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Doctoral thesis for a doctoral degree
at the Graduate School of Life Sciences,
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submitted by
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Dedicated to my family.

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"Knowledge is in the end based on acknowledgement." **-Ludwig Wittgenstein**

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ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
APS	Ammonium PeroxydiSulfate
BCA	BiCinchoninicAcid or 2-(4-carboxyquinolin-2-yl)quinoline-4-carboxylic acid
BSA	Bovine Serum Albumin
Ca^{+2}	Calcium divalent cation
CBB	Coomassie Brilliant Blue
CHT	Ceramic Hydroxyapatite
CMC	CarboxyMethylCellulose
CMC	Critical Micelle Concentration
CNS	Central Nervous System
CS	Canton S
CSP	Cysteine String Protein
Cy3	Cyanine3
CyO	Curly of Oster
D	Diffusion coefficient
DMA	N,N-Dimethylacrylamide
DMSO	DiMethyl SulfOxide
DTT	Dithiothreitol or (2S,3S)-1,4-Bis-sulfanylbutane-2,3-diol
ECL™	Enhanced ChemiLuminescence
EDTA	2,2',2",2"'-(Ethane-1,2-Diyldinitrilo)Tetraacetic Acid
EGTA	Ethylene Glycol-bis(2-aminoethylether)-N,N,N′,N′-Tetraacetic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPS-15	Epidermal growth factor receptor Pathway Substrate clone 15
FBS	Fetal Bovine Serum
Fc	Fragment crystallisable
GAL4	Galactose 4 (yeast transcription activator of galactose-induced genes)
GFP	Green Fluorescent Protein
HA	HydroxyApatite
HCS	Hybridoma Cloning Supplement
HEP	Hybridoma Express Plus
HEPES	2-[4-(2-HydroxyEthyl)Piperazin-1-yl]EthaneSulfonic acid
HRP	Horse Radish Peroxidase
HSP70	Heat Shock Proteins of 70 kDa
HT	Hypoxanthine-Thymidine
IAA	2-Iodoacetamide
IEF	IsoElectricFocusing
IgG	Gammaimmunoglobulin

IgM	Macroimmunoglobulin
MGUS	Monoclonal Gammopathy of Undetermined Significance
IP	ImmunoPrecipitation
IPG	Immobilized pH Gradient
κ	kappa chain
mAb	monoclonal Antibody
MS	Mass Spectrometry
MUNC	Mammalian UNCoordinated protein
NEPHGE	Non-Equilibrium pH Gradient gel Electrophoresis
NFDM	Non Fat Dry Milk
NMJ	Neuro-Muscular Junction
NP-40	Nonidet P-40 (Octylphenoxypolyethoxyethanol)
NSF	N-ethylmaleimide Sensitive Fusion protein
PAA	PolyAcrylAmide
PC	Phosphatidylcholine
PDB	Protein Data Bank
PE	Phosphatidylethanolamine
PFA	Paraformaldehyde (Polyoxymethylene)
pH	Power of Hydrogen
PIPES	1,4-PIPerazinediREthaneSulfonic acid
Rab	Ras-related in brain protein
Rhod-PE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)
RER	Rough Endoplasmic Reticulum
RPMI 1640	Roswell Park Memorial Institute 1640 medium
RT	Room Temperature
S	Svedberg unit
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
SNAP	SyNaptosomal-Associated Protein
SNARE	Soluble NSF Attachment protein Receptor
SRPK	Serine/Arginine Protein Kinase
TBCE	Tubulin Binding Chaperone E
TCA	TriChloroacetic Acid
TEMED	N,N,N',N'- TEtraMethylEthyleneDiamine
Tween 20	Polyoxyethylene (20) sorbitan monolaurate
twi	twist (mesoderm specific promoter)
UAS	Upstream Activation Sequence
VAMP	Vesicle Associated Membrane Protein

Chapter 1 INTRODUCTION

1.1 Nervous system to neurons to synapses to synaptic proteins

All living organisms have a fundamental property, i.e. the ability to extract, transform and use energy from the environment which enables them to carry out the various life processes like nutrition, locomotion, reproduction, etc which are performed by specialized organ systems. One such organ system is the nervous system, which has the ability to receive stimuli through sensory organs, process them and evoke required responses. The ability to sense stimuli and react to them was already present in single celled organisms (eg. movement towards the increasing gradient of nutrient concentration by chemotaxis in prokaryotes), but with increase in number of cells in metazoans there was need for the evolution of a more sophisticated organ system that could connect and co-ordinate the various cells as a single responsive system. Besides the various incoming sensory stimuli also needed a processing system to elicit the necessary responses that would enable the organism to respond to a given stimulus in the right way to ensure its survival. Further with increase in complexity in the nature of the incoming signals and the amount of processing required the need for a highly specialized organ system arose. This need was fulfilled by the evolution of the nervous system, which had specialized cells which could capture incoming stimulus, transmit these signals to the processing center and finally transmit the generated response to the effector cells. So this system had the unique ability of transmitting the signals between the cells within the body of the organism also over long distances, in the form of electrical and chemical signals. With its ability to process the input data into meaningful information and generate a suitable response as output, the nervous system was ‘nature’s computer’ with a processor and wires that connected it to all the input and output devices.

The simplest nervous system evolved in organisms of the phylum *Cnidaria* (eg. Hydra) as diffuse network of nervous cells which had long processes to physically connect to other cells over long distances and acted more or less in coordination. In higher animals the specialized nervous cells began to bundle their long processes into fibers and this further evolved into 'nerves', which could transmit the electrochemical messages more efficiently over large distances, analogous to wires carrying electrical signals. The electro-chemical nature of the signals also allowed faster transmission and hence better responsiveness to the system and the organism. Evolution of bilateral symmetry in life forms, led to organization of this system by clustering of sensory nerve cells with photo-, gustatory and olfactory receptors towards the anterior part of the body. This process of 'cephalization' of the most of the sensory organs gave evolution of the body part called 'head' which contained the clustered neurons within as the 'brain' and the bundled nerves extended to the posterior side as the 'longitudinal nerve cord'. The brain and the longitudinal nerve cord together comprised the 'central nervous system' (CNS) and is analogous to the computer's processing unit. The simplest CNS is found in the phylum *Platyhelminthes* (flat worms eg. Planarians). In more complex invertebrates such as Annelida the nervous system has the brain and the ventral nerve cord containing segmentally arranged cluster of neurons called ganglia. Both annelids and arthropods have a ganglion in each body segment (segmental ganglia). Other species in the animal kingdom, such as slow molluscs (eg. Chitons) have little or no cephalization, while cephalopod mollusks like squids have the most sophisticated nervous system among all invertebrates. Gradually in vertebrates the nervous system evolved more to gain the highest level of complexity in form of the human brain. Such complex nervous systems, have specific neural circuits dedicated to carry specific information to specialized centers in the nervous system which are responsible for processing of the

information. So the nervous system of higher organisms is a large aggregate of numerous processing centers which are connected to each other in a complex network, much more complex than the most complicated network of the circuit elements of even the most advanced microporcessor. The enigma of such complexity of the neuronal networks however could not discourage scientists from studying the organization of these networks. One of the first milestones in the elucidation of this network was the 'reticular theory' proposed to explain the morphology and functioning of the nervous system (Golgi, 1906), and was later replaced by the 'neuron doctrine' (Ramón y Cajal, 1906).

The unique electrical and physiological functions of the nervous system are due to the special structural and biochemical properties of the cells that constitute it. Cells of the nervous system are broadly classified into two major types:

a) Neurons b) Glia.

Neurons (coined by Ramón y Cajal) are electrically excitable cells, which can generate of a plasmalemmal 'all-or-none' action potential, capable of propagating through surface of the cell membrane as an ionic current due to sequential opening and closing of specific ion channels. The signal is further transmitted between two cells as a chemical or electrical signals. Hence neurons transmit the signals over long distances through a given network. They possess special electrical and chemical properties to carry out these functions. Glia on the other hand are nonexcitable cells of the nervous system. Various types of glia play diverse roles in genesis, development, nutrition, control, damage repair etc of adjacent neurons and their circuits. According to current estimates the adult human brain possesses around 100 billion neurons and around 9-fold more glial cells (Verkhratsky and Butt, 2007).

A typical neuron consists of a cell body (soma/ perikaryon) and long cellular processes called neurites which are of two major types namely dendrites and axons. Besides other common organelles the soma contains Nissl bodies, which are aggregates of rough endoplasmic reticulum (RER) and free ribosomes acting as sites of active protein synthesis. Dendrites are highly branched extensions of the soma that increase the surface area and can account for up to 90% of the cytoplasmic membrane area of a typical neuron (Martini and Bartholomew, 2010). Distinctive features of neurons are their axons, usually a single, long cylindrical process that carries the information received at the soma to other cells as electrical impulses. It lacks most organelles, but is rich in cytoskeletal elements and transport vesicles for delivering any required cellular machinery to the distal end of the neuron. It also contains mitochondria to deliver the energy for all its local activities.

Besides their special electrical and chemical properties, neurons also have specialized morphological features to carry out the functions of information transmission. The transmission of information between cells of a neural circuit occurs via points of contacts between cells. The specialized contacts where the signals are transmitted from one neuron to the other were named ‘synapses’ (from the Greek word *synaptein*, meaning to bind together) by Sir Charles Scott Sherrington (Sherrington, 1906). The majority of synaptic inputs from other neurons are usually received at the dendrites.

A typical chemical synapse has two parts: the presynaptic side (Murthy and De Camilli, 2003) comprising enlarged structures at the distal part of the axon called presynaptic terminals (by Ramón y Cajal) which abut the postsynaptic side comprising postsynaptic densities and receptors, etc (Sanes and Lichtman, 2001).

The two sides are separated by a narrow gap of 10-200 nm, called the synaptic cleft. Signal transmission across the synaptic cleft in the majority of synapses occurs chemically. In these chemical synapses, specific messenger molecules called neurotransmitters are contained in membranous sacs, which were discovered by electron microscopy and were called 'synaptic vesicles' (SVs) by Bernard Katz. The neurotransmitters are released by exocytosis (Fernandez-Chacon and Südhof, 1999) at specialized presynaptic sites, called active zones (Schoch and Gundelfinger, 2006) in response to Ca^{+2} influx caused by the incoming electrical signal. (Lin and Scheller, 2000; Pang and Südhof, 2010). However, chemical synapses are 'reliably unreliable' and every action potential is not converted into a secretory signal. Infact in most terminals, only 10%–20% of action potentials trigger an actual release (Goda and Südhof, 1997). The neurotransmitter molecules released by the fused SVs then diffuse across the synaptic cleft and bind to their corresponding receptors on the postsynaptic side, leading to an electrical or chemical signal in the postsynaptic cell. Thus the information travels down a neural network as a train of electro-chemical signals. In an electrical synapse (Meier and Dermietzel, 2006), on the other hand, the signal is transmitted only as current (ionic fluxe) through special ion pores called connexons, which form form a specialized form of tight-junction called gap junction. Electrical synapses constitute only a small proportion of all synapses in an adult brain. Other types of tight-junctions are more abundant in epithelial and muscular tissues (Meşe et al., 2007). Fusion of SVs in response to a membrane depolarization is called active release. The random fusion of single SVs in absence of any electrical stimulus is called spontaneous release (Fatt and Katz, 1952) and generates small postsynaptic currents called miniature postsynaptic current. Inspired by Max Planck's 'Quantum Theory', Katz described the event of fusion of an individual SV as discrete quanta of neurotransmission which is popularly referred to as the 'quantal release theory'

(Del Castillo and Katz, 1954). It was later replaced by the 'vesicle release theory' (Del Castillo and Katz, 1955). Today is it estimated that, an average 42 nm sized SV contains ~1500 neurotransmitter molecules that can be released simultaneously (Haucke et al., 2011). Infact the SV is often considered the most well characterized organelle of the cell (Takamori et al., 2006).

Each neuron with its highly branched structure, has the possibility to form numerous synpases with many adjacent cells. The synaptic connections of a typical circuit comprise a dense tangle of dendrites, axons terminals and glial (cellular) processes that are together called 'neuropils'**.** With a high cell density, numerous possibilities to intercommunicate and the inherent property of nervous system to comprise of complex neural circuits, the human brain is probably the 'most complex network system' in the known universe. Modern neuroscience aims to understand the functioning of this complex system. It was probably the increasing synaptic connectivity and the consequent increase in complexity of the brain that led to the evolution of higher brain functions like consciousness, perception, attention, cognition, thought, emotions, behavior, learning and memory etc. The molecular basis of these higher brain functions is still far from being well understood and a substantial part of the modern neuroscientific community continues to pursue this aspect. Learning and memory are among such higher brain functions, which have been widely studied at our department for quite some time. Learning can be simply be defined as the modification of existing (or acquisition of new) knowledge, behaviours, skills, etc., while memory can simply be defined as the ability to store, retain, and recall information and experiences. Memory is thought to arise as a 'memory trace' and then stored as an 'engram'. An engram is a hypothetical means by which memory traces are stored as biophysical or biochemical changes in the brain (and other neural tissue) in response to external

stimuli. One such theory of memory formation is the synaptic tagging and capture hypothesis (Redondo and Morris, 2011). The synapse has the ability to change its strength (level of activity) in response to stimuli by either use or disuse. Such activity-dependent modification of synaptic strength is called synaptic plasticity and is thought to be one of the possible mechanisms of formation of the traces leading to memory formation (Martin and Morris, 2002). Synaptic plasticity is thus considered one of the important neurochemical foundations of learning and memory (Kandel, 2000). Synaptic plasticity comprises both:

i) morphological changes in synapses, i.e. changes in number of synapses (formation of new synapses to strengthen an existing circuit or loss of existing synapses for abating/removing a circuit) and,

ii) functional changes in synapses i.e. increasing the rate of activity of a synapse or decreasing its rate of activity for a given amount of stimulus.

These structural and functional changes in synapses ultimately influence the fundamental process of synaptic neurotransmission. Hence the understanding of synaptic neurotransmission is required for the understanding of synaptic plasticity. Fusion of synaptic vesicles at the active zone is followed by retraction of the fused vesicle by endocytosis, such that it can be refilled with neurotransmitters and reused for neurotransmission (Heuser and Reese, 1973). This recycling of SVs has evolved for enable synapses to quickly prepare themselves for continued neurotransmission. This is of particular important for highly active synapses which need to maintain a high rate of transmission (Shupliakov and Brodin, 2010). The whole process of from SV fusion to its endocytic re-uptake and reuse is called the 'synaptic vesicle cycle' illustrated in the Fig. 1 below. Each of the steps including docking, priming, fusion and recycling are a highly co-ordinated process executed by the complex interplay of several proteins acting at the SV surface and active

zone (Jahn et al., 2003; Sudhof, 2004). Fusion itself is highly interesting process involving a wide repertoire of highly conserved proteins as shown in Fig. 2, notable among which are: SNAREs (Jahn and Scheller, 2006), and SM proteins (Carr and Rizo, 2010). The neuronal synapse is thus a highly active site of each neural unit and its proper functioning is indispensible for normal neural activity. The synapse contains many proteins which contribute to its structural and functional aspect. Significant among them are Piccolo, Bassoon, Rabs, Synapsins, CSPs, SNAPs, Syntaxins, VAMPs, etc some of which are shown below in Fig. 2. These proteins often exist as multiple isoforms each of which has its own functions (Richmond, 2007).

