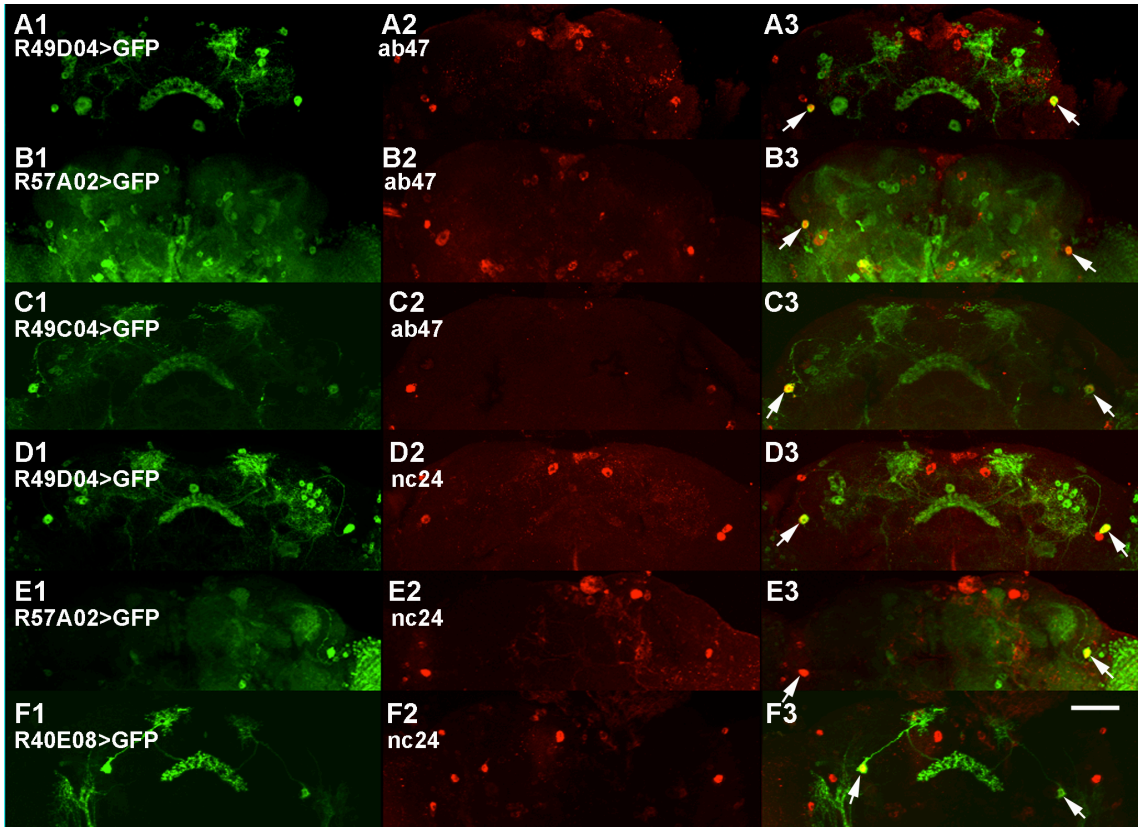

Figure 4.36. Examples for the co-localization of staining by mAbs ab47 (A-C) and nc24 (D-F) and GFP driven by selected Janelia Gal4 lines. Due to the variability of cell body positions in different individuals it is only suggestive that the Gal4 expression patterns overlap. Scale bar 100 µm. The arrows show the co-localization with the mAb.

Figure 4.36 shows some examples of co-localization in a subset of cell bodies from the Janelia farm Gal4 lines with the monoclonal antibodies ab47 (Figure 4.36 A, B and C) and nc24 (Figure 4.36 D, E and F). The discovery of three Gal4 lines with very different expression patterns overlapping apparently only in a pair of cell bodies labeled by either ab47 or nc24 suggests that it might be possible to generate a line expressing a transgene of choice only in these neurons by the split-Gal4 technique (Luan *et al.*, 2006).

4.2.5 Comparison between amon Gal4 lines and the monoclonal antibodies

Since it was shown that mAb nb168 binds to a neuropeptide or its precursor we wanted to test if other antibodies might also identify peptidergic cells. The amontillado gene codes for an enzyme responsible for the first step in the maturation of many neuroendocrine or neuromodulatory peptides from their precursor. Two amon Gal4 lines were tested for co-localization with stainings by the monoclonal antibodies. The co-localization results of all tested mAb/Gal4 pairs are summarized in the next figure.

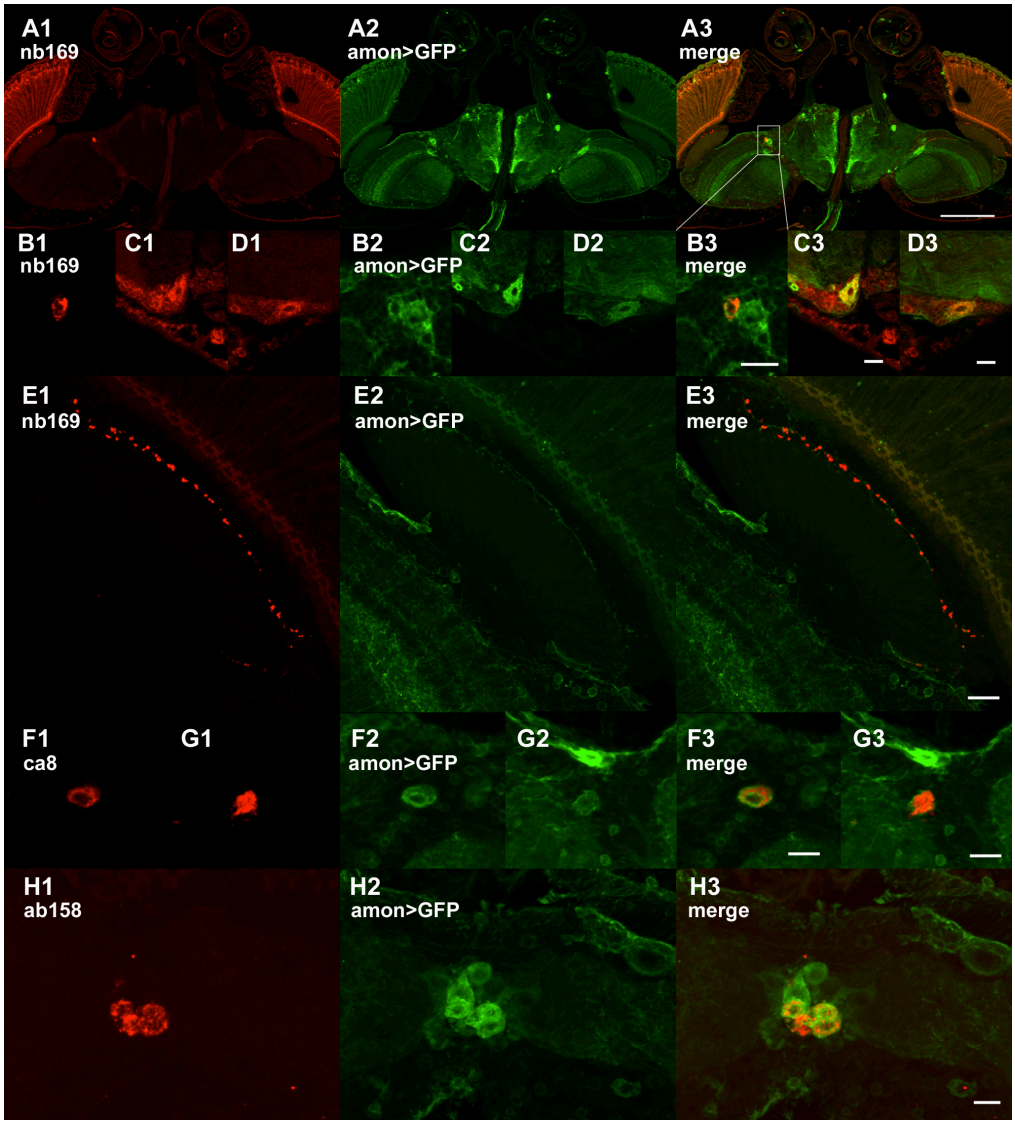

Figure 4.37. The antigens detected by mAbs nb169, ca8, and ab158 may be related to neuropeptides. (A-E) The cell stained by nb169 (red) (A, enlarged in B), and the giant cells in the caudal protocerebrum (C and D from different sections) all are positive for GFP (green) expressed under the control of 386Y amon-Gal4 which has been shown to selectively mark neuropeptide-containing cells.

(E) The neurite of a 386Y amon-Gal4>GFP-positive cell at the boundary between lamina cortex and neuropil apparently contains in discrete varicosities the nb169 antigen. **(F,G)** Examples are shown for co-localization of 386Y amon-Gal4-driven GFP and ca8 antigen in cells of the lateral protocerebrum. **(H)** Cells containing ab158 antigen in the pars intercerebralis, a region containing many neurosecretory cells, are also positive for amon-Gal4-driven GFP. Scale bar in A: 50 μm; in B-H: 10 μm.

Figure 4.37 shows the co-localization of nb169, ca8, and ab158 antibody staining with amon Gal4>UAS-mCD8 GFP expression, supporting the speculation that these three mAbs may perhaps bind to neuropeptide-related antigens. The same co-localization was checked in whole mounts (Figure 4.38).