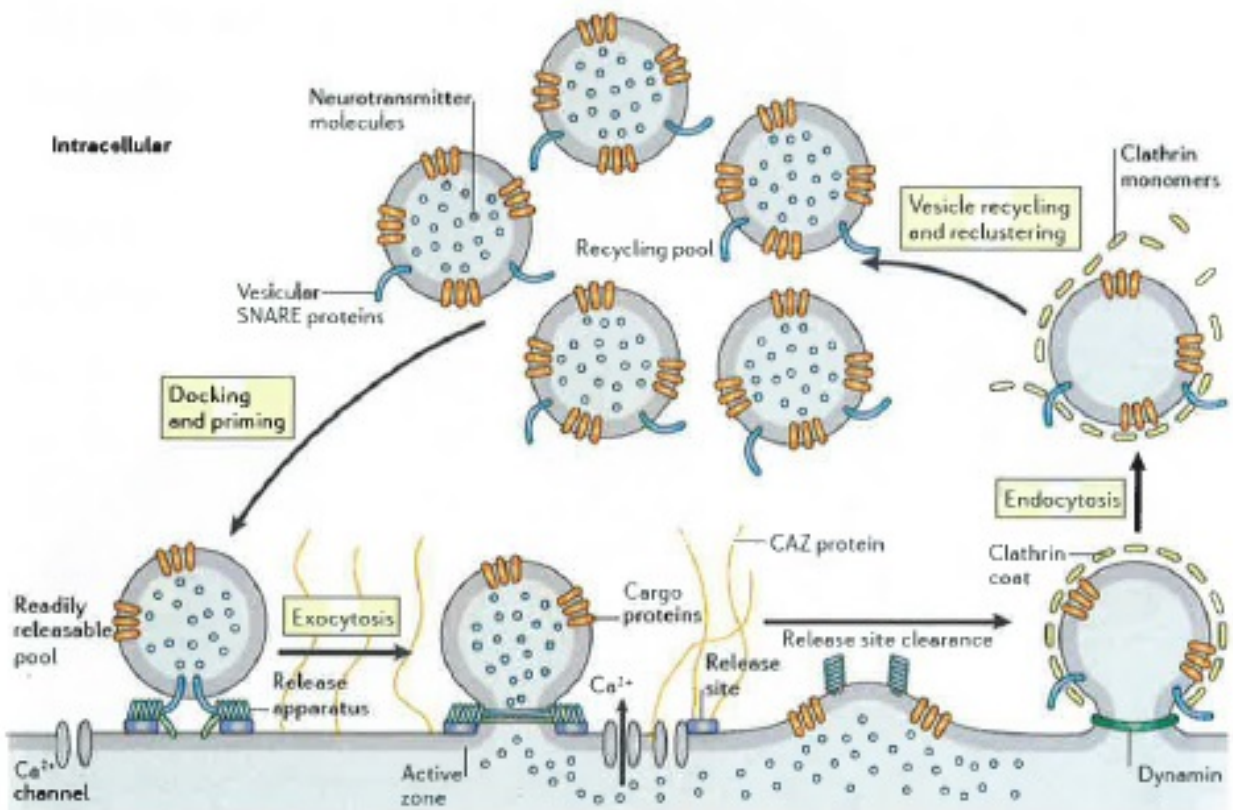


Fig. 1. Synaptic vesicle cycle (modified from Haucke et al., 2011).

Our group works on synaptic proteins in *Drosophila melanogaster*. The aim is to identify novel proteins in the synapses of *Drosophila melanogaster* and to understand their role in the structural organization and functioning of the synapse.

Synaptic proteins studied in our lab include Synapsins (Klagges et al., 1996), Bruchpilot (Wagh et al., 2006; Kittel et al., 2006), SAP-47 (Reichmuth et al., 1995), CSP (Zinsmaier et al., 1990), SRPK 79D (Nieratschker et al., 2009), TBCE-like etc. The identification of many such novel synaptic proteins was facilitated by the monoclonal antibodies of the Wuerzburg Hybridoma Library (Hofbauer 1991; Hofbauer et al., 2009). Besides synaptic vesicles that different kinds vesicles (endosomes, secertory vesicles, etc.) also occur in all types of cells, further makes the study of synaptic neurotransmission significant in elucidating the fundamental mechanism of membrane fusion (Söllner, 2004). Drosophila has been chosen as the experimental model organism for these studies because of its advantages.

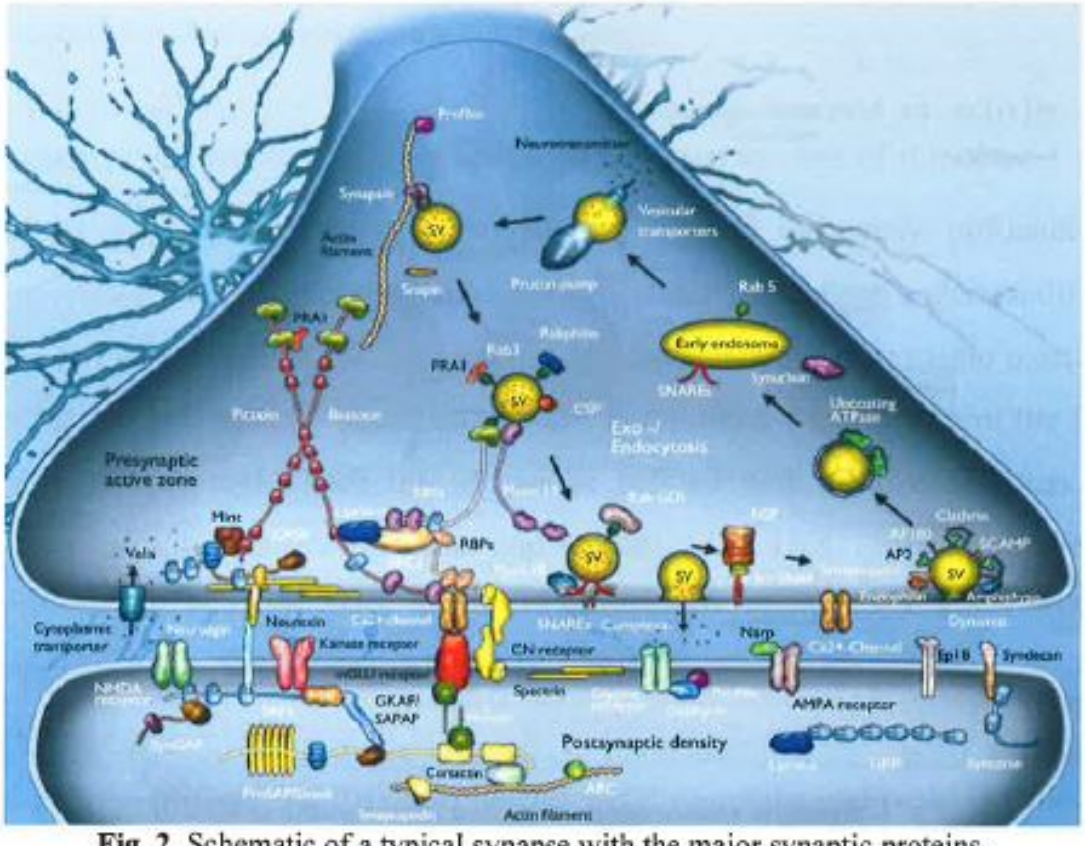


Fig. 2. Schematic of a typical synapse with the major synaptic proteins. (Adapted from http://www.sysy.com/flash-synapse/index2.html)

1.2 *Drosophila* as a model organism: *Drosophila melanogaster* is commonly known as the fruit fly and is a dipteran insect as illustrated below.

Kingdom	:	*Animalia*	Phylum	:	*Arthropoda*
Class	:	*Insecta*	Order	:	*Diptera*
Family	:	*Drosophilidae*	Subfamily	:	*Drosophilinae*
Genus	:	*Drosophila*	Subgenus	:	*Sophophora*
Species	:	*D. melanogaster*			

Charles W Woodworth began breeding fruit flies and proposed their use as model organism for genetic studies to William E Castle who further passed it on to Frank E Lutz who then introduced them to Thomas Hunt Morgan (Morgan, 1934). Features like short generation time, high reproductive rate, reasonable costs, ease of handling, etc made it an instant favorite. While the ethical constraints of vertebrate model organisms were not involved, most of the vertebrate genes were present due to high degree of evolutionary conservation making the fruit fly a valuable experimental model. Consequently it became the model which led to many seminal discoveries were made by Morgan's group in the 'Fly Room' of the Columbia University which enlightened almost all aspects of biology. The gradual development of various genetic tools (Ryder and Russell, 2003) for genetic manipulations like transgenic expression, miss-expression, mutagenesis, etc., facilitated *Drosophila* as one of the most powerful tools for genetic research. The possibility of spatio-temporal control of gene-expression using binary expression systems like the UAS-GAL4 (Elliott and Brand, 2008) further enhanced the possibilities in genetic research using *Drosophila*. Today more than a century after its introduction, *D. melanogaster* is one of the best established model organisms used in almost all facets of biological research (Arias, 2008) including neuroscience (Bellen et al., 2010). Inspite of being an invertebrate it is a suitable model to study vertebrate diseases (Botas, 2007; Doronkin and Reiter, 2008) including neurodegeneration (Lu and Vogel, 2009; Ambegaokar et al., 2010).

My doctoral project also aimed to identify and characterize synaptic proteins in the

fruitfly, *Drosophila melanogaster* with the help of monoclonal antibodies from the Wuerzburg Hybridoma Library.

1.3 Monoclonal Antibody of the IgM class: The Wuerzburg Hybridoma Library is a valuable resource as it contains more than 200 monoclonal antibodies. Monoclonal antibodies (mAbs) are one of the most important tools in modern biology and have come a long way from being mere analytical tools to becoming indispensible in diagnostics as well as therapeutics (Albrecht et al., 2009). They are used in treatment for a wide variety of diseases like cancers (Weiner et al., 2010), immunological disorders (Chan and Carter, 2010), respiratory diseases (Schachter and Neuman, 2009), infectious diseases (Lachmann, 2009). Today, mAbs represent over 30% of all biological proteins undergoing clinical trials and are the second largest class of biodrugs after vaccines (Elbakri et al., 2010) and with the advent of more efficient, genetically engineered antibodies (Cuesta et al., 2010; Li and Zhu, 2010) this trend is expected to grow (Aires da Silva et al., 2008; Nelson et al., 2010). Non-allowance of ascites in many countries and cumbersomeness of other methods makes hybridoma cell culture as the most popular methods even today.

Antibodies can be of different classes or isotypes as based on their heavy chain Fc regions (Mix et al., 2006). The different isotypes have different structural and chemical properties, which explains their specific and diverse roles in the immune system (Schroeder and Cavacini, 2010). IgM is one such class of antibodies which has been unique, right from their discovery as the horse anti-Type I *Pneumococcus* polysaccharide antibody which was heavier (19S) than the (7S) rabbit anti-Type III *Pneumococcus* polysaccharide antibody (Heidelberger and Pedersen, 1937). Due to its larger size it was called ‘macroglobulin’, leading to its nomenclature as IgM, while the 7S isotype is what we now call IgG (Cohen, 1965). Elevated levels of IgMs were detected by Jan Gösta Waldenström (Waldenstom, 1944) in a condition

which is now called Waldenström's Macroglobulinemia (Neparidze and Dhodapkar, 2009) and is now classified (McMaster and Landgren, 2010) under a wider group of diseases called IgM monoclonal gammopathy of undetermined significance (IgM-MGUS). Today it is known that IgMs are expressed as membrane-bound monomers (~180 kDa) on all naïve B1 cells even in the absence of an apparent antigen and constitute the major component of the 'natural' or 'innate' antibodies. So they are the first isotype of antibodies to be produced prior to the onset of class switch recombination (CSR) and somatic hypermutation (SHM). As a consequence their affinity is often lower than that of other isotypes. Upon antigenic stimulus (immunization or infection) IgMs are thus the first class of antibodies produced during a primary antibody response, hence forming the first line defense against invading pathogens and maintaining tissue homeostasis by regulating the clearance of cellular debris. Such IgMs are usually secreted as pentamers with a J chain and are around 970 kDa in size (Saltzman et al., 1994) or hexamers without the J chain and are around 1.15 MDa in size (Randall et al., 1990). The polymeric structure of IgM (Perkins et al., 1991), as seen in Fig. 3 illustrates 10 antigen binding sites, bestowing higher valency to IgMs and allowing them to bind to antigens with a wide range of avidities. This makes IgMs more efficient than other isotypes in causing agglutination or clumping, which facilitates the removal of foreign pathogens or antigens. The lower affinity and higher valency makes IgMs polyreactive, and enables them to recognize antigens with repeating epitopes, like bacterial surface polysaccharides (Cutler et al., 2007). Thus IgMs play a significant role in the immune system (Vollmers and Brändlein, 2006) and their properties have been utilized in immunotherapy of a wide range of diseases like arthritis (Odani-Kawabata et al., 2010), graft-versus-host diseases (Godder et al., 2007; Waid et al., 2009), neurological disorders (Rodriguez et al., 2009) or infectious diseases (Lu et al., 2011). The natural immunity of humans

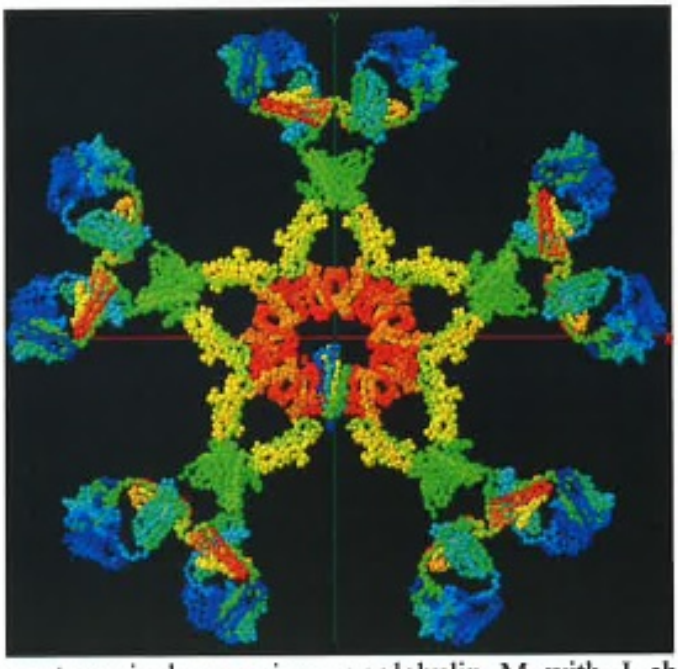


Fig. 3. Structure of pentameric human immunoglobulin M with J chain. This image was generated using the PDB file 2RCJ (Perkins et al., 1991) with the Jmol viewer (version 12.0.18).

against cancer cells consists almost exclusively of IgMs (Schwartz-Albiez et al., 2009; Vollmers and Brändlein, 2009) and so they have also been used in immunotherapy for cancers (Azuma et al., 2007; Bieber et al., 2007; Beutner et al., 2008). Interestingly IgMs have also been proposed as AIDS vaccines (Yang, 2009).

Infact, the first monoclonal antibody, Sp1 generated by the first hybridoma cell line was also an IgM (Köhler and Milstein, 1975). However IgMs remain to be the least studied and most enigmatic of all the isotypes. This is mainly due to the considerable differences in biochemical and structural properties of IgMs with respect to the other isotypes which makes it difficult to purify and study them by standard methods well established for the other isotypes. Due to their polymeric nature IgMs are around a megadalton in size and hence less soluble. So they tend to aggregate and are prone to precipitation at lower temperatures. Gel filtration chromatography is not suitable to purify IgMs, as it is based on diffusion and IgMs

with very low D (diffusion coefficient) values around 3.2±1.4 x 10^{-7} cm^2/s (Saltzman, 1994) reduce the capacity and resolution of the method. IgMs are denatured by extreme pH thus making affinity chromatography difficult. Solubility is also reduced at low conductance, thus making ion exchange chromatography unsuitable. Precipitation at low conductance was exploited in the method of euglobulin precipitation (García-González et al., 1988), based on which the technique of euglobulin adsorption chromatography (Bouvet et al., 1984) was developed. However, the method has problems like non-specificity leading to contamination with other co-precipitated proteins and low efficiency resulting in substantial loss of sample. Strong hydrophobic surfaces also denature IgMs thus ruling out hydrophobic interaction chromatography as an option for IgM purification. Furthermore IgMs do not bind well to protein A or G and hence they cannot be affinity purified like other isotypes, using these immunoglobulin-binding bacterial proteins (Ey et al., 1978). Protein L (Björck, 1988) does bind to IgMs with κappa-I type of light chain (Nilson et al., 1993), but it also binds to other isotypes with suitable light chains types (De Château et al., 1993) and hence is not useful for specific purification of IgMs from serum. Thus the unique biochemical and structural properties of IgMs makes them difficult to purify and study by standard methods established for other isotypes (Mahassni et al., 2009; Gagnon, 2009). The growing importance of IgMs and the difficulties in their purification called for effective yet simple methods for their purification. This would allow more detailed studies of their properties and functions and help to improve the availability and effectiveness of IgMs in various biological and medical applications. Hydroxyapatite (HA) column chromatography has been long used for purification of proteins by chromatography (Hjerten et al., 1956) and has found various applications in protein purification (Cummings, 2009). IgMs being more charged than IgGs bind to HA much more strongly than IgGs and contaminants,

thus HA column chromatography is a simple method for quick and easy purification of IgMs under mild conditions with a high recovery rate, yielding substantially purified, immunoreactive antibody in concentrated form (Henniker and Bradstock, 1993; Aoyama and Chiba, 1993). Upon selective desorption on ceramic hydroxyapatite (CHT), most IgMs typically elute as a characteristic peak between 200 and 300 mM phosphate with gradient elution (Gagnon et al., 2008), thus making it a standard method which needs minimal optimization. The compatibility of HA chromatography with the properties of cell culture supernatants allows the direct application of the hybridoma supernatants to the column with minor modifications but without sample dilution, thus reducing the overall time required.

Many of the mAbs of the Wuerzburg Hybridoma Library are also IgMs. Two such IgMs used in this doctoral work were produced by the cell lines na21 and ab52. Based on the above advantages, we here applied HA based selective desorption chromatography to purify the IgM, produced by the cell line na21 which was generated by serum-free hybridoma culture as described herein. This thesis will further describe the materials, methods and results from the experiments aiming to purify, characterize several mAbs and their target antigens.

Chapter 2. MATERIALS

2.1 Fly strains:

Drosophila melanogaster commonly known as 'fruit fly' was used as the model organism for this doctoral research work. Canton S available in the department was used as the wild-type strain unless otherwise mentioned. For the confirmation of the ab52 antigen, the EPS-15 mutant strain was kindly provided by Hugo Bellen. The genotype was: yw ; $\frac{eps15^{\Delta 29}}{CyO, twi>GFP}$; $\frac{+}{+}$

Other fruit fly species tested for presence of the na21 antigen were obtained from the Drosophila Species Stock Center, San Diego. The species tested were: *D. simulans*, *D. ananassae*, *D. willistoni*, *D. sechellia* and *D. yakuba*.

2.2 Buffers and reagents

2.2.1 SDS-PAGE

2.2.1.1 Mini Protean™ (BioRad®) electrophoresis system:

Gel percentage	12.5%	15%
Reagents	**(ml)**	**(ml)**
30% Acrylamide: bisacrylamide (29:1)	3.125	3.75
1.88 M Tris/HCl, pH 8.8	1.5	1.5
dH_2O	1.375	0.75
0.5% SDS	1.5	1.5
10% APS	0.04	0.04
TEMED	0.006	0.006

Table 1. Resolving gel (reagents for two mini gels)

Reagents	(ml)
30% Acrylamide: bisacrylamide (29:1)	0.5
0.635 M Tris/HCl, pH 6.8	0.6
dH_2O	1.3
0.5% SDS	0.6
10% APS	0.03
TEMED	0.004

Table 2. 5% Stacking gel (reagents for two mini gels)

SDS PAGE Running buffer (10x)	125 mM Tris (15.142 g) 960 mM Glycine (72.07 g) 0.5% (w/v) SDS (5 g) pH adjusted to 8.9 and final volume was made upto 1 liter by dH_2O (stored at room temperature)	
Lämmli buffer (2x)	120 mM Tris	(1.2 ml of 1 M stock, pH 6.8)
	20% (v/v) Glycerol	(2 ml of 100% Glycerol)
	4% (w/v) SDS	(4 ml of 10% stock)
	0.02% (w/v) Bromophenol blue	(0.2 ml of 1% stock)
	5.0% (v/v) β-Mercaptoethanol	(0.5 ml added fresh)
	Final volume made upto 10 ml with dH_2O	

2.2.1.2 Novex Mini XCell™ (Invitrogen®) electrophoresis system:

Gels : precast Bis-Tris NuPAGE™ gels.

Running buffer : 1x MES/ MOPS SDS running buffer from supplier.

Loading buffer : 1x LDS buffer with 1x Reducing agent from supplier.

2.2.1.3 Tricine SDS-PAGE

Components	**7.5%**	**10%**	**12.5%**	**15%**	**17.5%**
30% Acrylamide (Rotipur Gel A)	7.5 ml	10.0 ml	12.5 ml	15.0 ml	17.5 ml
2% Bisacrylamide (Rotipur Gel B)	3.0 ml	4.0 ml	5.0 ml	6.0 ml	7.0 ml
1.5 M Tris-HCl, pH 8.8	7.5 ml	7.5 ml	7.5 ml	7.5 ml	7.5 ml
dH_2O	11.5 ml	8 ml	4.5 ml	1.0 ml	-
Solution degassed for 5 min					
10 % SDS (w/v)	300 µl				
10 % APS (freshly made)	200 µl				
TEMED	20 µl				

Table 3. Resolving gel (8.5 x 16 x 0.1 cm = 30 ml)

Overlaid with dH_2O and allowed to polymerize for ~15 min.

Components	5% Stacking gel
30% Acrylamide	2.5 ml
2% Bisacrylamide	1.0 ml
0.5 M Tris-HCl, pH 6.8	3.7 ml
dH_2O	7.5 ml
Solution degassed for 5 min.	
10% SDS	150 µl
10% APS	200 µl
TEMED	10 µl

Table 4. Stacking gel (1.5 x 16 x 0.1 cm = 15 ml)

Allowed to polymerize for ~10 min.

Tricine Sample Buffer:

Tris	0.1 M	1 ml of 0.5 M stock
SDS	4%	2 ml of 10% stock
Glycerine	20%	1 ml of 99% stock
β-Mercaptoethanol	0.8 M	0.3 ml of 14M stock
Bromophenol blue	0.025%	0.1 ml of 1% stock
dH_2O	0.6 ml to make volume upto 5ml	

2.2.2 Western Blotting:

Transfer buffer (1x)	25mM Tris (6.04 g) 150 mM Glycine (22.52 g) 20% Methanol (400 ml) pH was adjusted to 8.3 and volume made upto 2 liter with dH_2O (stored at 4°C)
Washing buffer (1x TBST)	100 mM Tris (12.11 g) 1.5 M NaCl (87.66 g) 0.5% Tween 20 (5.0 ml) pH adjusted to 7.6 and volume made upto 1 liter with dH_2O to get the 10x stock.
Blocking solution (5% NFDM)	1 g Non fat dry milk powder dissolved in 20 ml of 1x TBST (per mini blot), briefly boiled, filtered.

Development method	ECL™ Western blot detection kit (Amersham GE®)
Detection system	Chemiluminiscence produced by the HRP coupled to the 2° Ab in presence of the luminal substrate was captured as exposures on X-Ray films which were developed using the developer and fixative solutions (Kodak).

2.2.3 Coomassie® Staining:

2.2.3.1 CBB R250:

- Staining solution

MeOH	45%	450 ml
Acetic acid	10%	100 ml
dH_2O	45%	450 ml
CBB R250	0.25%	2.5 g

The mixture was constantly stirred for 3 hrs for proper mixing and then filtered through filter paper.

- Destaining solution

MeOH	30%	300 ml
Acetic acid	10%	100 ml

Total volume was made upto 1liter with dH_2O

- Drying solution

MeOH	20%	200 ml
Glycerol	3%	30 ml

Total volume was made upto 1liter with dH_2O

2.2.3.2 Colloidal CBB G250:

- Fixative

ortho-phosphoric acid (H_3PO_4)	0.85 %	(1.5 ml of 85% stock)
MeOH	20%	30 ml

Final volume was made upto 150 ml with dH_2O

- Staining solution

MeOH	20%	30 ml
dH_2O	65%	90 ml
R250 solution	20%	30 ml Roti®-Blue

Roti®-Blue was added periodically while stirring for 3 hr.

- Destaining solution

MeOH	25%	250 ml

Final volume was made upto 1 liter with dH_2O

2.2.4 Silver Staining: (all solutions should be made fresh)

- Fixative

40% EtOH (20 ml)
10% Acetic acid (5 ml)
Final volume made upto 50 ml by dH_2O

- Wash — 30% EtOH (15 ml)
  Final volume made upto 50 ml by dH_2O
- Sensitizer — 0.02% $Na_2S_2O_3$ (0.01 g)
  Final volume made upto 50 ml by dH_2O
- Silver solution — 0.2% $AgNO_3$ (0.1 g)
  Final volume made upto 50 ml by dH_2O (cooled to 4ºC before use)
- Developer — 3% Na_2CO_3 (3 g)
  0.05% Formalin (50 µl of 35% Formaldehyde)
  Final volume made upto 100 ml by dH_2O
- Stopper — 0.05 M EDTA (0.931 g)
  Final volume made upto 50 ml by dH_2O
- Storage — 1% Acetic acid (0.5 ml Hac)
  Final volume made upto 50 ml by dH_2O

2.2.5 Destaining of silver stained gels: (all solutions should be made fresh)

- Solution A — 3.7 g of NaCl and 3.7 g CuSO4 in 45 ml of dH_2O by constant stirring to get an aqua marine solution, while constantly stirring add 25% NH_4OH to form a light bluish ppt, keep adding drop by drop till ppt turns dark blue and more till the ppt slowly dissolves to yield a clear, transparent dark blue solution.
- Solution B — 0.01 g $Na_2S_2O_3$ in 50 ml dH_2O
- Destaining solution — Mix equal volumes of solutions A and B just before use.
- Stopper — 10% Acetic acid
- Storage — 1% Acetic acid

2.2.6 Immunoprecipitation (IP)

IP lysis buffer (1X)
25 mM Tris (1.514g)
0.15 M NaCl (4.383g)
2 mM EDTA (0.372g)
2 mM EGTA (0.38g)
10% Glycerol (50 ml)
0.1% NP-40 (0.5 ml)

pH adjusted to 7.6 and final volume was made to 500 ml by dH_2O. To 10ml of the above IP buffer 1 tablet of protease inhibitor cocktail (Complete Mini™, Roche®) was added freshly before use and buffer was cooled to 4ºC before use.

2.2.7 Protein precipitation

2.2.7.1 TCA precipitation:

- TCA stock 72% (w/v) TCA
- Acetone ≥99% pure

2.2.7.2 Chloroform Methanol precipitation:

- Chlorofom ≥99% pure
- Methanol ≥99% pure

2.2.7.3 Ethanol precipitation:

- Ethanol ≥99% pure

2.2.8 Hybridoma cell culture:

- HT medium 20% v/v medium 199
  70% v/v RPMI 1640 with Glutamax
  10% v/v fetal bovine serum (FBS),
  1x HT (hypoxanthin-thymidin) cocktail
  1x Anti-Anti (antimicrobial agent)

  All media components (Invitrogen) were mixed freshly before use and filter sterilized through a 0.2 μm filter (Whatman)
- Staining solution Trypan Blue (Sigma)
- Serum free medium 50% v/v HT medium without FBS
  40% HEP (PAA Laboratories)
  10% v/v HCS (PAA Laboratories)

2.2.9 Capture ELISA for Isotyping:

- 1xPBS 10 mM phosphate buffer, 150 mM NaCl, pH 7.4
- Washing buffer 1x PBST (0.05% Tween 20)
- Substrate 5-Aminosalicylic Acid (1 mg/ml) in 0.02 M sodium phosphate, pH 6.8 + 0.01% H_2O_2 (v/v)
- Stopping solution 3 N NaOH

2.2.10 Subcellular fractionation:

- Homogenization buffer: 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA final pH 7.4 supplemented with 1 tablet (per 10 ml buffer) of protease inhibitors mix (Complete Mini™, Roche)

2.2.11 Two dimensional electrophoresis (IEF/SDS):

- Sample homogenizing mix:

Zoom® 2D Protein Solubilizer 1 or 2	91 µl
1 M Tris Base	0.5 µl
100x Protease Inhibitor Cocktail (Roche®)	1 µl
2 M DTT	1 µl
ddH2O	2 µl

- Alkylating agent: DMA (N,N-Dimethylacrylamide)
- Strip rehydration mix:

Zoom® 2D Protein Solubilizer 1 or 2	135-156 µl
2M DTT	1 µl
Ampholytes 3-10 (Servalyte™, Serva)	2 µl
ddH_2O	5 µl
1% Bromophenolblue	trace
Sample homogenate	1-22 µl
Final volume	165 µl

- Equilibration solution (400 µl per strip):

4x NuPAGE™ LDS Sample Buffer	final conc 1x
10x NuPAGE™ Reducing Agent	final conc 1x

- Alkylation solution:

1x NuPAGE™ LDS Sample Buffer	400 µl
125 mM Iodoacetamide	0.0093 g

- Strip overlaying solution : 0.5% agarose solution in SDS-PAGE running buffer
- SDS-PAGE running buffer: 1x MOPS/MES

2.2.12 Two dimensional electrophoresis (NEPHGE/SDS):

- Tube gels of length 11cm and diameter 3mm were casted overnight with:

	9 M Urea 4% acrylamide 2.5% NP-40 5% ampholytes pH 2-11 0.03% APS 0.2% TEMED
• Sample loading buffer 1	9.5 M Urea 0.5% SDS 5% β-mercaptoethanol 2% ampholytes pH 2-11
• Sample loading buffer 2	9.5 M Urea 5% NP-40 5% β-mercaptoethanol 2% ampholytes pH 2-11
• Sample overlaying solution	6 M Urea 5% NP-40 1% ampholytes pH 2-11
• Cathode buffer	20 mM NaOH
• Anode buffer	10 mM H_3PO_4
• SDS sample buffer	60 mM Tris-Cl pH 6.8 2% SDS 5% β-mercaptoethanol 10% Glycerin
• Tube gel overlaying solution	1% Agarose in SDS sample buffer
• 1x SDS-PAGE running buffer	Tris 3 g Glycine 14.4 g SDS 1 g Volume made upto 1 liter with dH_2O

2.2.13 Grape juice agar for egg laying:

1.5 g agar dissolved in 40ml dH_2O and boiled briefly + 1.5 g sucrose dissolved in 10ml of pure grape juice warmed upto 60ºC and mixed to the agar solution + 0.5ml

glacial acetic acid. Total volume ~ 50ml poured on empty petri plates and allowed to solidify under a hood for 5 minutes. Can be stored at 4ºC for upto a week or used for egg laying.