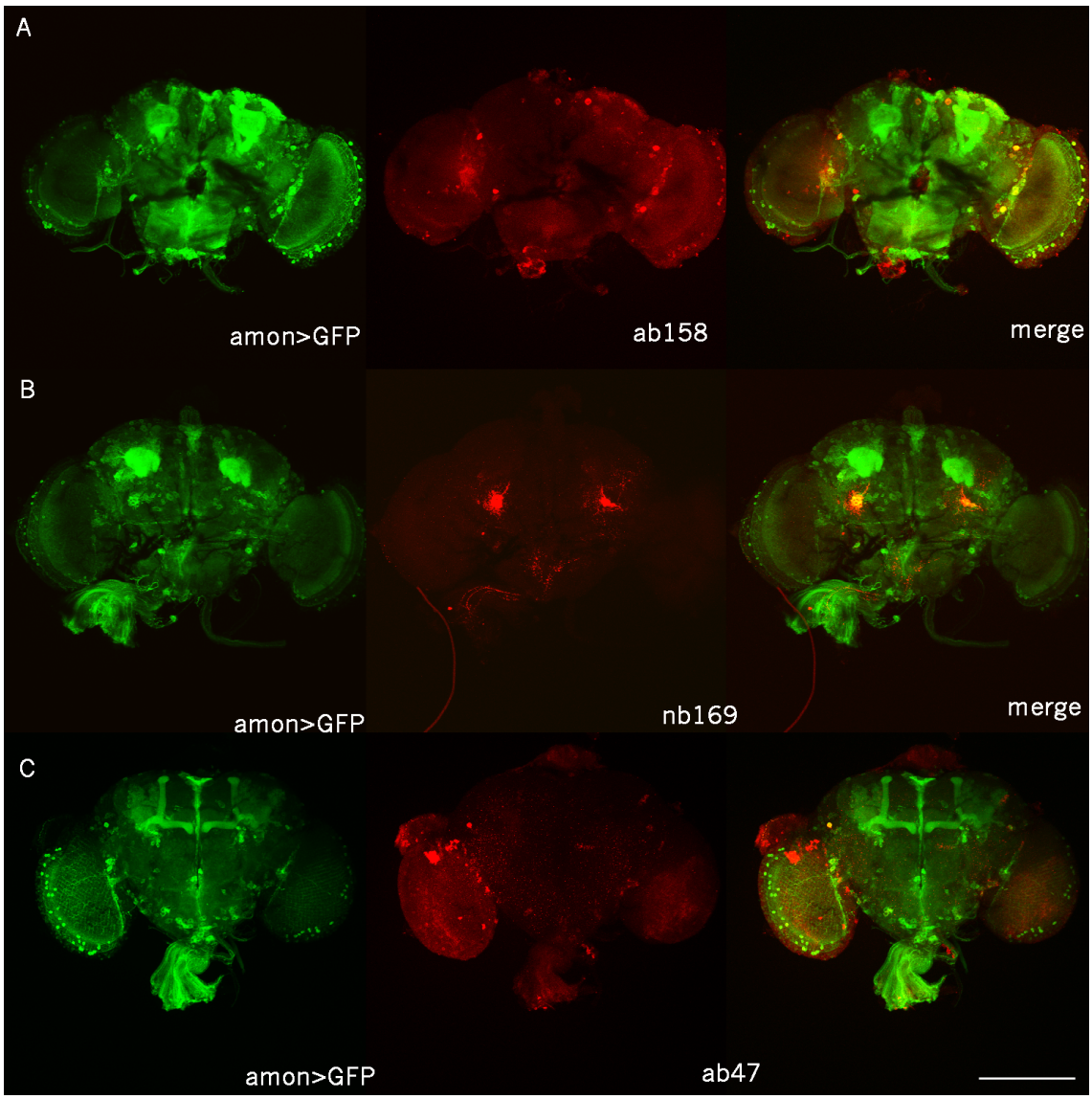

Figure 4.38. The antigens detected by mAbs ab158, nb169, and ab47 may be related to neuropeptides. Some subsets of cells stained by ab158 (A), nb169 (B) and ab47 (C) all are positive for GFP (green) expressed under the control of 386Y amon-Gal4 which has been shown to selectively mark neuropeptide-containing cells. Scale bar: 100 μm.

4.2.5.1 Amon Gal4 and nb168. Differences between larva and adult flies

It is known that PDF (Pigment Dispersing Factor) is a neuropeptide and, as it was shown above, nb168 antibody recognizes this peptide or its precursor. We decided to use nb168 as a control for our experiments with the amon Gal4 lines.

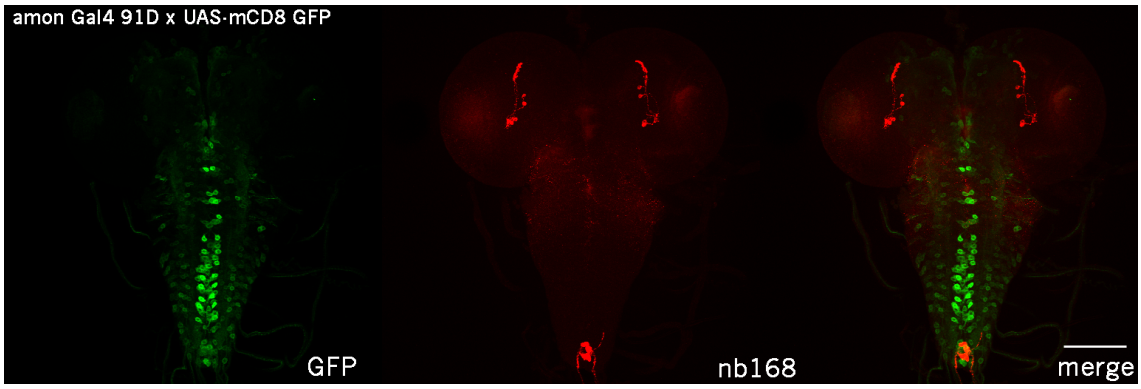

Figure 4.39. The antigen detected by the mAb nb168 in the larva brain whole mount do not co-localize with the GFP signal for the mature neuropeptides expressed by amon Gal4. There is no co-localization between the PDF signal detected by nb168 monoclonal antibody and GFP expressed under the control of 91D amon-Gal4 which has been shown to selectively mark neuropeptide-containing cells. Scale bar: 100 µm.

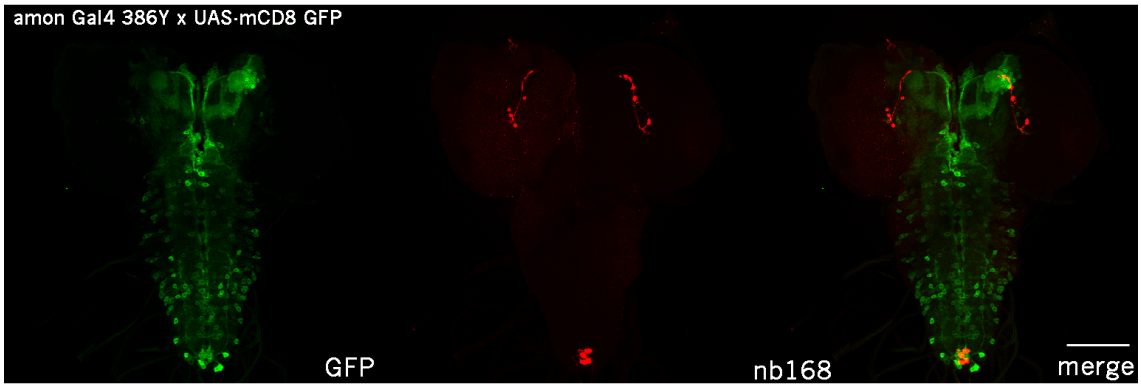

Figure 4.40. The antigen detected by the mAb nb168 in the larva brain whole mount do not co-localize with the GFP signal for the mature neuropeptides expressed by amon Gal4. There is no co-localization between the PDF signal detected by nb168 monoclonal antibody and GFP expressed under the control of 386Y amon-Gal4 which has been shown to selectively mark neuropeptide-containing cells. Scale bar: 100 µm.

In contrast to what we expected, it was not possible to find co-localization with nb168 antibody and the mature neuropeptides from the amon Gal4 lines in the larva brains (Figure 4.39 and 4.40). On the other hand, the co-localization present in adult brains (Figure 4.41) suggests that the maturation of these peptides does not happen in the larval stage.

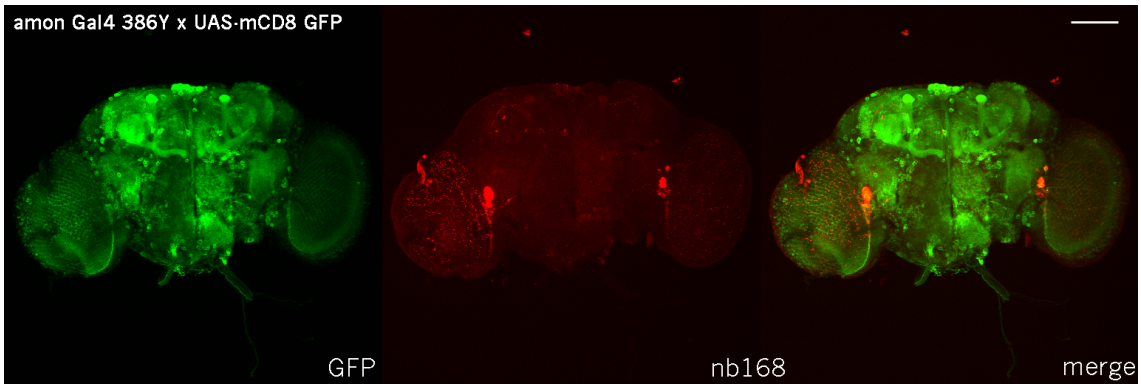

Figure 4.41. The antigen detected by the mAb nb168 in the adult brain whole mount co-localizes with the GFP signal for the mature neuropeptides expressed by amon Gal4. There is a clear co-localization between the cell bodies expressing PDF neuropeptide detected by nb168 monoclonal antibody and GFP expressed under the control of 386Y amon-Gal4 which has been shown to selectively mark neuropeptide-containing cells. Scale bar: 100 μm.

5. Discussion

5.1 Studies of synapsin phosphorylation

Synapsins are phylogenetically conserved phosphoproteins located in the presynaptic terminal of neurons (De Camilli *et al.,* 1982) and are differentially expressed in different parts of the brain indicating their complementary but not exclusive function. Furthermore, this phosphoprotein is known to regulate the exocytosis of synaptic vesicles and be involved in the regulation of neurotransmitter release interacting with synaptic vesicles and actin filaments (Benfenati *et al.,* 1989). When synapsin is phosphorylated, the neurotransmitter release is enhanced (De Camilli *et al.,* 1983). *Drosophila* synapsin null mutant larvae show a defect in the associative learning which is reduced to about 50% of wild-type CS levels (Michels *et al.* 2005, 2011). In adult flies deficits in early anesthesia-sensitive memory have been observed in flies lacking synapsin (Knapek *et al.,* 2010). Also, defects in short-term olfactory habituation are seen indicating that synapsin is necessary for the establishment of this form of non-associative memory (Sadanandappa, Blanco *et al.,* 2013). The synapsin gene model provided by the *Drosophila* genome project (flybase.org) predicts a single cDNA which due to alternative translation starts and partial UAG read-through results in four different protein isoforms: Syn-PA with a length of 537 amino acids; Syn-PE with a length of 597 amino acids; Syn-PF with a length of 981 amino acids; and Syn-PD with a length of 1041 amino acids (see scheme in the Appendix). However, the Edman degradation of the 70 kDa isoform showed an unconventional start of the translation with CTG 16 codons downstream of the predicted starts for Syn-PE and Syn-PD or 44 codons upstream of the predicted starts for Syn-PA and Syn-PF (Godenschwege *et al.,* 2004). Previous Western blot experiments also suggested the presence the four different synapsin isoforms of 70, 74, 80, and 143 kDa, respectively (Klagges *et al.,* 1996). It was demonstrated that the 143 kDa isoforms result from read-through of a UAG stop codon separating two large reading frames of the mRNA (Klagges *et al.,* 1996). While mass spectrometric analysis (Nuwal *et al.,* 2010) provided no evidence for alternative translation start, alternative splicing could produce additional isoforms (Klagges *et al.,* 1996). Very little, however, was known about the effects of synapsin phosphorylation in *Drosophila*. Seven novel phosphorylation sites were recently identified by mass spectrometry, which also showed that the N-terminus of *Drosophila* synapsin lacking the canonical AUG start codon contains methionine as the

first amino acid encoded by CUG. Phosphorylation at the PKA site (serine 6) of domain A was not observed in the mass spectrometry analysis (Nuwal e*t al.,* 2010).

However, since this PKA site is conserved in all species, we decided to generate a phospho-specific antiserum against this site. In this study, the specificity of two different *Drosophila* synapsin phosphoantisera generated by Eurogentec against phosphorylated serine 6 (S6) and serine 464 (S464) were tested. In addition, some transgenic flies for synapsin mutated at the PKA recognition site S6 and S533 in *Drosophila* were tested, as well as the CaMKII transgenic flies expressing either an inhibitory CaMKII peptide or CaMKII with the mutation at Thr 287 site; this with the aim to study the differences in synapsin phosphorylation. It was previously shown that PKA-dependent signaling has an effect in aged-related memory impairment in old flies (Yamazaki *et al.,* 2010). Here we attempted to study the effect of age on synapsin phosphorylation. In addition, preliminary studies of synapsin interaction partners were performed trying to understand the signaling pathway involved in synaptic vesicle release.

5.1.1 Specificity of the antisera

Previously, it was shown that polyclonal antisera can bind unspecifically (Kobayashi *et al.,* 1988). The antigens can show common antigenic sites. Some of the antibodies can have two completely independent binding sites (paratopes) for unrelated epitopes (Richards *et al.,* 1975). In addition, some antibodies have many overlapping paratopes that can potentially bind to a variety of related and unrelated epitopes. Antibodies generated for phosphorylation detection may be able to recognize different modified forms of the same molecule with great accuracy (Saper, 2009).

In collaboration with Eurogentec and Gerber's group, two antisera against peptides corresponding to phosphorylated synapsin at serine 6 (S6) and serine 464 (S464) sites were generated, respectively. These two sites where chosen because S6 is believed to be a conserved site for PKA/CaMKI/IV phosphorylation and S464 conforms to the phosphorylation consensus for CaMKII and was shown to be phosphorylated in *Drosophila* (Nuwal *et al.,* 2010). Additionally in this antibody production, two antisera against the two non-phosphorylated peptides were also generated. We tested the specificity of the antisera in Western blot and immunostainings. As seen in Figure 4.1, the

antiserum against non-phoshorylated synapsin at S6 position does not show any obvious differences between wild-type flies or synapsin null mutants. In conclusion, this antiserum may not be specific for synapsin. On the other hand, in addition to many unspecific signals a single specific band can be observed for the antiserum against non-phosphorylated synapsin at S464 position (Figure 4.2). Since the signal is missing in the Syn^{97} null mutants and has de same molecular weight as the signals of the monoclonal antibody 3C11 (anti-Syn), which recognize all synapsin isoforms regardless of their phosphorylation, it can be assumed that at least one synapsin isoform is recognized by this antiserum. Possibly, all other synapsin isoforms might be phosphorylated at that site.

The two antisera against phosphorylated synapsin at S6 and S464 were also tested. In Western blots of wild-type flies, two synapsin null mutants (Syn^{97} and Syn^{79}), $Sap47^{156CS}$ null mutants and the double mutants for synapsin and Sap47 were tested. We further investigated PSyn(S6) antiserum (see Figure 4.3), which only seems to detect two synapsin bands (see below). This indicates that this antibody could be specific for synapsin phosphorylated at S6. The signal of phosphorylated synapsin at S6 position in $Sap47^{156CS}$ null mutants is significantly reduced (quantification in Figure 4.3 b), indicating that Sap47 also influences synapsin phosphorylation at that specific site. In the Western blot of Figure 4.4, the three fused bands detected with the monoclonal antibody 3C11 (anti-Syn) can be seen, as well as a (fused) extra band in $Sap47^{156CS}$ null mutants. For PSyn(S464), a specific band can be detected in wild-type flies but this band is absent in the null mutants. Since apparently only one of the three isoforms is recognized by this antiserum, it seems to be specific for phosphorylated synapsin at S464 site. The figure also suggests that the extra synapsin isoform observed in the $Sap47^{156CS}$ null mutants is not phosphorylated at the S464 site. Therefore, Sap47 could play a role in regulating phosphorylation at that site.

It was shown that synapsin revealed 3 rows of spots with at least 21 different isoforms in 2D gels (Nuwal, 2010 (PhD thesis)). We wanted to test if both phosphoantisera recognize the same spots as the monoclonal synapsin antibody. For that purpose, preliminary 2D separation experiments were performed. The Western blots were developed two times, first with each phosphoantiserum and thereafter with the monoclonal synapsin antibody. Detection with PSyn(S6) shows an absence of the bottom row, indicating that these isoforms are not phosphorylated at S6, hence they could represent the non-phosphorylated forms of synapsin (Figure 4.5). For PSyn(S464) a

similar experiment produced inconclusive results (not shown) such that no speculations as to which synapsin isoforms could be phosphorylated at that site are possible. However, these were only preliminary experiments. Some more experiments regarding PSyn(S464) have to be done in order to clarify the phosphorylation of synapsin at that site.

Immunohistochemical analysis of wild-type flies and synapsin mutants stained with the phosphoantiserum PSyn(S6) were also performed in order to complement previous Western blot results. We decided to focus on PSyn(S6) due to the fact that the other antiserum (PSyn(S464)) does not show any significant difference compared to the monoclonal synapsin antibody. Figure 4.19 and 4.20 show the specific staining in some ring neurons of the ellipsoid body, which is missing for synapsin null mutants (Figure 4.21 and 4.22). That confirms once again the specificity of the antiserum, in particular also when used in immunohistochemistry.

In the Syn^{97} null mutant, weak staining in the optic lobe can be observed (Figure 4.22). This could be due to a cross-reaction of the antibody with other phosphoproteins or to autofluorescence of the eye pigment diffusing into the optic neuropil. To decide between these two possibilities, w;;Syn^{79} null mutant flies were also tested. The absence of staining in the eye and optic lobe of these flies indicates that the fluorescent signals in Syn^{97} were not due to other phosphoproteins but were caused by autofluorescence of the eye pigment that is present in the red-eyed of Syn^{97} null mutant but is lacking in the white-eye w;;Syn^{79} null mutants. The functional meaning of the presence of phosphorylated synapsin at S6 site in the ring neurons remains unclear.

Preliminary experiments were performed using the c232-Gal4 line which can drive GFP expression in some subsets of the ring neurons, in particular the R3 and R4d morphological subtypes that arborize in the distal part of the ellipsoid body required for spartial memory (Hanesch *et al.,* 1989; Renn *et al.,* 1999; Yang *et al.,* 2000; Neuser *et al.,* 2008; Seeling and Jayaraman, 2013). The corresponding transgenic flies were double stained with the PSyn(S6) antiserum and anti-GFP with the aim to determine if there is co-localization. Some of the cells showed co-localization, but the co-localization was only partial (Figure 4.26). Further experiments using other Gal4 lines which stain some subsets of ring neurons are necessary to clarify the cellular distribution of synapsin phosphorylation in this brain region.

5.1.2 PKA phosphorylates synapsin at S6 site

All synapsin proteins that have been investigated so far contain the conserved N-terminal phosphorylation consensus motif for PKA (Kao *et al.,* 1999). In *Drosophila*, the protein has two recognition sites for PKA, one is found at the domain A (PKA motif at S6) and the other between the domain C and the stop codon (PKA motif at S533). However, the PKA consensus motif at S6 is destroyed by pre-mRNA editing in most larval and adult mRNAs. The RNA modifying enzyme ADAR is responsible for an adenosine-to-inosine conversion which needs a special double stranded RNA that will form the editing region. By *in vitro* phosphorylation it was found that an N-terminal peptide containing the RRFS motif represents an excellent substrate for bovine PKA, whereas a peptide with the edited RGFS motif is not significantly phosphorylated by this enzyme. Thus, the editing mechanism induces an arginine residue to be replaced by glycine, leading to a loss of the target sequence for PKA (Diegelmann *et al.,* 2006).

In this study, the phosphorylation of synapsin at S6 and S464 was investigated using transgenic flies containing UAS-lines with mutated cDNA constructs encoding either synapsin with S6A and S533A mutations or the non-edited form of synapsin. These two transgenic lines were in a Syn^{97} null mutant background to exclude effects of intrinsic synapsin protein content (Michels *et al.,* 2001). They were crossed with elav Gal4 in Syn^{97} null mutant background, a pan-neuronal driver line. The fact that these flies express synapsin with a mutation at S6 and S533 or a non-edited form with the PKA consensus motif at S6 should not alter the phosphorylation at S464. As expected, the signals in the corresponding Western blot were comparable to wild-type flies (Figure 4.6). However, the phosphorylation at S6 is significantly altered for both transgenic lines as revealed by experiments using PSyn(S6). The flies with the mutated cDNA construct expressing S6A and S533A show a clear reduction of PSyn(S6) signals, comparable with synapsin null mutants, confirming one more time the specificity of the antiserum. On the other hand, flies expressing the non-edited form (synapsin containing the RRFS) PKA consensus motif at S6 show an increase in synapsin phosphorylation at that site (Figure 4.7, 4.8). As mentioned above, an RRFS motif is a good substrate for phosphorylation by bovine PKA. The significant increase in phosphorylation of the non-edited synapsin isoform suggests that PKA phosphorylation at the S6 *in vivo* could be regulated in a cell-specific manner by modulation of RNA editing.