2.2.14 Cryosectioning and Immunostaining

- Fixative: (made freshly before use)
    - 4% PFA: 2g dissolved initially in 25 ml dH_2O by heating upto 60ºC with constant stirring
    - 100 µl of 1 M NaOH was added and the solution turned clear
    - On complete dissolution it was cooled to RT
    - 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) M K_2HPO_4 and final volume made to 50 ml
    - Solution was chilled on ice before use

• Drosophila Ringer	NaCl	7.48g
	KCl	0.35g
	$CaCl_2$	0.2g
	Na_2HPO_4	0.105g
	KH_2PO_4	0.048g
	Final volume made upto 1liter with dH_2O	
• Wash and Cryoprotectant	25% Sucrose (25 g in 84 ml of above Ringer solution)	
• Embedding medium	16% CMC (1.6 g in 10ml of 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 1liter by dH_2O	

2.2.15 Adult brain whole mounts:

• Fixative	made freshly as described above in section 2.2.11.
• Drosophila Ringer	as described above in section 2.2.11.
• Wash buffer	1x PBST (0.5% Triton X-100).

- Blocking solution 5% Normal-Goat-Serum in 1x PBST.

2.2.16 Larval brain whole mounts: Same as above only with 0.3% Triton X-100.

2.2.17 Larval NMJ dissection:

- Ca^{2+} free saline (should be pre-cooled to 4°C):

NaCl	7.6 g
Sucrose	12.32 g
KCl	0.37 g
MgCl2	0.38 g
HEPES	1.3 g
EDTA	0.19 g

Final volume madeup to 1liter with dH2O.

- Fixative made freshly as described above in section 2.2.11.
- 2x PEM buffer:

PIPES	30.24g in 100ml dH2O	(dissolves at pH 7.0)
EGTA	0.76g in 100ml dH2O	(use conc. NaOH to adjust pH to 7.0)
$MgSO_4$	0.24g in 100ml dH2O	(No pH adjustment)

20 ml PIPES + 20 ml EGTA + 20 ml $MgSO_4$ + 40 ml dH_2O = 100 ml of 2x PEM buffer

- Blocking solution (should be freshly prepared):

1x PBS	4.64 ml
BSA (2%)	100 mg
Triton X-100 (0.2%)	10 µl
Normal Horse/Goat Serum (5%)	250 µl

Final volume made upto 5ml.

- Wash buffer 1x PBST (0.1% Triton X-100)

2.2.18 Proteoliposome formation:

- Phospholipid solution 20 mg Soyabean PC (Sigma) in 1ml of Chloroform:MeOH (2:1) + 300 µl of 1 mg/ml Rhod-PE (Avanti Polar Lipids Inc.)

2.2.19 Hydroxyapatite column chromatography:

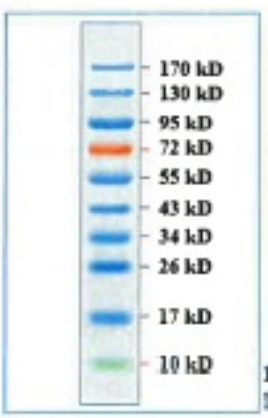
- Low salt (buffer A) 10 mM sodium phosphate pH 6.7

- High salt (buffer B) 500 mM sodium phosphate pH 6.7

2.2.20 Ion exchange chromatography:

- Low salt (buffer A) 30 mM HEPES ph 7.4, 100 mM NaCl, 4 M Urea, 1 mM EDTA, 1 mM EGTA
- High salt (buffer B) same as buffer A except 1 M NaCl

2.3 Protein M_r marker



Page Ruler™ prestained protein ladder (SM0671 Fermentas®)

Fig. 4. Separation profile of the marker.

2.4 Antibodies:

2.4.1 Primary antibodies:

1° Ab	Target protein	Dilution for WB	Sources*
3c11	Synapsins	1:100	WHL
na21	Protein X?	1:10	WHL
ab52	EPS-15	1:10	WHL
ab49	CSP	1:200	WHL
nc46	SAP-47	1:200	WHL
aa2	EPS-15	1:2	WHL
8C3	Syntaxin	1:1000	DSHB
BB70	HSP-70	1:1000	Axxora GmbH
Anti VAMP Clone 10.1	VAMP 1/2/3	1:3000	SYSY
ab5930	Mouse IgM μ chain	1:5000	Abcam

Table 5. Details about name, target proteins, dilution and sources of 1° Abs used.

*WHL = Wuerzburg Hybridoma Library, Wuerzburg, Germany.
DSHB = Developmental Studies Hybridoma Bank, Iowa, USA.
SYSY = Synaptic Systems, Goettingen, Germany.

2.4.2 Secondary antibody:

- α-mouse IgG-HRP (Biorad®) used at 1:7500 in 1x TBST for WB
- Biotinylated α-mouse IgG (Vector Labs) used at 1:200 in 1x PBST for IHC
- α-mouse IgG-Cy3™ (Invitrogen®) used at 1:1000 in 1x PBST for IHC
- α-mouse IgG-AlexaFluor488™(Invitrogen®) used at 1:1000 in 1x PBST for IHC

Chapter 3. METHODS

3.1 Fly rearing: Flies were reared in large (10 x 4.5 cm) or medium (9 x 3.5 cm) sized vials and maintained at 25°C with 60-70% relative humidity and 14/10 hr light/dark cycle. Flies were reared on food which had the following composition:

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

3.2 Collection of large amount of fly heads: For collection of large amount of fly heads, flies stock was expanded by frequently flipping stocks to new vials and having more progeny. Large sized polylpropylene cages 60 x 60 x 60 cm were also used for large scale fly culture. Adult flies were anesthetized with CO_2 or cold (4°C) treatment and collected in 50ml falcon tubes which were then snap frozen by dipping the in liquid N_2. Frozen flies could be stored at -80°C. To isolate the heads, frozen flies were vigorously vortexed to separate all jointed body parts and passed through a stack of two sieves. The upper sieve with 800µm mesh size retained thorax and abdomen while the lower sieve with 500µm mesh size retained the heads and smaller body parts passed through. Thus the isolated heads were collected from the lower sieve and used for further experiments.

3.3 Hybridoma cell culture and mAb production: Hybridoma clones were generated as described earlier (Hofbauer, 1991; Hofbauer et al., 2009). For mAb production, cryopreserved hybridoma cell lines were thawed slowly back to room temperature, diluted to 9 volumes of freshly made HT medium followed by sedimentation of cells by centrifuging at 1000 rpm for 2 minutes. The supernatant was discarded and the pellet was gently resuspended in 2 ml of HT medium and incubated in 24 well Nunclon™Δ plates (Nunc). 50 µl of FBS was added per well

to facilitate the initial growth of the thawed cells. Growth of cells was monitored daily under inverted microscope (Zeiss) and after 2-3 days, when cells proliferated, the cell suspension was used to inoculate 5 ml HT medium in 50 ml T-flasks (Grenier Bio) and further cultured for 2 days. Thereafter fresh medium was provided and after 2 more days, cell density was determined by Trypan Blue (Sigma) exclusion staining of cell suspension using a Neubauer-counting chamber (GLW). When the cell density reached ~10^5 cells/ml and the medium color turned yellow, supernatant was withdrawn, centrifuged at 2000 rpm for 5 min to pellet all cells and the cell-free supernatant was tested for the presence of antibodies to find the optimal dilution for a reliable signal in western blots and/or immunohistochemistry. Upon detection of an antibody signal, the antibody producing cells were further cultured for continued antibody production until the cell density reached ~10^6 cells/ml. At this stage they could be either split into more flasks or used to inoculate larger T-flasks (250 ml, 75 cm^2, Grenier Bio). Supernatant from larger flasks was withdrawn every 3rd day.

3.4 Production of serum free supernatant: For production of serum-free supernatant, concentrations of fetal bovine serum (FBS) were gradually lowered from 10% to 0% in 10 steps. At each step, concentrations of Hybridoma Express Plus (HEP) medium and Hybridoma Cloning Supplement (HCS) were increased by 4% and 1%, respectively, and cells were cultured for 3 days. After a month, the cells were growing in serum-free medium (50% HT medium, 40% HEP, 10% v/v HCS). For large scale production, serum-free cultures with a cell density of ~10^6 cells/ml were used to inoculate a two-compartment bioreactor, CELLine CL1000 (Integra Biosciences). The cells grew in 20 ml serum-free medium in the lower compartment separated by a 10 kDa semi-permeable cellulose acetate membrane, from 1 liter normal HT medium (with FBS) in the upper compartment. This

allowed diffusion of nutrients and small signaling molecules while preventing contamination of the culture by serum proteins. Serum-free supernatant was withdrawn every 7^{th} day.

3.5 Cryopreservation of cell lines: For cryopreservation, 6 ml of cell culture (late log phase) were centrifuged at 1000 rpm for 2 minutes and the cells were gently resuspended in HT medium supplemented with additional 10% FBS and 10% DMSO (cell culture tested, Sigma). The cells were frozen at -80^{o}C over night and thereafter stored in liquid nitrogen.

3.6 Characterization of the mAbs: For the characterization of mAbs their isotype was determined by capture ELISA using the ISO2-KT (Sigma) mouse monoclonal isotyping kit following the manufacturer's instructions. 5-Aminosalicylic acid (Sigma) was used as substrate (1mg/ml) in 0.02 M sodium phosphate buffer (pH 6.8) with 0.01% H_2O_2 v/v. The isotype was also confirmed by immunoassay based Isoquick Strips (Envirologix) following the manufacturer's instructions. For storage of the monoclonal antibodies, suitable sized aliquots of the culture supernatant were snap frozen in liquid nitrogen and stored at -20°C. However in case of IgM antibodies, which tend to aggregate upon repeated freezing and thawing, they were either stored as small frozen aliquots or larger aliquots were stored at 4°C by adding 0.02% NaN_3 (w/v) as antimicrobial agent.

3.7 Protein quantification: Protein concentrations of samples were determined by the BCA Protein Assay Kit (Pierce®) following manufacturer's instructions. A_{562} was measured using the Sunrise™ Basic (Tecan) 96-well microplate reader.

3.8 Protein precipitation:

3.8.1 TCA precipitation: To a protein sample solution, $1/5^{th}$ volume of 72% (w/v) TCA stock solution (e. g. 0.25 ml TCA stock + 1 ml sample) was added and mixed well by brief vortexing. The mixture was incubated on ice for 30 min

followed by centrifugation at 14000 rpm for 30 min at 4^{o}C. The supernatant was discarded and the pellet was washed with 0.5 ml of ice-cold Acetone. Sample was again centrifuged at 14000 rpm for 10 min at 4^{o}C and supernatant was discarded. The pellet was air-dried at 37^{o}C for 5 min (or till no more acetone was visible) and was ready to be dissolved in Lämmli buffer for SDS-PAGE.

3.8.2 Chloroform Methanol precipitation (for removal of salts & detergents): To the protein sample (say 0.1 ml) 4 volumes (0.4 ml) of Methanol was added and mixture was mixed well by vortexing for 1 min. Then 1 volume (0.1 ml) of Chlorofom was added followed by vortexing as above and addition of 3 volumes (0.3 ml) of ddH_2O again followed by vortexing as above. The sample was centrifuged at 14000 rpm for 1 min at RT to get a protein layer at the interface between the upper aqueous and lower organic phases. Upper aqueous phase was discarded and 4 vol (0.4 ml) of Methanol was added, followed by vortexing as above. The sample was again centrifuged at 14000 rpm for 2 min at RT and as much supernatant as possible was removed without disturbing the pellet. The pellet was air-dried at 37^{o}C for 5 min (or till no more liquid was visible) and was ready to be dissolved in Lämmli buffer for SDS-PAGE.

3.8.3 Ethanol precipitation: To protein sample solution 10 volumes of pre-chilled (-20^{o}C) Ethanol was added and mixed well by brief vortexing. The mixture was incubated at -20^{o}C for 3 hr followed by vortexing and centrifugation at 14000 rpm for 5 min at 4^{o}C. The supernatant was discarded and the pellet was washed with 70% Ethanol followed by centrifugation at 14000 rpm for 2 min at 4^{o}C. The supernatant was discarded and the pellet was air-dried at 37^{o}C for 5 min (or till no more liquid was visible) and was ready to be dissolved in Lämmli buffer for SDS-PAGE.

3.9 Sub-cellular fractionation of fly head: Frozen heads collected from the lower

sieve as described earlier in section 4.2 were pulverized in a mortar-pestle, which was prechilled to -80°C. The powder was dissolved in homogenization buffer (section 2.2.10) 10 ml per g of fly head. The sample was thoroughly mixed to get a uniform homogenate which was then incubated on ice for 5 minutes. Thereafter it was spun twice at 13000 rpm for 15 min each, at 4°C to pellet the exoskeleton, cell debris, nuclei. The 'post-nuclear supernatant' (S1) was re-spun in a 60Ti rotor, using an L8 ultracentrifuge (Beckman Coulter®) at 100000g for 1 hr at 4°C to get the 'cytosolic fraction' as the supernatant (S2) and the 'total membrane fraction' as the pellet (P2) which were then tested on Western blots. Anti HSP70 antibody (BB70, Axxora GmbH) was used as a cytosolic marker (1:1000) while anti-Syntaxin antibody (8C3, DSHB) was used as a membrane marker (1:1000).

3.10 Subfractionation of total membrane fraction: The total membrane pellet was resolved by density gradient centrifugation. 5-25% continuous gradient was prepared in 13.2ml Ultra-Clear® polyallomer, open top tubes (Beckman Coulter®) using a gradient mixer (made in house at workshop) with 25% and 5% sucrose solutions (w/v) in homogenization buffer (section 2.2.10). Total membrane pellet (P2) from 10 ml of S1 (10000 fly head equivalents) was resuspended in 0.5 ml of the same homogenization buffer by mild homogenization and pipetting and then overlaid upon the gradient. The samples were centrifuged in a SW41Ti rotor, using an L8 ultracentrifuge (Beckman Coulter®) at 39000 rpm (~ 187813 g) for 4.5 hr at 4°C. Fractions were carefully withdrawn from top gradually by a pipette.

3.11 Generation of proteoliposomes:

3.11.1 By simple dilution below CMC: For generation of proteoliposomes P2 was dissolved in 1% (w/v) CHAPS followed by ultracentrifugation at 100000g for 1 hr at 4°C to get supernatant (S3) and a pellet (P3). S3 was then diluted 10 times to bring the CHAPS concentration to 0.1% (w/v) which is below its critical micelle

concentration (CMC). Any proteoliposome formed were separated as pellet (P4) by another round of ultracentrifugation at 100000g for 1 hr at 4°C.

3.11.2 By gel filtration chromatography: The total membrane pellet P2 was dissolved in 3% (w/v) CHAPS followed by ultracentrifugation at 100000g for 1 hr at 4°C to get supernatant (S3) and a pellet (P3). S3 was used to dissolve phospholipids (Soybean phosphatidylcholine, Sigma) containing Rhodamine-Phosphatidylethanolamine (Avanti® Polar Lipids Inc.) as a fluorescent marker. The sample was resolved by gel filtration chromatography, through a Sephadex™ G50 (GE) column to separate the proteoliposomes from the monomeric detergent molecules. Fractions eluted around the Rhodamine-PE peak were collected and analyzed by Western blot.