Previous studies have provided evidences that synapsin phosphorylation regulates the size of the vesicle pool and, in consequence, controls the neurotransmitter release (Arbergenova and Bykhovskaia, 2007). Besides, it was also shown that synapsin is necessary in GABAergic local interneurons for short-term olfactory habituation. This synapsin-mediated enhancement indicates that GABA release after 30 minutes of stimulation by high odor concentration attenuates excitatory signals which result in the reduction of the behavioral response (Sadanandappa, Blanco *et al.,* 2013). The results showed in Figure 4.17 indicate that synapsin phosphorylation at S6 position is also necessary in GABAergic local interneurons. Conversely, the dephosphorylation of synapsin at S6 and/or S533 sites can potentially account for the spontaneous recovery of the olfactory response after short-term olfactory habituation.

In support of what was shown in Western blots and behavioral experiments, transgenic flies in Syn^{97} background expressing pan-neuronal (by elav Gal4) the cDNA with S6A and S533A mutations (Figure 4.23, 4.24 and 4.25) were used to prove the specificity of the antiserum PSyn(S6). The wild-type flies as well as the transgenic synapsin rescue (Figure 4.25) exhibit selective staining in the ellipsoid body, but this staining is absent in the transgenic fly with S6A and S533A mutation as well as in the Syn^{97} null mutant. Taken together, the specificity of the antiserum PSyn(S6) for synapsin phosphorylation at S6 can be assumed also in immunohistochemical applications.

In conclusion, PSyn(S6) is a specific and good antiserum to study the phosphorylation of synapsin at S6, since it shows a reduction in synapsin phosphorylation when the S6 site is mutated and a hyperphosphorylation when the kinase target motif containing S6 is not modified from RRFS to RGFS by RNA-editing. As the RRFS motif is a good substrate for PKA phosphorylation, we speculate that this hyperphosphorylation is effected by PKA. Furthermore, synapsin phosphorylation mediated facilitation of GABA release from LNs is necessary for the non-associative memory formation (Sadanandappa, Blanco *et al.,* 2013).

Vertebrate studies have shown that the mutated form of synapsin at S9 (homologous to S6 in *Drosophila*) leads to a decrease in the synapse number and that the phosphorylation at this site regulates the formation of both glutamatergic and GABAergic

synapses at various developmental stages (Perlini *et al.,* 2011). Future experiments could be done to explore similar effects in invertebrates using the *Drosophila* transgenic synapsin S6A flies and the specific PSyn(S6) antiserum.

5.1.3 Not only PKA phosphorylates synapsin at S6 site

PKA is composed of a tetramer of two catalytic subunits (PKAc) and two regulatory subunits that can be autophosphorylated. When cAMP binds to the regulatory region, the tetramer disassembles, resulting in two dimers. The catalytic subunits are now active and capable to phosphorylate synapsin (Ueda and Greengard, 1977). In *Drosophila*, the *DCO* gene encodes the PKA-C1 catalytic subunit and it was shown that the mutants have behavioral and learning defects (Skoulakis *et al.,* 1993; Levine *et al.,* 1994). As has been discussed above, PKA potentially could phosphorylate synapsin at S6 site.

In this study we wanted to analyze if only PKA has a role in synapsin phosphorylation at the S6 site or if other kinases are also involved. For that purpose, preliminary experiments were performed using Gal4/UAS PKA transgenic flies either expressing a PKA-C1 RNAi construct (UAS-PKA RNAi), a mutated PKA-C1 with strongly reduced catalytic activity (UAS-PKA-C1 K75A), or an intact PKA-C1 mutant (UAS- PKA-C1 W224R) (Kiger *et al.,* 1999, Kiger and O'Shea, 2011). The ectopic expression of PKA-C1 K75A has dominant effects whereas UAS-PKA-C1 W224R apparently is unable to compete with endogenous PKA-C1 (Kiger and O'Shea, 2011). These transgenic flies were crossed to elav Gal4, a pan-neuronal driver line. For all three lines, unaltered synapsin S6 phosphorylation could be observed comparing the progeny (last lane of the blots) with the parents (Figures 4.9, 4.10. and 4.11). However, due to the fact that we had no specific antibody for PKA, it was not possible to verify if the transgenic lines we used were effective for PKA knock-down or the expression of the mutant PKA. Experiments using some other transgenic lines for PKA (Drain *et al.,* 1991; Cheng *et al.,* 2012), as well as mutants for the *dunce* gene, which encodes a cAMP-specific phosphodiesterase (Ronald *et al.,* 1981) or the *rutabaga* gene, which encodes a calcium-dependent form of adenylyl cyclase (Livingston *et al.,* 1984) could also be performed to test the importance of cAMP and PKA in synapsin phosphorylation. As seen before, the synapsin S6 site is edited in most synapsin mRNAs and *in vitro* experiments indicated

that the edited synapsin isoform is not a good substrate for PKA phosphorylation. The lack of differences in PSyn(S6) signals between the wild-type, the parents and the progeny could be due to that fact. Some other kinases could phosphorylate synapsin at that site.

5.1.4 CaMKII phosphorylates synapsin at S6 site but not at S464 site

The synapsin S6 site peptide sequence RRFS is not only a consensus sequence for PKA phosphorylation, it also conforms the CaMKII target consensus RxxS. Since it has been shown that the PKA consensus RRFS is edited to RGFS in most pre-mRNAs (Diegelmann *et al.,* 2006) which still conforms the CaMKII consensus motif RxxS, it was tested whether CaMKII is important for synapsin phosphorylation at this site.

Structure and function of vertebrate CaMKII has been described in the Introduction. In *Drosophila*, the autophosphorylation occurs at Thr 287 position making the enzyme independent of Ca^{2+}/calmodulin (Griffith *et al.,* 1993). Activation of the kinase may lead to modifications of synaptic physiology, including Ca^{2+}-dependent vesicular release. Inhibition of CaMKII disrupts synaptic processes (Wang *et al.,* 1994).

In this study two transgenic flies for CaMKII were tested: the UAS line which expresses an inhibitory peptide for CaMKII (UAS-CaMKII Ala), and the UAS line which expresses a mutant CaMKII protein incapable of achieving calcium independence (UAS-CaMKII T287A) (Griffith *et al.,* 1993). These transgenic flies were crossed to elav Gal4. Notice that another fly line that expresses a calcium-independent CaMKII protein under UAS control (UAS-CaMKII T287D) could not be used in this study, because no progeny could be obtained after crossing them to the elav Gal4 line. Further experiments using the temperature sensitive tubulin $Gal80^{ts}$ suppressor could be done. When Gal80 is ubiquitously expressed under the control of the tubulin 1α promoter, at 18ºC the Gal4 activity is repressed in all tissues. Gal80 repression of Gal4 is relieved by a simple temperature shift, providing an exact control of expression (Lee and Luo, 1999).

With our phospho-specific antisera two sites that conform to the CaMKII target consensus (RxxS) were tested for phosphorylation by this kinase, S6 and S464. In Western blots of heads from flies expressing the inhibitory peptide for CaMKII, no

reduction could be observed in synapsin phosphorylation at S464 compared to the parents or the wild-type flies detected with PSyn(S464) antiserum (Figure 4.13). Since we do not have any transgenic fly line expressing a mutation for this site it becomes very difficult to prove the specificity of the antiserum. On the other hand for synapsin phosphorylated at S6, a reduction of almost 80% could be observed in the Western blot signal detected with PSyn(S6) antiserum (Figure 4.12). This reduction was seen both for transgenic flies expressing an inhibitory peptide for CaMKII as well as for transgenic CaMKII flies expressing T287A mutation which prevents prolonged activation of the kinase by autophosphorylation of this site (Figure 4.14). Accordingly, CaMKII seems to phosphorylate serine in position 6. Furthermore, in both Western blots a strong signal of higher electrophoretic mobility for the progeny is detected with the anti-Syn antibody (mAb 3C11). This signal might correspond to dephosphorylated synapsin. The relatively large shift could indicate perhaps additional post-translational modifications effected by CaMKII in synapsin not only at the S6 site.

If indeed CaMKII regulation of synapsin was required for its *in vivo* function, one would expect that CaMKII function in LNs would be necessary for STH. Using the temperature sensitive tb-Gal80^{ts} system, the UAS-CaMKII Ala, and UAS-CaMKII RNAi transgenic lines were tested. When the temperature was maintained at 18ºC throughout the development and test, flies showed normal STH. However, when the temperature was shifted to 29ºC for four days prior to the experiment to induce the CaMKII inhibitory peptide or the CaMKII RNAi expression in LN1 neurons, flies displayed reduced STH (Figure 4.18).

Taken together, these *in vivo* CaMKII results demonstrate that the predominant form of synapsin is a potent substrate for CaMKII. Thus, the expression of an inhibitory CaMKII peptide in neurons not only reduces the phosphorylated form of synapsin detected using the PSyn(S6) antiserum, but also blocks the olfactory habituation. These results are functionally consistent with what was already observed in vertebrates, whereas the structural basis for the regulation changes from vertebrates to invertebrates because invertebrate synapsins lack the domain D important for CaMKII phosphorylation in vertebrates (Huttner *et al.,* 1981). The consideration of all available data on olfactory STH and LTH (Das *et al.,* 2011; Sadanandappa, Blanco *et al.,* 2013) has lead to a hypothesis of the possible PKA/CaMKII signaling pathway that can be seen in Figure 5.1.

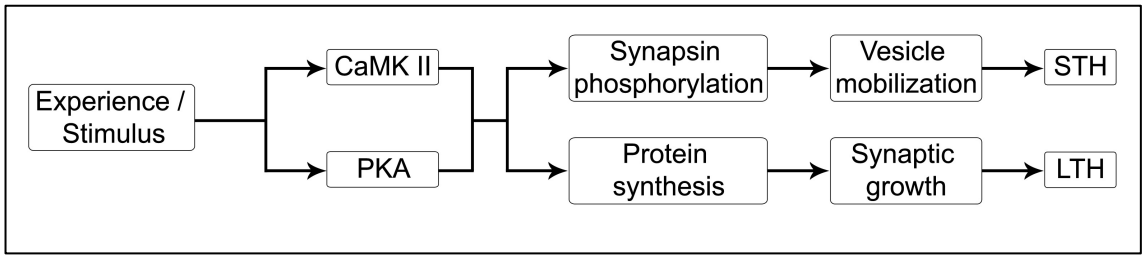

Figure 5.1. Schematic synaptic mechanism for PKA and CaMKII in *Drosophila* (Sadanandappa, Blanco *et al.,* 2013).

Furthermore, we cannot exclude the action of other kinases like MAPK or ERK, in the phosphorylation of *Drosophila* synapsin at S6, S464 and other sites. Phosphorylation of synapsin by these kinases has already been demonstrated in vertebrates (review Cesca et al., 2010).

5.1.5 Synapsin and the Age-Related Memory Impairment

Similar to humans, as *Drosophila* age, they suffer a decrease in memory, referred to as age-related memory impairment (AMI). In *Drosophila*, AMI consist of a specific reduction in memory dependent on the *amnesiac* gene (Tamura *et al.,* 2003) which has been proposed to regulate cAMP levels in the mushroom bodies (review Heisenberg, 2003). It was shown that heterozygous mutations in the *DCO* gene -which encodes the major catalytic subunit of cAMP-dependent protein kinase A (PKA)- act as a strong AMI suppressor (Yamazaki *et al.,* 2007). Another recent study showed that increasing cAMP and PKA activity in the mushroom bodies causes premature AMI. However, since total PKA activity does not change upon aging, an age-related increase in PKA-dependent signaling is assumed to contribute to AMI (Yamazaki *et al.,* 2010).

One aim of this study was to test whether synapsin phosphorylation could be the downstream target of PKA signaling and contributes to AMI. For that purpose several sets of experiments were performed. Flies from the same large culture vial were separated into two groups, one group was frozen as young (1-4 days old) flies and kept at -80°C, the other group was allowed to age (22-31 days old) before freezing. The Western blots show large variations in the signals that were obtained during all the repetitions (Figure 4.15 f). In general, PSyn(S6) signals reflecting phosphorylated synapsin at S6 significantly increase upon aging; however, the same is true for the total synapsin (detected with mAb 3C11). It

was also seen that the loading control protein level also increased upon aging in most of the experiments. This may lead to the conclusion that the brain size changes and therefore the amount of protein increases with age. Although the phosphorylation of synapsin at S6 site increases, it does not significantly increase when it is compared to the total synapsin (Figure 4.15).

Analyzing these results all together, it was not possible to detect a clear effect under our conditions of the age of the flies on synapsin phosphorylation at the S6 site which might be caused by PKA-dependent signaling. Further experiments in which the conditions are changed might be necessary to reappraise this conclusion.

5.1.6 Synapsin null mutants do not show shorter lifespan

It is known that Syn^{97} null mutants are viable and fertile; however, they show a phenotype affecting complex behavior, as well as defects in associative learning in larvae, and in short-term olfactory habituation in adults (Godenschwege *et al.,* 2004; Michels *et al.,* 2005; Sadanandappa, Blanco *et al.,* 2013), but it remained open whether the lifespan of these null mutants changes compared to wild-type flies. Our experiments indicate that lifespan of Syn^{97} null mutants was indistinguishable from those of wild-type flies (Figure 4.27). By contrast, $Sap47^{156CS}$ null mutants and the double mutants showed a decrease in the lifespan. Since we worked only with a small number of flies, further experiments are needed in order to clarify whether these differences in the $Sap47^{156CS}$ null mutants and the double mutants are robust. We can conclude that the observed defects in associative learning and STH are not due to premature aging which should be associated with shorter lifespan of Syn^{97} null mutants.

5.1.7 Synapsin and Sap47 do not interact with each other directly

Previous studies by N. Funk, T. Nuwal (unpublished) had revealed that Western blots of $Sap47^{156CS}$ null mutant flies show an extra band not observed in blots of wild-type flies developed with anti-synapsin antibody (mAb 3C11). Quantification of the total synapsin in fly heads using ELISA showed that synapsin levels are approximately 2 fold increased in $Sap47^{156CS}$ null mutants when compared to wild-type flies. When the same samples were treated at 37°C with alkaline phosphatase (AP), the additional Western

band was no longer present. This suggested that this extra band might be a result of synapsin phosphorylation (T. Nuwal, 2010 (PhD thesis)). However, this interpretation must be considered with caution in view of our phosphatase experiment (Figure 4.30) which indicated that high temperature alone without AP significantly changes the isoform pattern of synapsins in Western blots. The fact that synapse-associated proteins contain a BSD domain (**B**TF2-like transcription factor, **S**ynapse-associated protein and **D**OS2-like proteins) present in several protein families of highly different function (Doerks *et al.,* 2002) does not provide any idea on the relation between synapsin and Sap47. Although no biochemical evidence exists for an interaction between Sap47 and DNA, Sap47 could regulate a transcription factor for the *synapsin* gene. Thus, Sap47 could downregulate synapsin in wild-type flies and an increase in *synapsin* gene transcription in Sap47^{156CS} null mutants could be due to the absence of Sap47 protein. Furthermore, it was also shown that the BSD domain containing the signal transducer and Akt1 interactor, BSTA (also known as SYAP1, homolog to Sap47 in mammals), promotes phosphorylation of Akt1 via BSTA-Akt1 complex formation, and boosts adipogenesis (Yao *et al.,* 2013).

To determine a direct interaction between synapsin, as well as Akt1 and Sap47, immunoprecipitation of Sap47 using mAb nc46 was done from wild-type and Sap47^{156CS} null mutant flies. The immunodetection on Western blots with synapsin antibody (mAb 3C11) or phospho-Akt antibody should determine if these proteins bind to Sap47. Neither Western blot nor silver staining analysis revealed a direct interaction between the proteins. Considering this, we can exclude a direct stoichiometric high affinity binding of Sap47 to synapsin or to Akt under our experimental conditions (Figure 4.28). In addition, no direct stoichiometric interaction partners of Sap47 could be found in silver staining, even though the experiment worked because it showed an enriched Sap47 protein in wild-type flies which is absent in Sap47^{156CS} null mutants (arrows Figure 4.28 b). These observations of course do not exclude an indirect interaction between Sap47 and synapsin, possibly via kinases.

5.1.8 Variability of synapsin protein

Post-translational modifications (PTM) are chemical modifications of proteins which regulate activity, localization and interaction with other molecules. They are commonly employed to regulate cellular activity. Furthermore, PTMs occur at distinct amino acid sites. Normally, they are mediated by enzymes (kinases, phosphatases, transferases and ligases). The most common types of PTM are: (a) Phosphorylation, a reversible event, is mediated by kinases and phosphatases at serine, threonine or tyrosine residues. Phosphorylation causes conformational changes that regulate catalytic activity of the protein; (b) Glycosylation, the attachment of sugar moieties to proteins has important effects on protein folding, conformation, distribution, stability and activity; (c) Ubiquitination, a reversible process is carried out by a set of three enzymes. Mono-ubiquitination can alter the fate of the protein affecting the cellular sub-localization, function or its degradation; (d) S-Nitrosylation, reversible reactions used by cells to stabilize proteins, regulate gene expression and provide nitric oxide donors. It includes transcriptional and post-transcriptional regulation of protein expression (Gaston *et al.,* 2003); (e) Methylation, the addition of a methyl group to a protein. This event typically takes place on arginine or lysine amino acids residues; (f) N-acetylation is an irreversible or reversible mechanism which transfers an acetyl group to nitrogen; (g) Lipidation is a process used to target proteins to membranes. There are four types of lypidation and each type gives proteins distinct membrane affinities; (h) Proteolysis, where proteases break peptide bounds resulting in smaller polypeptides or amino acids, is often involved in protein maturation.

Vertebrate synapsins also contain different PTMs. It was shown that synapsin I and synapsin II contain N-acetylglucosamine residues in O-linkage to the protein (Lüthi *et al.,* 1991; Kang *et al.,* 2013). This modification can play an important role in the modulation of phosphorylation or protein interaction. Synapsin I, for instance, contains at least seven O-GlcNAcylation sites including serine and threonine amino acids. They are clustered in the same regulatory domain as the phosphorylation sites, but in different Ser/Thr residues (Cole and Hart, 1999). Furthermore, learning and long-term potentiation increase synapsin fucosylation which plays an important role in the regulation of synaptic proteins and neuronal morphology (Murrey *et al.,* 2005). In addition, different serine phosphorylation sites were observed in synapsin Ia, IIa and IIb by mass spectrometry

analysis (John *et al.,* 2007; Multon *et al.,* 2007). On the other hand, very little is known about the PTM in *Drosophila* synapsin, except phosphorylation (Nuwal *et al.,* 2010).

During the course of the thesis, a high variation in the isoform pattern of synapsin in different blots could be observed. For instance, notice the shift in the electrophoretic mobility of synapsin in the Western blots of the progeny elav Gal4>UAS-CaMKII Ala or elav Gal4>UAS-CaMKII T287A (Figure 4.12 and 4.14), which could be due to a dephosphorylation of the protein. Therefore, we asked the question if synapsin protein also varies between different wild-type strains. The five different wild-type strains chosen were collected at different latitudes, for they show differences in the allele of *timeless* gene, a cardinal component of the circadian clock. Flies can have two major alleles of *timeless* (*tim*) gene, *ls-tim* (longer *tim*) and the truncated *s-tim* (shorter *tim*) genes (Sandrelli *et al.,* 2007; Tauber *et al.,* 2007). In the Western blot of head homogenates developed with anti-synapsin antibody, large differences in the synapsin isoforms could be observed for each strain, while the loading control Sap47 (mAb nc46) was not altered (Figure 4.29). Consequently, it seems that synapsin protein could suffer PTMs due to the variability in the genetic background of each fly strain. The PTMs that cause these changes should be studied in further experiments. Also, preliminary experiments using alkaline phosphatase treatment were performed in order to study which of the synapsin isoforms are modified by phosphorylation. Surprisingly, we found that only the increase in the temperature of the sample drastically changes the isoform pattern, possibly by affecting endogenous phosphatases which might dephosphorylate synapsin. An effect of the the alkaline phosphatase treatment is not clearly detectable in this experiment (Figure 4.30). Future experiments using different phosphatases have to be performed in order to specify which phosphatases dephosphorylate synapsin.