3.12 Immunoprecipitation for enrichment of na21 antigen: CS fly heads were isolated as described earlier in section 4.2. Heads were homogenized in IP lysis buffer (1 ml per gm of fly head) in a prechilled glass homogenizer (Kontes). Homogenate was centrifuged at 13000 rpm twice for 15 mins each at 4ºC to get rid of the debris. 5 µl aliquot of the supernatant was stored at -20 ºC as an IP input control. To another aliquot undiluted mAb na21 was added (0.5 ml supernatant per ml of tissue lysate) and incubated for 3 hr with gentle rotatory mixing at 4ºC. 0.1 ml of mAb 3C11 was added to 0.5 ml lysate as positive control. 0.1 ml of protein G agarose beads were washed with the IP lysis buffer for 15 min and then pelleted by a short spin. The mAb-lysate mixture was added to the beads and incubated overnight with gentle rotatory mixing at 4ºC. Next morning the beads were spun down at 13000 rpm for 3 min. Supernatant was discarded and beads were washed with 0.5 ml of IP lysis buffer. Washed beads were spun down and the supernatant was discarded. The beads were washed three times more as above and after the final wash 50 µl of 2x Lämmli buffer was added. 2 fresh heads were also

homogenized in 20 µl of 2x Lämmli buffer. All samples were incubated at 95ºC for 3 min, followed by a short spin and then loaded on separate lanes of a 12.5% PAA gel and resolved by SDS-PAGE.

3.13 Resolution of proteins by electrophoresis:

3.13.1 One dimensional SDS-PAGE with self made gels: 1D SDS-PAGE was based on Lämmli's system (1970). In brief, 0.75 mm thick, 12.5% PAA gel was casted as per recipe described in section 3.2.1.1. Samples were heat denatured at 95ºC for 3 min, followed by a short spin and then loaded on separate lanes. The Mini Protean™ (Bio-Rad) apparatus was used. Samples were initially stacked at 60 V and then upon entering the resolving gel, run at 100 V till the smallest (10 kDa) marker was ~1 cm above the bottom.

3.13.2 One dimensional SDS-PAGE with pre-cast gels: 1 mm thick, 12% Bis-Tris, precast gels (NuPAGE™, Invitrogen®) were used. Samples were prepared with the 4x LDS sample buffer and 10x Reducing agent (both Invitrogen®), both diluted to get final concentrations of 1x of each. Samples were heat denatured at 70ºC for 10 min, followed by a short spin and then loaded on separate lanes. The X-Cell Sure Lock™ (Invitrogen®) apparatus was used and samples were resolved at 100 V constant voltage till the smallest (10 kDa) marker was ~1 cm above the bottom. PageRuler™ Prestained Protein Ladder (Fermentas SM0671) was used as the molecular weight marker.

3.13.3 Tricine SDS-PAGE: For better resolution of proteins in the lower molecular weight range, Tricine SDS-PAGE (Schägger and von Jagow, 1987) was used. In brief, 15% PAA gels were casted as per recipe described in section 2.2.1.3 and fly eggs collected over grape-juice plates were homogenized in 2x Tricine sample buffer and resolved by SDS-PAGE. The stacking was done at 60 V followed by separation at a constant current of 25 mA.

3.13.4 Two dimensional electrophoresis (IEF/SDS): Proteins from fly head homogenate were resolved by 2D electrophoresis using the Zoom® 2D (Invitrogen) setup. In brief, 100 freshly isolated CS fly heads were homogenized in 100 µl of Zoom® 2D Protein Solubilizer 1 (Invitrogen) containing 1x protease inhibitors (Complete-Mini™, Roche®). The homogenate was then centrifuged at 13000 rpm for 15 min at 4°C, to get rid of exoskeleton, cell debris, and nuclei. 1 µl of 99% DMA (Sigma) was added to the post-nuclear supernatant and incubated on a rotary shaker at room temperature for 15 min to alkylate the proteins. Thereafter 1 µl of 2 M DTT was added to quench any excess DMA and the sample was ready for loading. 25 µl of this homogenate, equivalent to 25 fly heads was mixed with rehydration mixture as follows:

Zoom® 2D Protein Solubilizer 1/2	137 µl
2 M DTT	1 µl
Bromophenolblue)	traces
Servalyte 3-10 pH ampholyte (Serva)	2 µl
Sample (homogenate)	25 µl
Final volume	165 µl

Immobilized pH gradient (IPG) (Zoom®, Invitrogen) strips of pH range 3-10 were rehydrated overnight at 18°C with this sample following manufacturer's instructions in the Zoom® IPG Runner™ cassette. Next day the sample in the rehydrated strips was resolved by isoelectric focusing (IEF) with a Zoom® Dual power supply unit (Invitrogen), while keeping the power limited to 0.1 W per strip and using the voltage regime shown in Table 6 (below). Thereafter the strips were incubated in the equilibration solution (1x NuPAGE™ LDS sample buffer with 1x NuPAGE™ Reducing Agent, both Invitrogen) and alkylation solutions (1x NuPAGE™ LDS sample buffer) with 125 mM IAA (Sigma) both for 15 min, respectively, with gentle shaking. Thereafter the strips were loaded in the IPG well of 4-12% Bis-Tris NuPAGE™ (Invitrogen) 2D PAA gel and overlaid with agarose

Step	Voltage (V)	Time (min)	Equivalent 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 6. Voltage regime for isoelectric focusing.

solution (0.5% in 1x MOPS running buffer). The second dimension was run at 100 V at 4°C to avoid over heating. Besides the molecular weight marker, 2 fly heads, freshly homogenized in 1x LDS sample buffer (Invitrogen) and heat denatured at 70ºC for 10 min was also loaded on the 1D well, to serve as a 1D reference to the 2D separation profile. After the 2nd dimension electrophoresis, proteins were either blotted from the gel onto nitrocellulose membrane for detection by Abs or the gel was stained to visualize the proteins.

3.13.5 Two dimensional electrophoresis (NEPHGE/SDS): For resolution of larger amounts of proteins, Non-Equilibrium pH Gradient gel Electrophoresis (NEPHGE) was performed as per O'Farrell et al. (1977) with some modifications. In brief, glass tubes with length 12cm and inner diameter 3mm were rinsed with isopropanol and Na-Silicate followed by baking in an oven at 110°C for 1 hr. One end of the tubes was sealed with parafilm and tube gels of length 11 cm were casted overnight based on the recipe described in section 2.2.11. Soluble (cytosolic) fraction S2 was obtained as described earlier in section 3.8 and precipitated with 9 volumes of chilled acetone for 3 hours at -20°C. The sample was centrifuged at 10000 g for 10 min at 4°C to pellet the precipitated proteins. The supernatant was discarded, the pellet was air-dried and resuspended in sample loading buffer 1. Upon dissolution, an equal volume of buffer 2 (9.5 M Urea, 5%

NP-40, 5% β-mercaptoethanol, 2% ampholytes pH 2-11) was added. Sample equivalent to 100 heads was loaded on top of the tube gel. The sample was overlaid with 40 µl of overlaying solution (6 M Urea, 5% NP-40, 1% ampholytes pH 2-11). Electrophoresis was carried out in the Model 175 Tube Cell (Bio-Rad) setup at 200 V for 15 min, followed by 300 V for 30 min and finally at 400 V for 120 min. 10 mM H_3PO_4 and 20 mM NaOH were used as anode and cathode electrophoresis buffers respectively. As a marker for highly basic proteins, Cytochrome C having a pI >11, was loaded on one of the tube gels as a control for the progress of the 1st dimension. At the end of the run, the NEPHGE gel with the sample was slowly withdrawn from the glass tube, equilibrated for 20 min with the SDS sample buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% Glycerol) and overlaid with 1% agarose in SDS sample buffer on a 12% PAA gel. 10 freshly homogenized fly heads were also loaded in an adjacent lane, to serve as a 1D reference to the 2D profile. Electrophoresis was carried out at 15 mA for 16 hr. The tube gel with Cytochrome C was cut into 0.5 cm pieces and each piece was incubated overnight with 3 ml ddH_2O at 4°C. The pH of the solutions was measured next morning to determine the pH gradient along the length of the gel.

3.14 Western blotting: Proteins from gels (both 1D and 2D) were transferred onto 0.45 µm nitrocellulose membranes (Protran®, Whatman). Mini gels were transferred using the Mini Trans-Blot™ (BioRad®) wet-blot apparatus with 1x transfer buffer (Towbin et al., 1979), described in section 2.2.2 for 1hr at 100 V. Larger gels were transferred using the PerfectBlue™ (peqLAB) semi-dry blotting apparatus with the same buffer at 2 mA of current per cm^2 of gel area for 1hr. Thereafter membranes were stained with Ponceau S (Sigma) solution to check for success of protein transfer. Membranes were then blocked with 5% milk solution at RT for 2 hr with gentle shaking. Thereafter membranes were incubated overnight

with primary mAb at suitable diluted (section 2.4.1) in 1x TBST at 4ºC with gentle shaking. Next morning membranes were incubated at RT for 30 min before being washed three times with 1x TBST, for 5 min each. Then membranes were incubated with corresponding secondary antibody and washed again as above. Finally membranes were incubated with the a freshly prepared mixture (1 ml per mini-blot) of the equal volumes of the two substrate reagents from the ECL™ kit (Amersham GE®) for 2 minutes in darkness. Thereafter excess detection reagent was decanted off and signals from the blot were captured as exposures on X-ray films for various exposure times in a dark room. The signal was developed using the development and fixation solutions (Kodak).

3.15 Partial blotting of NEPHGE 2D gel followed by Western blot for detection of ab52 antigen: After 2DE, the gel was blotted for 20 minutes only, using the semi-dry blotting apparatus as described above in section 3.10.5. Due to the reduced time, only part of the protein content of the gel was transferred onto the membrane, while the rest was retained in the gel, which was then silver stained for MS compatibility as described later in section 3.14.3 The blot was then blocked and developed with ab52 (diluted 1:10) as described above in section 3.10.5. Overnight exposure was done to get a strong signal for the antigen along with weak non-specific signals on the blot, which would serve as landmarks for comparison with the silver-stained gel. Images of the over-exposed blot and the silver-stained gel were digitally superimposed with Photoshop (Adobe) to pinpoint the protein spot in the silver stained gel that corresponded to the signal in the Western blot. This spot was then excised and analyzed by mass spectrometry (MS) as described in Hofbauer et al., (2009).

3.16 Staining of proteins in gels:

3.16.1 Coomassie® R250 staining: After electrophoresis, the part of the gel to be

stained was cut out from the glass plates and washed in dH_2O for 3 min. The gel was then incubated in Coomassie® Brilliant Blue R250 staining solution (section 2.2.3.1) for 30 mins at RT. The dye was decanted for reuse and the gel was washed in dH_2O for 2 mins. The gel was then incubated with destaining solution for 2 hrs at RT. Thereafter destaining solution was discarded and fresh solution was added and left overnight until the gel matrix was almost destained and only specific protein bands were visible in minimal background. The gel's image was then stored (before any band was to be cut out or the gel was to be dried). For preservation, the gel was incubated in drying solution for 6-8hrs. Then it was wrapped with transparent cellophane sheets and dried under vacuum and heat.

3.16.2 Coomassie® G250 staining: After electrophoresis, the part of the gel to be stained was cut out from the glass plates and washed in dH_2O for 3 min. The gel was then incubated with fixative for 30 minutes followed by staining in colloidal Coomassie® G250 staining solution (section 2.2.3.2) overnight at RT. Next day staining solution was decanted off and gel was destained for 2 hr or more till until the gel matrix was almost destained and only specific protein bands were visible in minimal background.

3.16.3 MS compatible silver staining: All solutions were made fresh and using high purity chemical in Milli-Q™ H_2O. After completion of electrophoresis the part of the gel to be silver stained was cut out from the glass plates and washed in dH_2O for 3 min. Thereafter the gel was incubated overnight at 4ºC in the fixative (section 2.2.4). Next morning gel was washed in 30% EtOH twice for 20 min each. The gel was then washed in Milli-Q™ H_2O for 20 min. The gel was then sensitized for 1 min followed by washing three times in Milli-Q™ H_2O for 20 seconds each. The gel was then incubated in prechilled, $AgNO_3$ solution at 4ºC for 20 mins. The gel was again washed three times in Milli-Q™ H_2O for 20 seconds each. The gel

was transferred in a fresh container and rewashed in Milli-Q™ H_2O for 1 min. A small volume of developer was then added to the gel to remove excess stain from the gel and the coloured developer was discarded. More developer was then added to develop the bands until suitable intensity was observed. The reaction was stopped by discarding the developer and adding stopping solution. Finally the gel was washed briefly in Milli-Q™ H_2O and preserved in 1% acetic acid at 4ºC.

3.16.4 Destaining silver stained gels: For destaining of silver stained gels, the gel was incubated in the freshly prepared destaining solution (section 2.2.5) with mild shaking at RT. A mini-gel is destained in about 15 min. Destaining was stopped by adding the stopping solution. The gel could now be preserved in the storage solution or washed three times in Milli-Q™ H_2O for 10 min each, after which it is like a fixed gel, ready to be restained with silver (beginning from sensitization step) or Coomassie (beginning from staining step). Staining gels for the second time after destaining apparently gives better staining probably because of removal of some of the impurities.

3.17 Cryosections and immunostaining: Cryosection of adult fly heads were made as previously described (Buchner et al., 1986). In brief, adult flies were anesthetized and immersed in freshly prepared, ice-cold fixative (section 2.2.14). Their proboscis and ventral air sacs in the head were removed under a stereomicroscope, followed by fixation for 3 hr at 4ºC. Thereafter flies were washed once in cryoprotectant solution and incubated in the cryoprotectant overnight at 4ºC. The desired body part (head or whole body) was then embedded in a drop of the embedding medium (section 2.2.14) which was placed on a metal holder and the specimen was oriented for horizontal sections. The holder with the preparation was immersed in melting nitrogen (slush, obtained by evacuation of liquid nitrogen in a desiccator) for rapid freezing to reduce tissue damage by ice

crystal formation. Thereafter 10 µm thick cryosections were cut at -26ºC with a cryotome (2800 Frigocut® E, Reichert Jung). Series of consecutive 10 µm sections were collected on subbed glass slides (Menzel Gläser), thawed on the slide and air-dried. The slides were blocked for 2 hr at RT in a humid chamber with normal serum (1:66 in 1x PBST pH 7.6) from the species in which the secondary antibody was generated (Vectastain®, Vector Labs). Thereafter the sections were incubated with 1° Ab suitably diluted in 1x PBST at 4ºC overnight in a humid chamber. Next morning slides were brought back to RT for 30 min and excess 1° mAb was drained off, the slides were washed once briefly and then twice for 10 min each with 1x PBST. The slides were incubated with biotinylated α-mouse IgG secondary Ab (diluted 1:200 in 1x PBST) at 37ºC for 1 hr and the signal was developed as Avidin-Biotin Complex (Mouse ABC Kit, Vector Labs) using 3,3'-diaminobenzidine (DAB, Linaris) following the manufacturer's instructions. After washing in PBS the sections were permanently mounted with Kaiser's gelatin (Merck). The sections were imaged in transmission brightfield mode (Leica DMR).

3.18 Collection of eggs: For collection of eggs, grape juice agar plates were made as described in section 2.2.13. Adult flies were anesthetized and collected over the solidified agar on plates. An empty fly vial with holes made in its bottom and having a vial stopper stuffed inside and pushed to the bottom was inverted and placed on the petri plate such that their mouths overlapped (as shown in Fig. 5 above). The two were then sealed with cellotape and the flies were allowed to recover. The vials were incubated in 25ºC and flies were allowed to lay eggs on the agar surface. Plates were withdrawn after 8 hr and flies were anesthetized with CO_2 on the vial stopper and plates were carefully replaced with fresh plates. The eggs laid on the plates could now be scraped off gently with a wet brush for further experiments.