5.2 Identification and structural characterization of interneurons of the *Drosophila* brain by monoclonal antibodies of the Würzburg Hybridoma Library

5.2.1 Antibody description

Around 1000 clones were produced by injecting mice with homogenized *Drosophila* heads and fusing their spleen cells with myeloma cells. The result of this process is the creation of the Würzburg Hybridoma Library. This library contains about 200 mAbs, which selectively stain specific structures in the brain of *Drosophila melanogaster* (Hofbauer *et al.,* 2009). To better understand how the *Drosophila* brain works, it is necessary to identify the proteins localized in the brain and their functions. In this section, we used ten different antibodies to structurally characterize interneurons that contain the corresponding (unknown) antigens. No reliable Western blot signal from brain homogenate could be obtained with these antibodies, defeating attempts to identify the antigens by protein purification and mass spectrometry (Wagh *et al.,* 2006; Halder *et al.,* 2011). Z-projections of wild-type brain whole mount preparations stained with the 10 different monoclonal antibodies were presented (Figure 4.31). The monoclonal antibody (mAb) ab47 stains the fan-shape body and few cells of the central brain, as well as a few cells in the *pars intercerebralis*. The mAb ab158 stains large cells in the *pars intercerebralis* with innervation to the fan-shape body. In addition, arborizations in the medulla are also stained. The mAb ab135 stains cell bodies in the *pars intercerebralis* and an aborization in the tangential layers of the medulla. The mAb nb139 stains the cells in the *pars intercerebralis* and a fine arborization in the tangential layers of the medulla. The mAb nb168 shows the same staining pattern as the previously described nb33 antibody; it stains large and small ventral lateral neurons (lLNv, sLNv), clock neurons containing PDF. The mAb ca8 stains the protocerebral bridge and a few cells in the *pars intercerebralis*. The mAb nc24 stains the fan-shape body and the ring neurons in the central brain, as well as few cells near the auxiliary medulla and shows an arborization in the medulla. The mAb fb20 stains the fan-shape body in the central brain, as well as a few cells near the auxiliary medulla, and shows an aborization in the medulla. The mAb nc53 labels cell bodies in the central brain. Finally, the mAb nb169 stains a pair of large cells in the central brain which send out an arborization network to the rest of the central brain.

Three to four smaller neurons near the auxiliary medulla are also stained (Figure 4.31). Only the mAbs ca8, nb168 and nb169 showed larva staining (Figure 4.32).

5.2.2 The mAb nb168 co-localizes with PDF

The molecular circadian clock relies on oscillations in the activation of particular genes with a period of ≈24h at certain times of the day. However, the highest concentration of essential molecules is found in lateral neurons of the central nervous system. Pigment dispersing factor (PDF) plays a role in the regulation of insect biological rhythms. The cell bodies of *Drosophila* PDH-immunoreactive neurons are located at the anterior part of the medulla; their axonal arborizations modulate the neuronal activities in the optic lobe and relay the circadian information to the midbrain (Helfrich-Förster, 1997). The similarity of the mAb nb168 staining with PDF neurons was obvious. In the test for co-localization with anti-PDH antibody the results showed a clear co-localization between these two antibodies, leading to the speculation that the mAb antibody recognized PDF or its precursor protein (Figure 4.33). Since the mAb nb168 and nb33 were obtained in the same fusion experiment using B-lymphocytes from a single mouse, it is possible that they derive from a common B cell and thus recognize the same epitope. The fact that the staining with nb168 or nb33 is absent in PDF mutants strongly supports this speculation.

5.2.3 Janelia Gal4 collection

Flies of the Janelia Gal4 collection express Gal4 under the control of defined 3 kb DNA fragments from either flanking non-coding or intronic regions of associated genes which have patterned expression in the adult brain. Thus, these drivers express Gal4 in more restricted patterns than was observed with standard enhancer trap lines (Pfeiffer *et al.,* 2008). For this study, we decided to screen subsets of the Janelia Gal4 collection as revealed by UAS-GFP by looking for similarities in the staining patterns obtained with the 10 monoclonal antibodies. We selected 20 Janelia Gal4 lines (Table 4.4) and by performing double stainings we investigated whether there was any overlap detectable. Frequently, however, in spite of apparent similarity of staining patterns in the neuropil, the GFP-positive and the mAb-stained cell bodies were distinct albeit often in close proximity. In some cases, a small subset of mAb-stained cells also expressed GFP driven by a Gal4-line. For instance, the staining for ab47 and nc24 (Figure 4.36) suggest that

Gal4 expression from different Gal4 lines may overlap as their GFP reporter is observed in mAb-stained cells at similar positions. Hence, the split Gal4 system becomes an important tool in future experiments if one wants to analyze the function of these cells, e.g. by transgenic expression of a calcium sensor. The split Gal4 system is a selective genetic manipulation for targeting gene expression in specific cells defined by the overlap of two different Gal4 lines. The separate DNA binding domain (DBD) and the activation domain (AD) of Gal4 are fused to a heterodimerizing leucine zipper motif and independently targeted using two different promoters ('hemi-driver'). Only when they are expressed in the same cell, the two leucine zipper will bind to the one another resulting in the formation of a functional Gal4 activator (Luan *et al.,* 2006).

5.2.4 Amon Gal4

Since it was shown that one of the monoclonal antibodies (nb168) binds to a neuropeptide or its precursor, possibly, other antibodies could also bind to neuropeptides. In general, neuropeptides are cleaved from precursor proteins by proteolytic enzymes. *Amontillado* gene is predicted to encode a member of the Kex2 family of proteases and is orthologous to PC2 in mammals, which functions in proteolytic processing of neuropeptides (Siekhaus and Fuller, 1999; Wegener *et al.,* 2011). Amon Gal4 386Y carries a P-element enhancer-trap insertion within 320 bp of the amon 3' end (Taghert *et al.,* 2001). On the other hand, amon Gal4 91D is a promoter construct containing 464bp of the 5' regulatory region (bp -331 to + 133) and it is located on the second chromosome. In this study, the co-localization of ca8, nb169, ab158 and ab47 antibody staining with amon>GFP expression supports the speculation that these four mAb may perhaps bind to neuropeptide-related antigens (Figure 4.37 and 4.38). Surprisingly, the positive control used (nb168) does not show co-localization with amon>GFP in larvae (Figure 4.39 and 4.40), whereas a clear co-localization is observed in adults (Figure 4.41). These results lead to the speculation that the amon Gal4 enhancer trap construct may be missing interactions with some larval enhancer of the *amon* gene. Alternatively, expression of the amontillado gene in these cells is seen only in adults, perhaps because the processed PDF is not required in larvae.

5.2.5 The nb169 antibody

The antibody nb169 is of particular interest. It stains two very large cell bodies in each brain hemisphere and an arborization in the whole central brain. In addition, it shows three to four smaller cell bodies near the auxiliary medulla in the region between the optic neuropil and the central brain (Figure 4.31, 4.35 A2). The distribution of the corresponding antigen in frozen sections which stains the two very large cell bodies at the level of the noduli, as well as the labeling of the cell bodies in the visual system with fine arborization and varicosities in the accessory medulla, in a tangential layer of the medulla and in the cell body layer of the lamina, reminds us of the serotonin staining done earlier (Buchner *et al.,* 1988). Fluorescent double staining with anti-5HT serum and nb169 revealed that although nb169 labels a set of cells similar to serotonergic neurons, they are different neurons. In the lamina, both antibodies label distinct but similar axons with parallel arborization patterns (Figure 4.34 A-G). In the larval ventral ganglion mAb nb169 selectively stains five neurons on each side (Figure 4.32 and 4.34 K). Screening patterns of GFP expression driven by subsets of the Janelia Gal4 collection described above two lines were selected (R33G11 and R49C04) that drive GFP expression in two large cell bodies in the caudal protocerebral cortex. Double staining shows co-localization of GFP in these two lines with nb169 staining. Thus, the two antibodies label the same perikarya, while GFP expression was also prominent in other structures (Figure 4.34 and 4.35). The co-localization of the nb169 antigen and GFP driven by R33G11 is also seen in the larva (Figure 4.34). On the other hand the GFP, driven by R49C04, shows no such co-localization in the larval ventral ganglia. The Gal4 line R33G11 contains a regulatory DNA fragment of the gene CG18405, *Sema-1a.* Gal4 lines driven by other regulatory fragments of this gene show no expression in these cells, such that the relation of the antigen recognized by nb169 to the *Sema-1a* gene remains obscure. The discovery of two Gal4 lines with very different expression patterns overlapping apparently only in the pair of giant neurons labeled by mAb nb169, suggests that it may be possible to generate a line expressing a transgene of choice only in these two giant neurons by the split Gal4 technique (Luan *et al.,* 2006). As seen above, the co-localization of nb169 and amon>GFP supports the speculation that this mAb may perhaps bind to a neuropeptide-related antigen (Figure 4.37 and 4.38).

6. References

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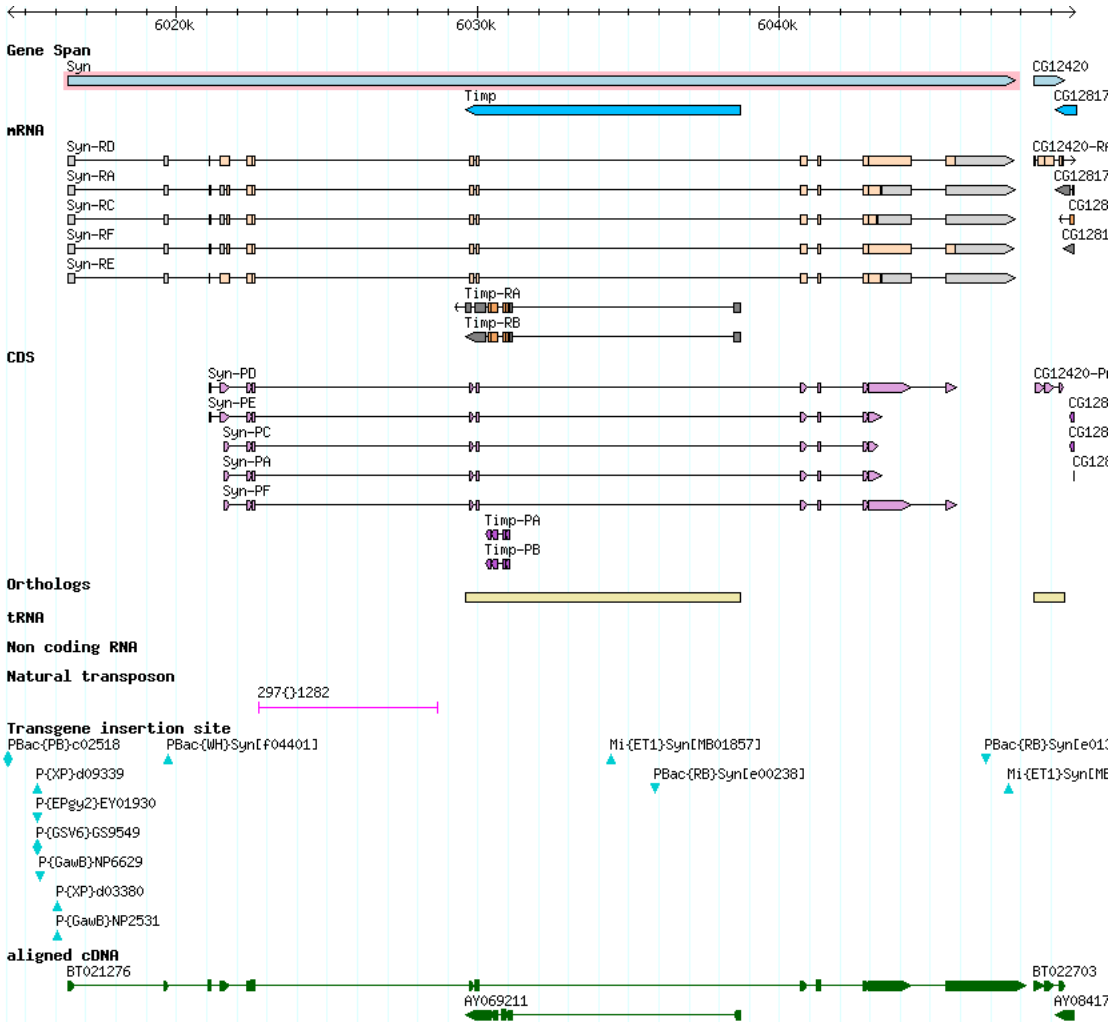
7. Appendix

7.1 Synapsin gene



Source: Flybase

7.2 Synapsin genspan

Caps; Transcripts: bold; CDS underlined; aligned ESTs italics; aligned cDNAs: red, deleted in Syn97

A*GTGAAAATTTCATCACCGTCGTTGACAGTTCAGCCAAAATTCTCTAGCTCCACAAAATT*
CGCCCAGCCAAATCTCAAAATTATCTAAACTACATTTTTTGTTCACTTTTGATCATTAAA
AGTCATCGCTTAACGTTCATCGGCCATTCGAGTGGAAATTTGTGAGCTCGAGCAGGAATA
*TTCAAGTGAATTTTTAGCTCTTTTCG*GTAAAGAAAAACGCAATTACCCAAAAATCGAAAC
ACGGCTAAAGTGGAAAAAAAAATATATAAGAAATTTCTGTATCAGCTAGTCAGCGTTGTT
TGCCCCCAGTGTGAGTGCAAGTGTCTGTGTGTATTTTGTGTGTGTGCGAGTGTGTGTGGG
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CCCGAGGTTAGAGAGGCACCATCGCTTTATTGAATTACAAGTAAAACAAAGAGCGCGACT
TAGCTGGCGATTAAAGAGTCCTCAAAAGGGGAGAGGTGGAGGGGAAGTCGGAAAAAAAAG
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GCTTTAGATTCAACTTGTTTTTGATCTTAGCTATTGGTTCTGGGTAACTGAAACTATTTT
TAAAACGAATTATCTTAGTTAAAGAACATCAGAAAATGCTTTCTGATATATCCAATAAGC
*TTAAATTTATACGCACAAGCCCAATTTTATTTCTTGAGTTCT*AAAGTGGCAAAATCTTTA
TAGCTTAACTAAAAGCCATAAAAGTCTTTGACCCCAGACGGAAATAGCTGCCACAATTTG
CGTGAGCCAACGGTACAATTTACTCTGAAATGCTTGGCAGTTTTAATTACCATCGAAAAG
ACATTACGCCCTGTTTTATGACAAGCCAACGCACATATCAACACAAAGCATTCTCGTAAG
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GGATGTAGAGTCCTGCATTTACCCATTCCGCAACTCGTTCTCGCATTGCAGTTTATATAA
AACGGTTGACAGAAAATGCAACTGCTTAAGCTGCCATAAAAGGAAACTTTAATTTGCCAC
TGACGTTGATGGATTGTGTTGCAACTTCGCTCTTACCGGGAAAATAGCCCAAGCCACCCA
CAAGATCGGGGGGTTAAGCCGGGCCATAAAGTTTTTCCAGCACAGATTTGTGCTGGGTAC
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AGACAGAACGGCGTCCCTGCGTATACGTAATCTTCTTAGCCACAGCAACTCATTATTAGA
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7.3 List of Tables

7.4 List of Figures

7.5 List of Abbreviations

ADAR	Adenosine Deaminase Acting on RNA
ADP	Adenosine Diphosphate
AL	Antennal lobe
Ala	Alanine
ANOVA	Analysis of Variaciance
AP	Action potential
APS	Ammonium Persulfate
ATP	Adenosine Trisphospate
BDNF	Brain-derived neurotrophic factor
BRP	Bruchpilot
BSA	Bovine Serum Albumin
BSTA	BSD domain-containing signal tranducer and AKT interactor
Ca^{2+}	Calcium
Ca^{2+}/CaM	Calcium-calmodulin
Cav	Calcium channels
CaMKII	Calcium-calmodulin dependent protein kinase II
CAMS	Cell-Adhesion Molecules
cAMP	Cyclic Adenosine Monophosphate
Cdk-*5*	Cyclic-dependent Kinase *5*
CMC	Carboxymethylcellulose
CS	Canton S
CSP	Cysteine String Protein
DAB	Diamino benzidine
DMP	Dimethylpimelidate
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphates
DSHB	Developmental Studies Hybridoma Bank
DTT	Dithiothreitol
EB	Ethyl Butyrate
EDTA	Ethylenediamine Tetraacetic Acid
EFC/F-BAR	Extended Fer-CIP4 Homolog Domains
EGTA	Ethylene Glycol Tetraacetic Acid

ENTH	Epsin N-terminal Homology Domain
Eph	Ephrin
EPS15	Epidermal Growth Factor Receptor Substrate 15
EPSP	Excitatory postsynaptic potential
EtOH	Ethanol
GDP	Guanosine Diphosphate
GFP	Green Fluorescent Protein
GTP	Guanosine Triphosphate
Hyd	Hydrophobic
HRP	Horseradish Peroxidase
IF	Immunofluorescence
Ig	Immunoglobulin
IP	Immunoprecipitation
IPSP	Inhibitory postsynaptic potential
kb	KiloBases
kDa	KiloDalton
mAbs	Monoclonal Antibodies
MAPK	Mitogen-Activated Protein Kinase
MB	Mushroom Bodies
MES	2-(N-morpholino) Propanesulfonic Acid
MOPS	3-(N-morpholino) Propanesulfonic Acid
mRNA	Messenger Ribonucleic Acid
NGS	Normal Goat Serum
NMDA	N-methyl-D-aspartate
NMJ	Neuro Muscular Junction
NSF	N-ethylmaleimide-sensitive fusion protein
OSN	Olfactory sensory neurons
PBST	Phosphate Buffered Saline with Tween 20
PCR	Polymerase Chain Reaction
PDF	Pigment Dispersing Factor
PDZ	Postsynaptic density proteins, Drosophila disc large tumor suppressor, Zonula occludens-1 protein
PFA	Paraformaldehyde
PKA	cAMP-dependent Protein Kinase A

PIPK1γ	Phosphatidilinositol Phosphate Kinase Type 1 γ
PTM	Post-Translational Modifications
PVDF	Polyvinylidene Fluoride
RP	Resting potential
SDS	Sodium Dodecyl Sulphate
Sap47	Synaptic Associated Protein of 47 KDa
Ser	Serine
SNAP	Synaptosomal Associated Protein
SNARE	SNAP Receptor
STH	Short Term Habituation
SV	Synaptic Vesicles
Syn	Synapsin
SynCAM	Synaptic Cell Adhesion Molecule
TBE	Tris/Borate/EDTA
TBST	Tris Buffered Saline and Tween 20
TEA	Triethanolamine
TEMED	Tetramethylethylenediamine
Thr	Threonine
TIMP	Tissue Inhibitor of Mealloproteinase
Tyr	Tyrosine
UAS	Upstream Activation Sequence
VAMP	Vesicle Associated Membrane Protein
WB	Western Blot
ºC	Celsius
µm	Micrometers

7.6 Affidavit/ Eidesstattliche Erklärung

Affidavit

I hereby declare that my thesis entitled "***Studies of synapsin phosphorylation and characterization of monoclonal antibodies from the Würzburg hybridoma library in Drosophila melanogaster"*** is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I verify that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg.....

Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation "***Untersuchungen der Phosphorylierung von Synapsin und Charakterisierung monoklonaler Antikörper der Würzburg Hybridoma Library in Drosophila melanogaster"*** eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg.....