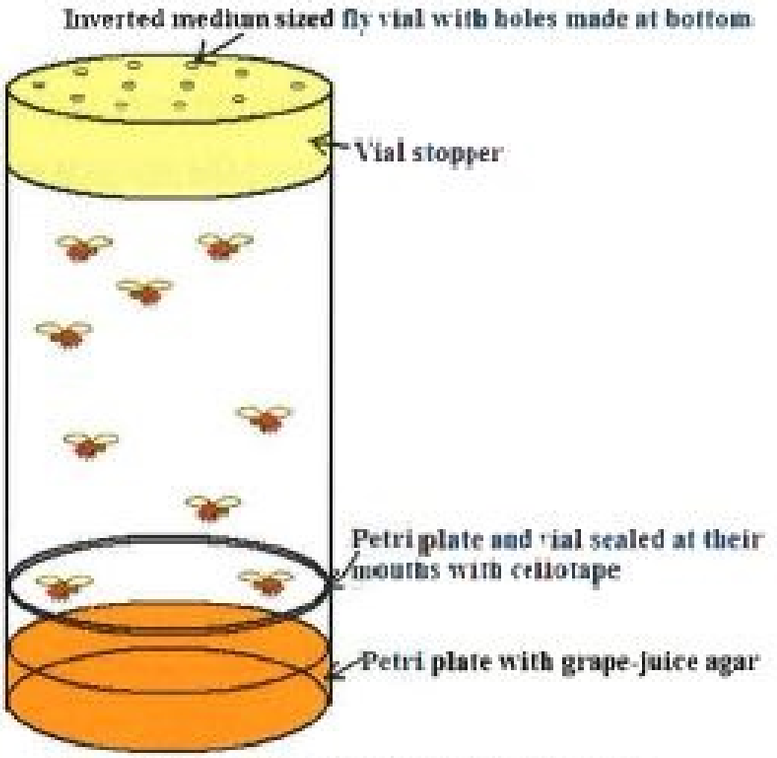


Fig. 5. Assembly of egg collection chamber.

3.18.1 Selection of EPS-15 homozygous mutants: For screening EPS-15 mutants the embryos from the heterozygous eps15 mutant line (balanced over a CyO chromosome with a GFP marker) were observed under a stereomicroscope while being illuminated with blue light which was able to excite the GFP fluorescent emission. The heterozygous embryos were removed allowing only the GFP negative homozygous mutants to develop. Only few of the embryos developed to late 3rd instar larval stage and only a single one developed to become a male (though comparatively smaller in size). This fly's head was later tested on the Western blot with respect to a CS fly head for absence of signal with the ab52 antibody.

3.19 Adult brain whole mounts: For preparation of adult brain whole mounts, adult flies were anesthetized with CO_2 and collected over a glass petri plate and kept on ice to keep them immobile. An adult fly was placed onto a silica dish with its dorsal side down and fixed with a pin through its abdomen as shown below in

Fig. 6. The fly was bathed in prechilled Ringer solution as described in section 2.2.14. The proboscis and ventral air sacs were removed under a stereomicroscope. The head capsule was dissected by pulling apart the eyes on either side to expose

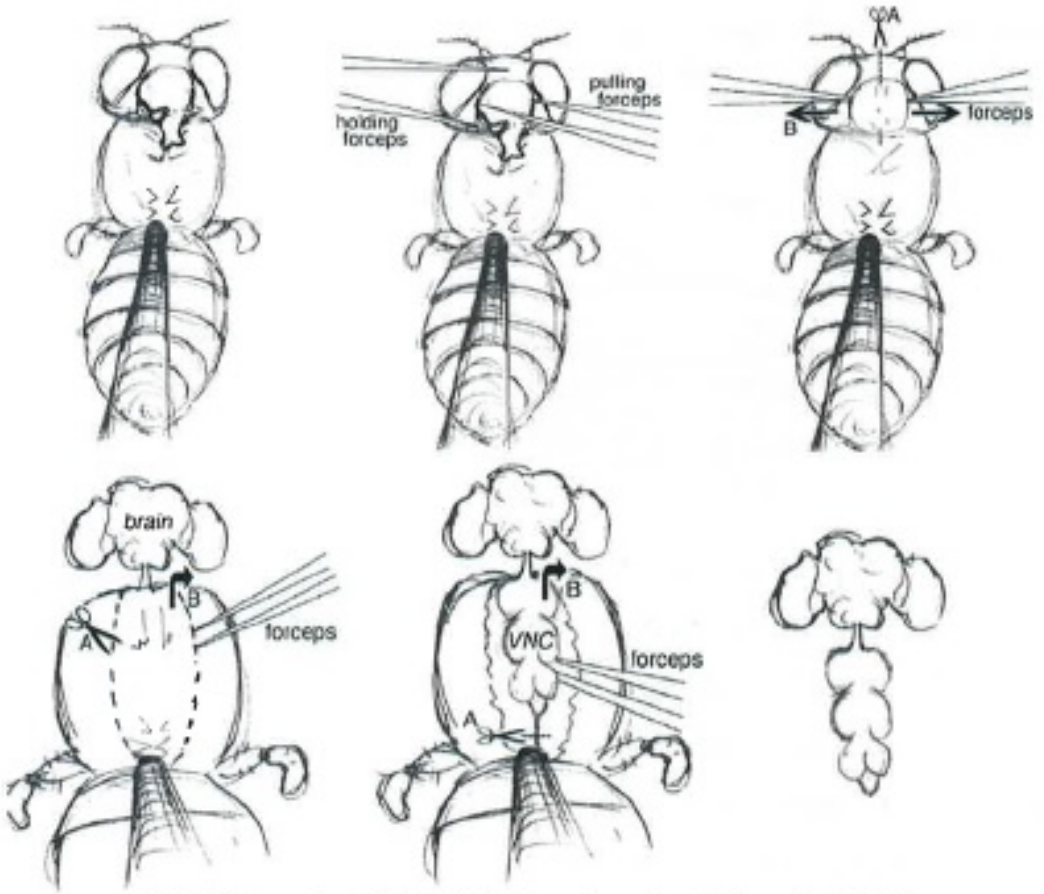


Fig. 6. Schematic of adult CNS dissection adapted from Ito, 1999.

the brain. Fat bodies and tracheal tissues sticking to the brain were removed followed by fixation of the brain for 1 hr at RT. Thereafter fixed brains were washed three times in 1x PBST (section 2.2.15) for 10 min each at RT. Then brains were blocked for 2 hr in RT followed by overnight incubation with 1° Ab (suitably diluted in blocking solution) at 4°C. Next day brains were brought to RT for 30 min before being washed 6 times in 1x PBST for 30 min each at RT. Thereafter brains were incubated overnight with corresponding 2° Ab (section 2.4.2) diluted suitably in blocking solution at 4°C. Next day brains were washed as earlier and

mounted on Vectashield™ (Vetor Labs) fluorescent mounting medium and stored in darkness at 4°C.

3.20 Larval brain whole mounts: For preparation of larval brain whole mounts, freely moving 3rd instar larvae were collected using a brush and placed in pre-chilled Ringer solution in glass petri plate and kept on ice to keep them immobile. Thereafter a larva was placed on silica dish under a stereomicroscope and was pinched with 2 forceps at a position around 1/3rd of its body length from the anterior tip. The body is then gently ripped open by pulling the forceps apart as shown in the schematic below. The anterior part of the body was slowly opened up and surrounding tissues were removed. Thereafter the cleaned brains were fixed (recipe described in section 2.2.16) for 30 min at RT. Thereafter fixed brains were washes thrice in 1x PBST for 10 min each at RT. Then fixed brains were blocked for 90 min in RT followed by overnight incubation with 1° Ab (suitably diluted in blocking solution) at 4°C. Next day brains were brought to RT for 30 min before being washed 6 times in 1x PBST for 30 min each at RT. Thereafter brains were incubated overnight with corresponding 2° Ab (section 2.4.2) diluted suitably in blocking solution at 4°C. Next day brains were washed as earlier and mounted on Vectashield™ (Vetor Labs) fluorescent mounting medium and stored in darkness at 4°C. Both adult and larval brains were imaged as soon as possible using a confocal laser scanning microscope (SP2, Leica). The confocal stacks were analyzed and 3D

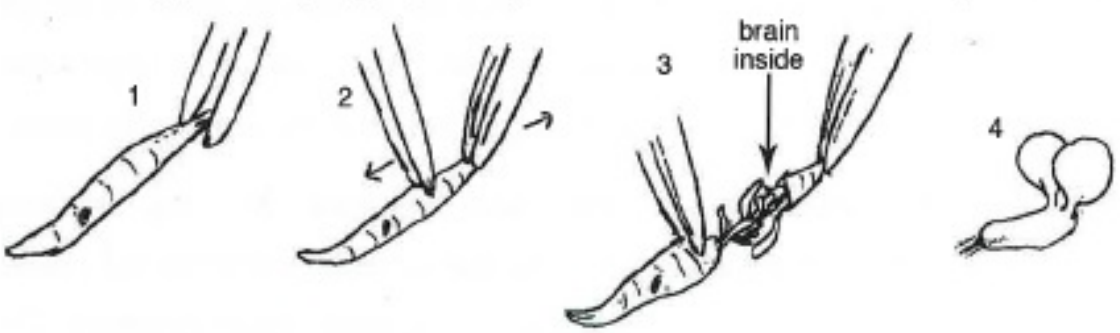


Fig. 7. Schematic of adult CNS dissection adapted from Ito, 1999.

reconstructions were generated using the Fiji (Schindelin, 2008; Schmid et al., 2010) based on ImageJ (Collins, 2007).

3.21 Purification of IgM by CHA column chromatography: Low and high salt buffers (section 2.2.19) were made as per the Henderson-Hasselbalch equation (Henderson, 1908; Hasselbalch, 1917). Buffers were filter sterilized (0.45 μm, Millipore) and degassed before use. 40 ml of serum-free cell culture supernatant was equilibrated to room temperature by gentle mixing on the rotary shaker and then clarified through a 0.2μ filter (Whatman). A 5ml ceramic hydroxyapatite column (Bio-Scale Mini™ CHT Type II 40μm, Bio-Rad) was used with a Äkta Purifier (GE Healthcare) FPLC system for the purification. The column was first rinsed with 10 column volumes of the high salt buffer at a flow rate of 1ml/min to wash out all bound substances, followed by equilibration with 10 column volumes of the low salt buffer at 1 ml/min. Thereafter sample was loaded onto the column at 0.2 ml/min. The flow through was collected and then the column was washed with 100ml of the low salt buffer at 0.5 ml/min. Finally the IgM was eluted using a linear gradient from 10mM to 500mM over a volume of 40 ml at 1ml/min. 0.4 ml eluate fractions were collected and resolved by SDS-PAGE as described in section 3.10.2 and blotted on nitrocellulose membrane as described in section 3.11. The blots were stained with Ponceau S to estimate the protein content and developed with anti-IgM antibodies (section 2.4) to check for the elution profile of the IgM. The purified IgM from specific fractions were also used to detect the target antigen in Western blot of wild type fly head homogenates and cryosections of fly heads.

3.22 Partial enrichment of the na21 antigen by ion exchange chromatography: Partial enrichment of the endogenously expressed na21 antigen from head homogenates was tried using ion exchange chromatography. Total membrane pellet P2 was resuspended in 0.4% CHAPS followed by

ultracentrifugation at 100000g for 1 hr at 4°C. The supernatant was then diluted by 7.5 volumes to reduce the detergent concentration well below its CMC (thus to prevent the formation of micelles) using 4 M Urea as a chaotrope. The sample was then dialyzed overnight against the homogenization buffer (section 2.2.9) supplemented with 4 M Urea through a cellulose membrane (ZelluTrans, Carl Roth) with MWCO 4000-6000. Next morning the dialyzed sample was again centrifuged as above and the clarified supernatant was loaded onto a Mono Q™ (GE) column which was already washed and equilibrated with the low salt buffer A (section 2.2.20). Proteins were eluted with a linear gradient of the high salt buffer B. Fractions were precipitated with TCA and tested on Western blots.

Chapter 4. RESULTS AND DISCUSSION

This doctoral project involved identification and characterization of antigens recognized by monoclonal antibodies from the Wuerzburg Hybridoma Library (Hofbauer 1991; Hofbauer et al., 2009). The monoclonal antibodies that gave a clear signal on Western blots and hence could be characterized not only based on anatomical stainings but also in terms of their biochemical properties were ab52 and na21. Some other monoclonal antibodies were also studied but they failed to give a clear signal on Western blots. Hence these monoclonal antibodies could be only studied by immunohistochemistry to describe their staining pattern and thus to illustrate the localization of their target antigens. This section is therefore divided into three major parts: ab52 (4.1), na21 (4.2) and other mAbs (4.3).

4.1 Monoclonal antibody ab52 (Halder et al., 2011):

Migration pattern of antigen recognized by ab52 on 1DE:

The monoclonal antibody ab52 recognized a single distinct band around ~100 kDa on Western blots of freshly homogenized, wild-type (Canton S) fly heads as shown in Fig. 9a. The monoclonal gave a clear signal at dilution 1:10 with a single fly head loaded per lane.

Staining pattern of the mAbs ab52 on cryosections: Immunohistochemical studies based on staining of cryosections of adult fly heads showed ubiquitous staining of the synaptic neuropil. The central brain, optic lobe and all other neuropils were strongly stained, indicating that the antigen is expressed throughout the brain and localized in the synaptic neuropils as shown in the Fig. 9c.

Characterization of the mAb isotype: Upon isotyping, ab52 was found to be an IgM with a kappa (κ) type light chain. In general IgMs, are unsuitable for

enrichment of their target antigens by immunoprecipitation (IP). However IP was nonetheless pursued with mAb ab52, but as expected, it was not successful. This could also be partly due to the low antibody concentration of the antibody in the supernatant, evident from the fact that a clear Western blot signal is achieved only when the supernatant is used at dilution 1:10 or less.

Subcellular fractionation of the ab52 antigen: To find out more about the antigen, subcellular fractions from fly head homogenate were generated by utracentrifugation at 100000 x g (as described in section 3.9). When tested on Western blots, (Fig. 9b) a signal for ab52 was obtained exclusively in the cytosolic supernatant (S2) indicating that the antigen is soluble under the conditions of homogenization. CSP, a protein attached to the synaptic vesicles by a palmitoyl membrane anchor (Gundersen et al., 1994) is recognized by ab49 only in the total membrane pellet P2. Hence ab49 can be used as the membrane marker, thus demonstrating the effectiveness of the fractionation.

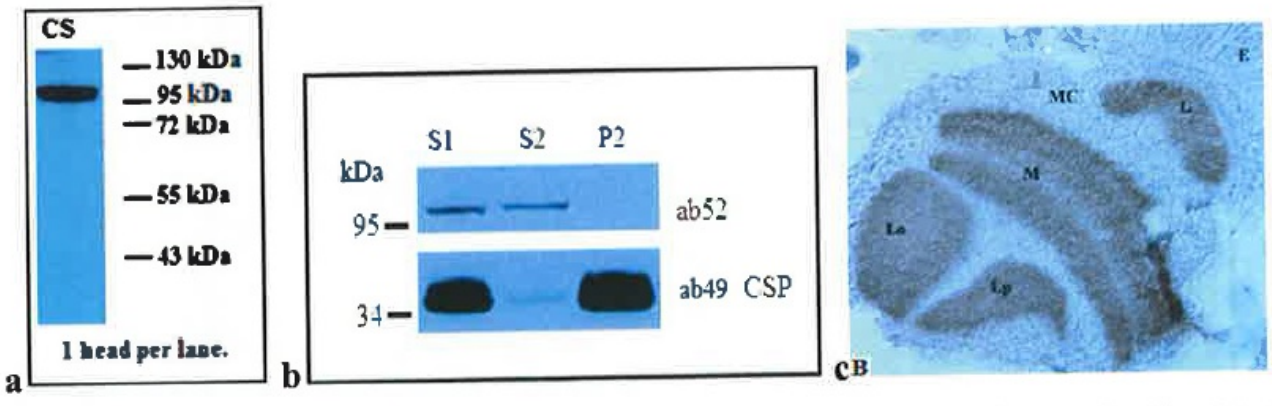


Fig. 9. (a) Western blot of a single Canton S fly head showing a single band recognized by ab52. **(b)** Western blot of subcellular fractions of wild-type (CS) fly heads showing that the protein detected by mAb ab52 is exclusively present in the cytosolic supernatant. S1 = postnuclear supernatant; S2 = cytosolic fraction; P2 = total membrane fraction (10 head equivalents loaded per lane). The synaptic vesicle protein CSP recognized by the mAb ab49 was used as the marker for the total membrane fraction. **(c)** Cyrosection of adult fly head showing ubiquitous staining pattern with mAb ab52 (dilution 1:5) in the optic neuropil. E = eye; L = lamina; MC=medulla cell bodies; M = medulla; Lo = lobula and Lp = lobula plate.

Migration pattern of antigen recognized by ab52 on 2DE: Conventional one dimensional SDS-PAGE (1DE) was not suitable to purify the antigen sufficiently to identify it. However, the soluble nature of the antigen allowed us to resolve it as a distinct spot by two dimensional electrophoresis (2DE). The Zoom® 2D setup (Invitrogen) was used as described in section 3.13.4. Proteins resolved in the gel were blotted as described in section 3.14. The blot was incubated with the monoclonal ab52 (dilution 1:10). Monoclonal antibodies 3C11 (dilution 1:100) which recognizes Synapsins and nc46 (dilution 1:200) which recognizes SAP-47 respectively, were used as positive controls. The antigen recognized by ab52 was detected on the blot as a single distinct spot around the size expected from one dimensional SDS-PAGE at a pI around 6 as shown in Fig. 10.

When the same gel after blotting was stained with silver, it was however not possible to detect a spot corresponding to the signal on the Western blot, probably due to the fact that very little amount of protein remained in the gel after the blot. Also only a small amount of protein (50 µg) can be loaded on the small-sized IPG

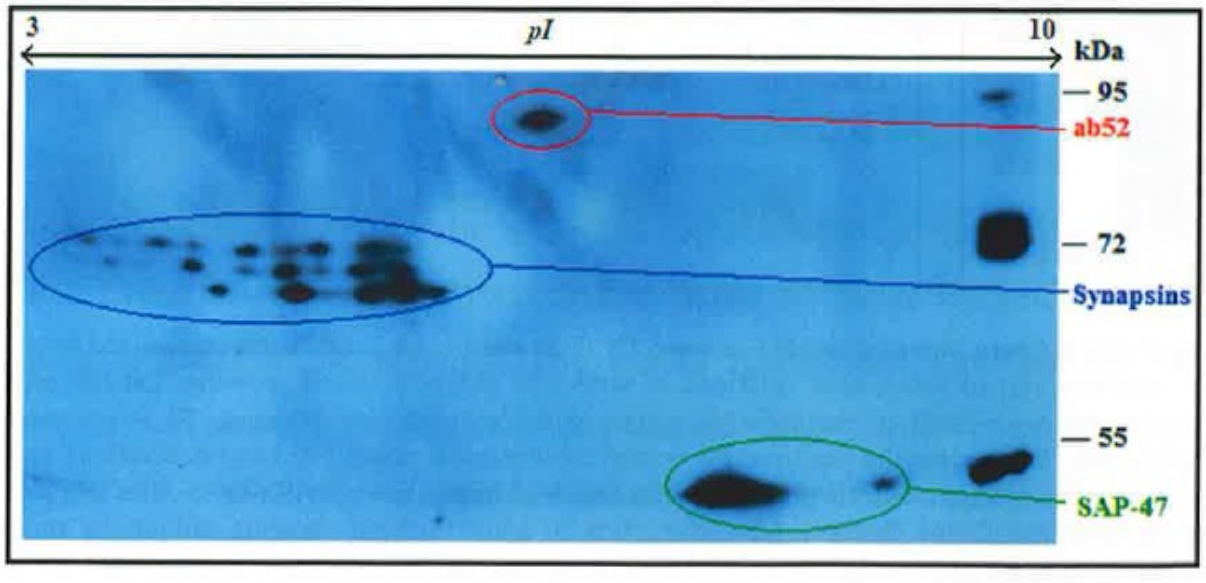


Fig. 10. Western blot of fly head proteins resolved by 2DE (IEF/SDS), showing the antigen recognized by ab52 as a distinct spot around pI 6. Synapsins recognized by 3C11 and SAP-47 recognized by nc46 were used as positive controls. Synapsins were seen as a group of ~22 spots.

strips in order to obtain optimal resolution. Hence the ab52 antigen could possibly load higher amounts of proteins (see below). It was also found that Synapsin was be resolved as a single, distinct spot by using largersized 2DE gels with capacity to not only resolved into the three major bands along the vertical axis (due to their Mr) between 70-90 kDa, as seen in 1D blots (Klagges et al., 1996) but each band was further resolved along the horizontal axis as an array of spots extending towards the acidic side of the pH gradient. The spots differed in their pI values and hence were probably post-translationally modified versions of Synapsins for each band (cf. Disussion).

Enrichment of the antigen of mAb ab52 by 2DE (NEPHGE/ SDS-PAGE): The observation that the antigen recognized by the mAb ab52, can be resolved by 2DE as a single, distinct spot, was followed up by using larger sized 2DE gels to allow loading of more sample. For better resolution of proteins, NEPHGE was performed

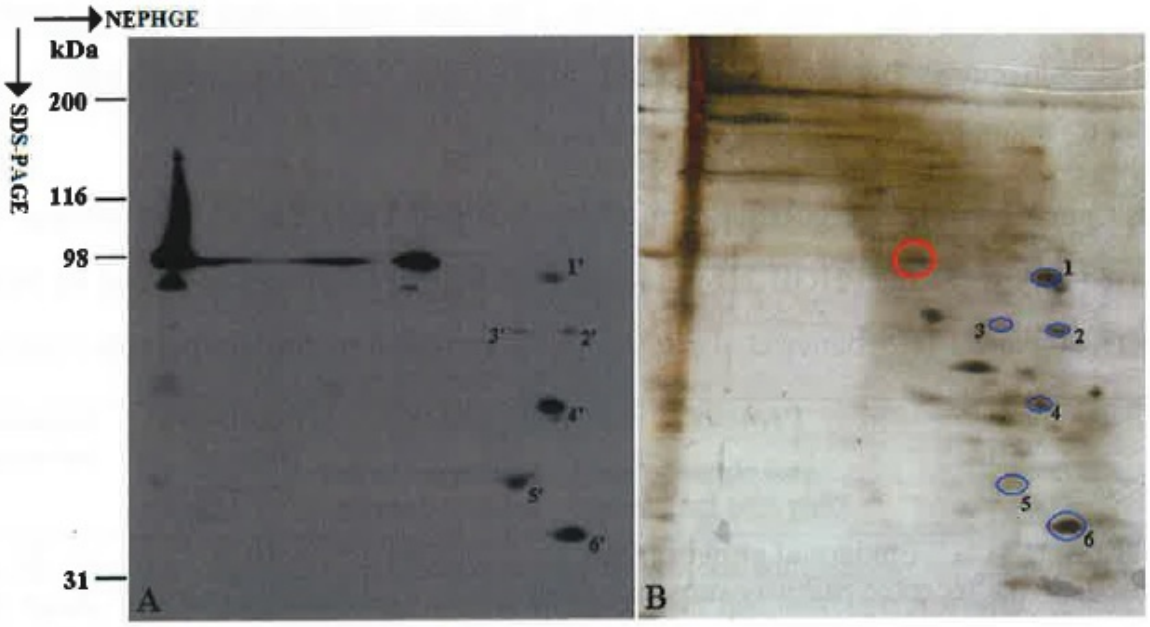


Fig. 11. (A) Signals from Western blot after 2hr exposure. The signal from the monoclonal antibody ab52 can be clearly seen as distinct a spot ~100kDa, with a corresponding strong signal in the 1D lane, which was loaded with 10 freshly homogenized fly heads. Non-specific signals were numbered as landmarks 1'- 6'. (B) Silver stained NEPHGE gel with protein spots corresponding to the non-specific Western signals numbered 1 - 6 and the spot corresponding to the Western signal from the monoclonal antibody ab52 (encircled in red).

after O'Farrell et al., (1977). Proteins from the cytosolic fraction (S2) were precipitated with 9 volumes of chilled acetone for 3 hr at -20°C, redissolved in the lysis buffers 1 and 2 and resolved as per their pI in tube gels by NEPHGE (section 3.13.5), followed by SDS-PAGE. The separated proteins were blotted for only 20 min, to transfer only part of the protein content onto the membrane, while retaining the rest of it in the gel. The proteins in the gel were visualized by MS-compatible silver staining (Fig. 11B). The blot was then incubated with mAb ab52 (1:10) and developed with a long exposure of 2 hours to obtain, besides the specific signal for the ab52 antigen, some non-specific spots (Fig. 11A) as landmarks relative to the specific signal. The non-specific spots on the Western blot were numbered 1'- 6' and their corresponding spots on the gel were numbered 1- 6. Images of the silver stained gel and blot were digitally superimposed to align the pairs of spots and thus pinpoint the silver-stained spot (encircled in Fig. 11B), corresponding to the mAb ab52 antigen signal in the Western blot. The spot was excised and analyzed by mass spectrometry (by Dr. Urs Lewandrowski in the Sickmann group at the Rudolf Virchow Center of the University of Wuerzburg).

Mass spectrometric identification of the antigen candidates: The protein spot excised out of the NEPHGE 2D gel (encircled in Fig. 11B) was analyzed by MS as described earlier (Hofbauer et al., 2009) and was found to contain peptide patterns

Accession	Protein	MW	Cumulative Mascot scoring	Sequence coverage
Q8MMD3	Epidermal growth factor receptor pathway substrate clone 15*	119761	1039	35
Q9VUC1	Hsc70Cb*	89016	483	13
P16568	Protein Bicaudal D	89127	115	3

Table 7. Proteins identified in the gel piece encircled in Fig. 11B. *These protein identifications featured several different accession entries which belonged to the same protein but isoforms were not resolvable. Accessions are given in Swiss-Prot format.

that could be matched only to three proteins of the Drosophila proteome (Table 7). Since Hsc70Cb and Bicaudal D have not been reported to localize to the synaptic neuropil or the peri-active zone where the mAb ab52 antigen is detected, these proteins presumably are false positive hits leaving EPS-15 as a strong candidate.

aa2 also recognized EPS-15, comparison of migration pattern aa2 and ab52 antigens on 1DE: Interestingly another mAb from the Wuerzburg Hybridoma Library, namely aa2 was already shown to recognize the antigen EPS-15 (Chen, 2009; Hofbauer et al., 2009). To check whether the antigens recognized by aa2 and ab52 display identical properties, proteins from freshly homogenized, CS fly heads were resolved by 1D SDS-PAGE, followed by Western blotting. The blot of a single lane was vertically cut into two halves. One half of the lane was incubated with mAb aa2 and the other with mAb ab52 (Fig. 12) and both were developed together. The developed blots suggest that the antigens recognized by the two antibodies have the same electrophoretic mobility and hence maybe the same protein, EPS-15.

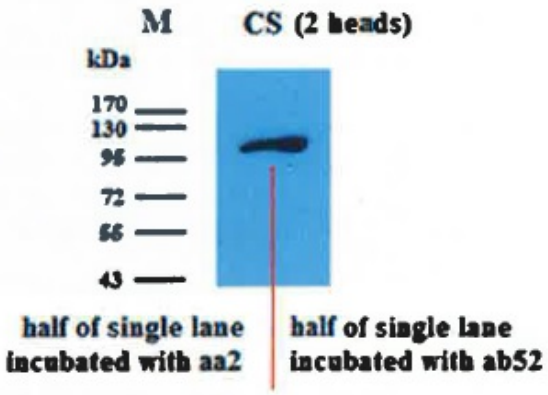


Fig. 12. Blot of a single SDS gel electrophoresis lane loaded with homogenate from 2 wild-type (CS) heads. The blotted membrane was vertically cut in two halves; one was developed with mAb aa2 (left), the other with mAb52 (right). The signals at identical Mr suggests that both mAbs probably recognize the same antigen.

Migration pattern of the antigens recognized by aa2 and ab52 on 2DE: Since aa2 and ab52 seemed to recognize the same antigen on 1DE and since the antigen

recognized by ab52 was found to be a soluble, cytosolic protein, we repeated 2DE experiment to compare the Western blots signals of the 2DE profile for both the mAbs aa2 and ab52. CS fly head homogenates were resolved by 2DE (IEF/SDS-PAGE) followed by partial Western blot of the same gel, consecutively on two separate membranes to get duplicate blots of the same 2DE separation profile. Development of the blots incubated with aa2 and ab52 separately, revealed signals for both antibodies as a single, distinct spot with identical patterns having Mr ~100 kD in the pI range 6-7 as shown in the Fig. 13. This further indicated that both the mAbs indeed may recognize the same antigen.

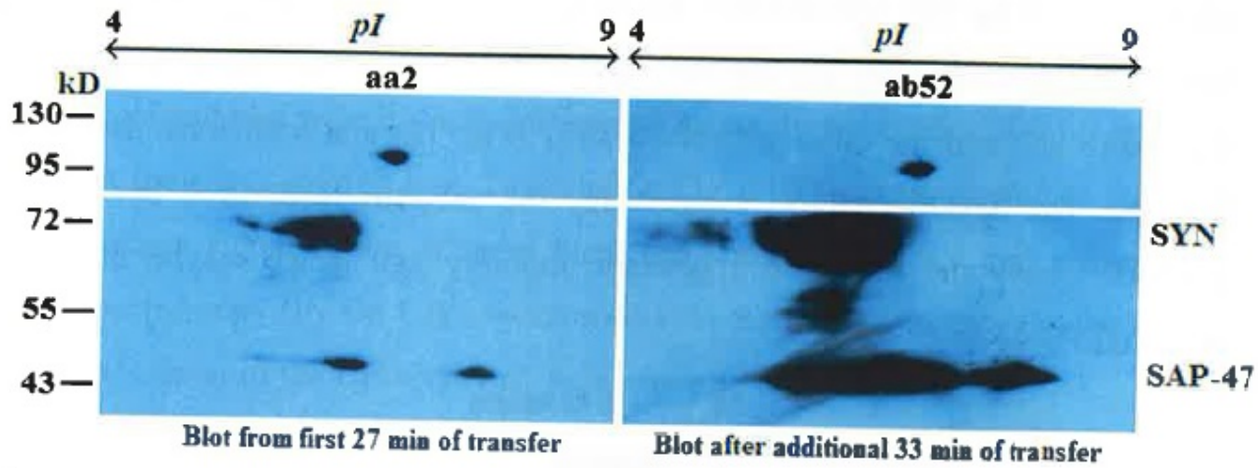


Fig. 13. Two sequential Western blots from a single 2DE gel loaded with sample equivalent to 25 fly heads. The two membranes were cut along the horizontal white line, the upper parts were developed with mAbs ab52 (left, dilution 1:10) or aa2 (right, dilution 1:2), the lower parts were stained with mAbs 3C11 (anti-SYN, 1:100) and nc46 (anti-SAP47, 1:200) as controls for both blots.