Datum Unterschrift

8. Curriculum vitae

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Sep-Oct 2009 Erasmus exchange program, University of Würzburg, Germany

Publications:

1) **Blanco Redondo Beatriz,** Bunz M, Halder P, et al.; Identification and Structural Characterization of Interneurons of the Drosophila Brain by Monoclonal Antibodies of the Würzburg Hybridoma Library, *Plos One,* 2013 8 (9):e75420.doi:10.1371/journal.pone.0075420
2) Sadanandappa Madhumala K, **Blanco Redondo Beatriz,** et al.; Synapsin function in GABA-ergic interneurons is required for short-term olfactory habituation, *The Journal of Neuroscience,* 2013 33 (42):16576-16585;doi:10.1523/JNEUROSCI.3142-13.2013

Abstracts:

1) **B. Blanco Redondo**, M. K. Sadanandappa, Birgit Michels, Bertram Gerber, K. VijayRaghavan, Mani Ramaswami, Erich Buchner (October 2013). Studies of synapsin phosphorylation in *Drosophila melanogaster*. Poster presentation at the 2013 meeting Neurobiology of Drosophila, Cold Spring Harbor Laboratory, New York City, USA
2) **B. Blanco Redondo**, A. Hofbauer, B. Michels, S. Diegelmann, B. Gerber, M. Sendtner, E. Buchner (October 2012). Studies of synapsin phosphorylation and characterization of monoclonal antibodies from the Würzburg hybridoma library

in *Drosophila melanogaster*. Poster presentation at the 7th International Symposium organized by the students of the Graduate School of Life Sciences of the University of Würzburg, Germany

3) **B. Blanco Redondo,** A. Hofbauer, B. Michels, S. Diegelmann, B. Gerber, M. Sendtner, E. Buchner (September 2012). Studies of synapsin phosphorylation and characterization of monoclonal antibodies from the Würzburg hybridoma library in *Drosophila melanogaster*. Poster presentation at the International Symposium of the Neurofly 2012 in Padova, Italy
4) M. K Sadanandappa, I. Twick, **B. Blanco Redondo**, A Dervan, J Anthony Lee, B. Gerber, V. Rodrigues, E. Buchner, K. VijayRaghavan. Ramaswami M. (September 2012). Synapsin functions in GABAergic interneurons for short-term olfactory habituation. Poster presentation at the International Symposium of the Neurofly 2012 in Padova, Italy
5) **B. Blanco Redondo,** A. Hofbauer, B. Michels, S. Diegelmann, B. Gerber, M. Sendtner, E. Buchner (April 2012). Studies of synapsin phosphorylation and characterization of monoclonal antibodies from the Würzburg hybridoma library in *Drosophila melanogaster*". Poster presentation at the International Symposium "Molecular models of diseases of the nervous system" organized by Sonderforschungsbereich 581 in Würzburg, Germany
6) **B. Blanco Redondo**, A. Hofbauer, B. Michels, S. Diegelmann, B. Gerber, M. Sendtner, E. Buchner (October 2011). Studies of synapsin phosphorylation and characterization of monoclonal antibodies from the Würzburg hybridoma library in *Drosophila melanogaster*. Poster presentation at the 6th International Symposium organized by the students of the Graduate School of Life Sciences of the University of Würzburg, Germany
7) **B. Blanco Redondo,** A. Hofbauer, B. Michels, S. Diegelmann, B. Gerber, M. Sendtner, E. Buchner (July 2011). Characterization of monoclonal antibodies from the Würzburg hybridoma library and studies of synapsin phosphorylation in *Drosophila melanogaster*". Poster presentation at the 22nd Neurobiology PhD Student Workshop, Department of Zoology, University of Bonn, Germany
8) **B. Blanco Redondo**, E. Asan, N. Funk, V. Nieratscheker, T. Nuwal, N. Nuwal, A. Schubert, M. Jauch, D. Wagh, E. Buchner (May 2011). Synapsin, SAP47, Bruchpilot, SRPK79E: Functional analysis of presynaptic proteins and their interactions at the neuromuscular junction of *Drosophila*. Poster presentation at the Department of Clinical Neurobiology. Herrman und Lilly Schilling Stiftung Meeting, Würzburg, Germany

Invited talks:

1) "Studies of synapsin phosphorylation in the brain of Drosophila". Invited by Prof. Dr. Heinrich Reichert, University of Basel (Nov 2012)
2) "Studies of synapsin phosphorylation in the brain of Drosophila". Invited by Prof. Dr. Bertram Gerber, University of Magdeburg (Nov 2012)
3) "Studies of synapsin phosphorylation in the brain of Drosophila". Invited by Prof. Dr. Stephan Sigrist, University of Berlin (Oct 2012)

Workshops and seminars:

1) Olympus Microscopy Workshop (22-23 March, 2012) organized by Institute for Clinical Neurobiology, University Hospital Würzburg, Germany
2) Workshop at Graduate School of Life Science (GSLS): Becoming a Better Academic Writer (1-2 July, 2011); Poster Presentation (15 July, 2011); Cover

Letter & CV (4-5 July 2013); Writing Scientific Publications (9-10 July 2013), University of Würzburg, Germany

3) Monthly Drosophila meeting, (Personal oral presentation, once per year), Biocenter of Würzburg, Germany
4) Weekly PhD Journal Club, Progress Report, Lab Meeting (Presentation every 6 weeks) University of Würzburg, Germany

Grants:

1) Graduate School University of Würzburg and the DAAD-initiative "A New Passage to India" funded by BMBF (2925 €). (TIFR National Center for Biological Sciences (NCBS) Bangalore, India. February-April 2013) (Awarded on a competitive basis)

Memberships:

1) International Brain Research Organization (IBRO)
2) Graduate School of Life Science Würzburg (GSLS)

Technical experience:

1) **Western blot:**
   Immunoprecipitation, 2D gel electrophoresis, Silver and Coomassie staining, 16-BAC gels, protein precipitation
2) **Molecular Biology:**
   Reverse Transcriptase PCR
3) **Cryostat**
4) **Immunohistochemistry**
5) **Confocal Microscopy (Leica SP5, Olympus FV1000):**
   Analysis of the brain and neuromuscular junction (NMJ) in Drosophila
6) **Cell culture:**
   Hybridoma in Drosophila, Motoneuron cell culture in mice
7) **In vivo behavioral assays:**
   Short- and long-term olfactory habituation behavioral (Y-maze) in Drosophila and larva behavioral studies

9. Acknowledgments

First and foremost, the person I place my sincere gratitude is Prof. Dr. Erich Buchner for his kind supervision. It was a great pleasure to work in your group. Thanks for rely on me and help me with everything since I arrived to Würzburg. I'm thankful for giving me the opportunity to visit other countries and interact with other researchers in meetings and exchanges programs. Together with Sigi, you have become a family for me. It is an honor for me to be your last PhD student. Thank you for everything!

I'm also thankful to my co-supervisor Prof. Dr. Michael Sendtner. Thanks for giving me the opportunity to join your group and work in your department. I'm very grateful for all the suggestions and help I have received from you. I also want to express my gratitude to Prof. Dr. Eduardo Soriano, my third supervisor. Thanks for helping me, as far as possible, from Barcelona. I'm especially thankful to the DAAD STIBET for helping me during the last months of my PhD and also to the DAAD-initiative "A New Passage to India" for giving me the opportunity to visit India and join the NCBS lab in Bangalore during two months.

I cannot forget those who were my supervisors during my stay in India, Prof. Dr. VijayRaghavan and Prof. Dr. Mani Ramaswami. I'm very grateful for the welcome received and for being treated as one of your students. Thank you for all the scientific talks and meetings we had. It was a great experience in which I learned a lot.

To the people from the *Drosophila* meeting, it was very nice to hear about all the projects from the drosophilists in Würzburg. I'm grateful for discussing my projects with you and for the nice inputs I got from you. Special thanks go to the group from Prof. Dr. Thomas Raabe (Thomas, Anna, Kati, Benjamin and Stephanie); it was very nice to discuss my results with you and feel part of your group. Thanks a lot for all your help.

An enormous thanks goes to Madhu with whom I shared lot of experiences in the last year. Together, we visited six different countries in less than one year (Italy, Germany, Switzerland, France, Ireland and India). Furthermore, we had the possibility to collaborate together and publish two papers in a record time! I keep an Indian friend in my memory with an excellent family that I hope to visit soon. Next destiny?

Kati, you have been a big support for me in the MSZ. Thanks for sharing lunchtime, dinners, parties, “fast food days”, and for all advises and help you gave me. Over the months you have become a very good friend.

धन्यवाद to all the NCBS family, especially Elda, Sudeshna, Indu, Ankita and Swati. You treated me like one of you from the beginning and showed me a lot from your culture. Thank you!

I will also have good and nice memories about all Sendtner’s lab. Thanks for sharing with me progress reports, journal clubs, baby journal clubs, summer barbeques and Christmas dinners. I cannot mention everybody but I would like to thank especially Simi, Lena and the Kopfklinik people for the great time we spent together.

I would like to thank all the students from Buchner’s lab. First the PhDs who helped me at the very beginning: Alice, Mandy, Tulip, Nidhi, Alex and specially Partho and the students who were helping me during these years: Kathrin, Marta, Marlene and Dominique as well as the technicians Gertrud, Katharina and Viera. I’m very thankful for all your help.

Gracias a mi compi de piso, Paola, por ayudarme con las correcciones y por el buen tiempo que hemos pasado conviviendo juntas, me llevo una amiga.

Itsaso y Laura, que se han convertido en mi familia aquí. Itsaso, gracias por todo!! Tus visitas al hospital, nuestras comidas, nuestros viajes y nuestras locuras. Nuestro PhD acaba con una incorporación nueva, la pequeña Leyre, ¡la adoro! Laura, la meva xurri i germaneta petita, gràcies per tots els moments bojos que hem compartit. Mil gracias también a todo los demás por los momentos vividos: Sarah, Jessica, Teffy, Elisa, Adrián, Ruben y Patrick. I gràcies també als meus amics de Barcelona per recolzar-me en tot moment, especialment a “Les Nenes”.

Mi agradecimiento más especial va hacia mi familia. Gracias por apoyarme en todo, por aceptar que viva fuera de casa durante muchos años y por nunca quejaros por nada. Sé que echáis de menos el "huracán Za" (que arrasa por donde pasa) tanto como yo os echo de menos a vosotros, pero sé que siempre os tendré y siempre me tendréis. Gracias además por mostrar tanto interés por mi trabajo, aunque no entendáis nada. ¡Os quiero muchísimo!

And to you, Christian, cariño. I could not be more grateful for having moved to Würzburg and for having met the most important person in my life during my PhD. We have been one year between 6000-10000 kilometers far away from each other but soon it will be over and we will be together. I'm very very very thankful for your support during this difficult year and for being close to me despite the distance. Don't forget that Ich liebe dich unendlich!