Staining pattern of the mAbs aa2 and ab52: On cryosections of adult heads these two antibodies equally stain all synaptic neuropil as shown for aa2 in Fig. 14A. Immunohistochemical experiments of larval NMJ preparations also showed similar staining patterns for both ab52 and aa2 (Fig. 14B and 14C). To finally prove that ab52 recognizes EPS-15, we tested homozygous *Eps15* null mutant escapers on Western blot with ab52. The *Eps15* mutant fly stock kindly provided by Hugo Bellen had the following genotype:

$$yw\ ;\ \frac{eps15^{\Delta 29}}{CyO,\ twi{>}GFP}\ ;\ \frac{+}{+}$$

Flies were allowed to lay eggs on grape-juice-agar plates (section 3.18) and then homozygous *Eps15* mutant embryos were retained by selecting the GFP negative

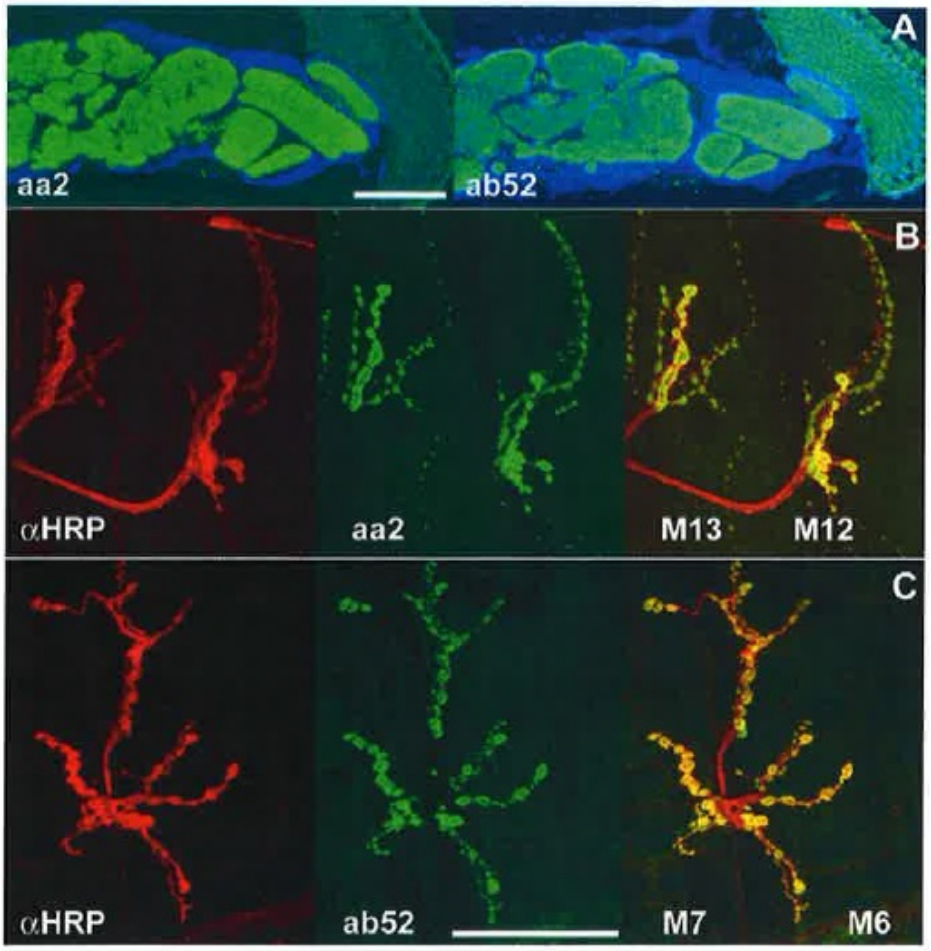


Fig. 14. (A) Cryosections of an adult fly head were probed with mAb aa2 (left) and mAb ab52 (right). Both antibodies stain all synaptic neuropil (green) but not the surrounding the cell body layer whose nuclei are stained with DAPI (blue). (B, C) Synaptic boutons of larval motor neuron terminals stained with anti-HRP (left, red) and mAbs aa2 (B, middle, green), or ab52 (C, middle, green). The overlays in the right column demonstrate that the epitopes recognized by both mAbs are present in all boutons (here shown for muscles M12/13 (B) and muscles M6/7 (C)) but not in the axons. Scale bars in A: 100 μm; in C for B and C: 50 μm)

ones lacking the the GFP-labeled CyO chromosome. Out of the few selected embryos only a single one survived to become a small sized, adult male. This adult

"escaper" was tested again for its genotype by checking for absence of the GFP signal under a fluorescence microscope in comparison to the heterozygous mutant stock as shown in Fig. 15. When this individual fly's head homogenate was tested on Western blot, it gave no signal with the mAb ab52, while SAP47 used as a loading control was present in both (Fig. 16), along with faint non-specific, background signals. This demonstrated beyond any reasonable doubt that mAb ab52 recognizes the protein EPS-15 of *Drosophila*.

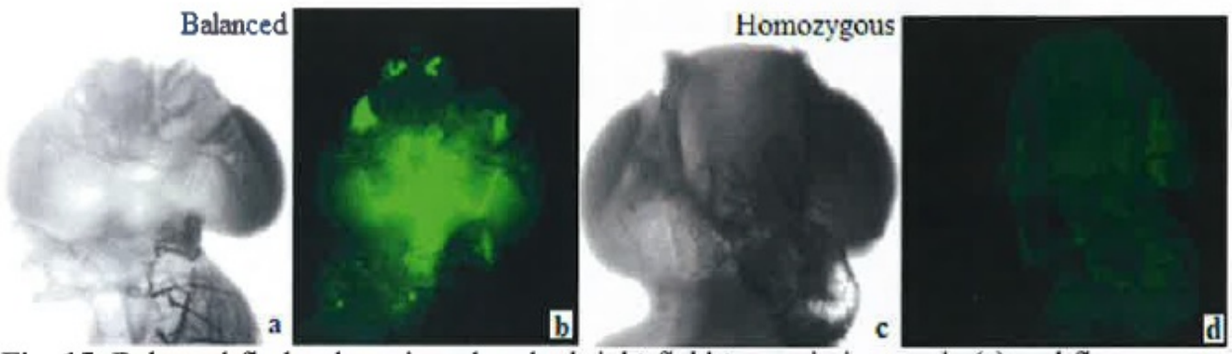


Fig. 15. Balanced fly head as viewed under bright-field transmission mode (a) and fluorescence mode (b) showing GFP expression from the balancer CyO chromosome. Homozygous escaper fly head as viewed under bright-field transmission mode (c) and fluorescence mode (d) showing lack of any GFP expression.

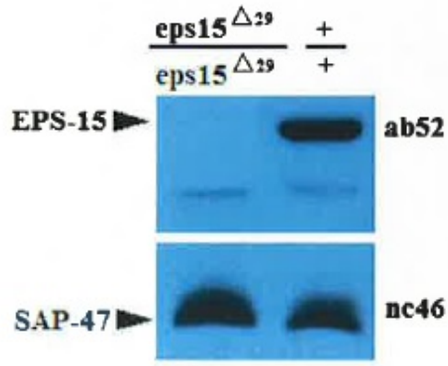


Fig. 16. Western Blot with mAb ab52 showing the absence of the wild type signal in the Eps15 null mutant. SAP-47 recognized by mAb nc46 was used as the loading control.

This was further confirmed by immunostainings of the larval CNS of the homozygous null mutant escapers (Fig. 17A-B). The staining pattern of the guinea pig anti-Eps15 (red) antiserum (kindly provide by H. Bellen) (1:300) in 1x PBST perfectly matches (yellow) that of aa2 (green) in synaptic boutons on muscle M13

(Fig. 17C).

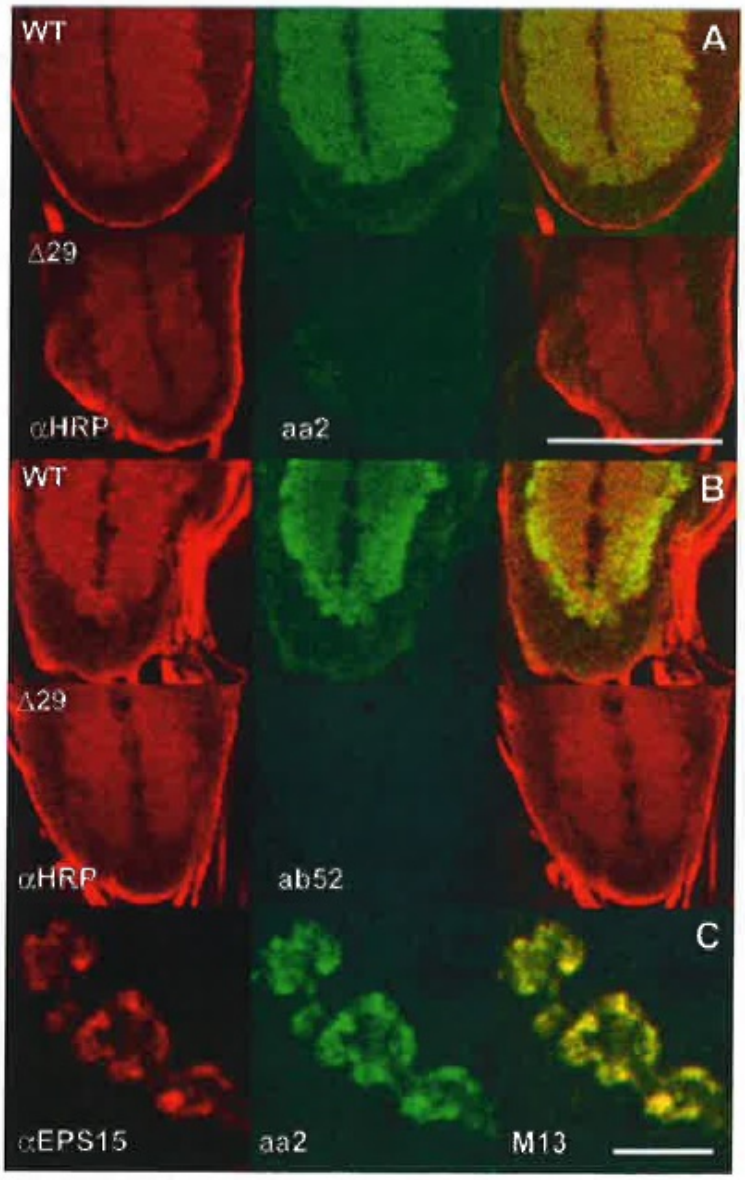


Fig 17. Immunohistochemical staining of larval synaptic neuropil with mAbs aa2 (A) and ab52 (B) is present in wild type (WT) but absent in eps15$^{\Delta 29}$ null mutants (Δ29) and perfectly matches the distribution of Eps15 in synaptic boutons, here shown on muscle M13 (C). Scale bar in A for A and B: 100 μm; in C: 5 μm.

4.1.1 Disscussion

We conclude that the two of the mAbs of the Würzburg Hybridoma Library: aa2 and ab52 recognize the same protein, Eps15 of *Drosophila*. As the two mAbs are

of different isotypes, they apparently are produced by two distinct hybridoma cell lines and can not be sublcones from a common parent cell. aa2 being an IgG1 is more suitable for applications like IP and hence has already been contributed to the Developmental Studies Hybridoma Bank (DSHB) or easy distribution to the entire research community. However ab52 being an IgM is only suitable for immunostainings and Western blots.

EPS-15 in *Drosophila* has been characterized as a protein of the peri-active zone required for normal synaptic bouton development and synaptic vesicle recycling (Majumdar et al., 2006; Koh et al., 2007).

An interesting by-product of the 2DE analysis of the ab52 antigen was the observation of the conspicuous train of synapsin isoforms (Fig. 10).

Protein isoforms differing by a single charge yield such a 'train of spots' on 2DE (Anderson and Hickman, 1979). Hence 2DE is able to resolve proteins with even single charge differences and thus can demonstrate minute differential post-translational modifications in isoforms of a given protein that lead to differences in their pI values. Phosophorylation is one such post-translational modification known to lower the pI of proteins, often seen as a shift of the spots by 1 to 2 pH units per phosphoryl group added, towards the acidic end of the pH gradient (Yamagata et al., 2002). The observed shift is due to the very low pK value of the PO_4 group and its repulsive interactions to SDS, reducing the number of SDS molecules that can bind to the protein (Immler et al., 1998). Since every additional phosphorylation on a protein will cause a further shift, an increasing number of phosphorylations would thus lead to 'train of spots' (Görg et al., 2004). Since vertebrate Synapsins are known to have multiple phosphorylation sites (Cesca et al., 2010), its differentially phosphorylated forms can lead to such trains of spots

horizontally shifted towards the acidic end in 2DE (Kang et al., 2010). Fig 10 may have been the first demonstration of the existence of multiple phosphorylated forms of *Drosophila* Synapsins seen by the 'train of spots' in a 2D gel. It was however not possible to get well stained spots in a stained gel, corresponding to such train of spots on a blot, probably due to the low protein loading capacity of the small sized 2D gels. This observation was however reproducible (Racic, 2010). Recently immunoprecipitation of *Drosophila* Synapsins using the mAb 3C11, followed by separation of major isoforms by 1D SDS-PAGE and mass spectrometric analysis of the 1D bands showed the presence of multiple phosphorylations in Synapsins (Nuwal et al., 2011). It is also noteworthy that an additional, heavier band for Synapsin was observed) in *Sap47* null mutant ($Sap47^{156}$) flies which was not observed in samples treated with alkaline phosphatase (N. Funk unpublished; Nuwal, 2010). This could be a hint for a possible interaction of the two proteins SAP-47 and Synapsins, in a phosphorylation dependent manner. Also $Sap47^{156}$ mutant larvae were recently shown to have a 50% reduction in odorant-tastant associative learning ability (Saumweber et al., 2011). Synapsins are assumed to tether the synaptic vesicles to the cytomatrix of the presynaptic terminal and phosphorylation is believed to play a role in the recruitment of vesicles to the various vesicle pools (Fdez and Hilfiker, 2006). The demonstration of hyperphosphorylated *Drosophila* Synapsins, calls for a detailed study of the various phosphorylated forms of Synapsins and their possible role in synaptic vesicle recruitment, learning and memory. Around 22 spots of synapsins were observed in the blot above and 2D-PAGE using larger gels with higher protein loading capacity and greater resolution ability could probably lead to an increase in the number of distinct spots. Hence 2DE would probably be the best-suited technique for further study of the various phosphorylated forms of Synapsins.

4.2 Monoclonal antibody na21:

Migration pattern of antigen recognized by na21 on 1DE:

The monoclonal antibody na21 recognized a single antigen as a distinct band at Mr ~9 kDa on Western blots of freshly homogenized, CS fly heads as shown below, in Fig. 18a. The monoclonal gave a signal at dilution 1:10 with a single fly head loaded per lane. Besides the head, the antigen was also detected in other body parts like: thorax, abdomen in both genders, with high abundance in female abdomen (due to its presence in oocytes as shown later). The antigen was found to be present in all life stages right from embryo to adulthood (Fig. 18b).

To check whether the protein is conserved, various species of fruitflies were also tested on Western blots and a distinct band at ~9 kD was found in all of them as well (Fig. 18c).

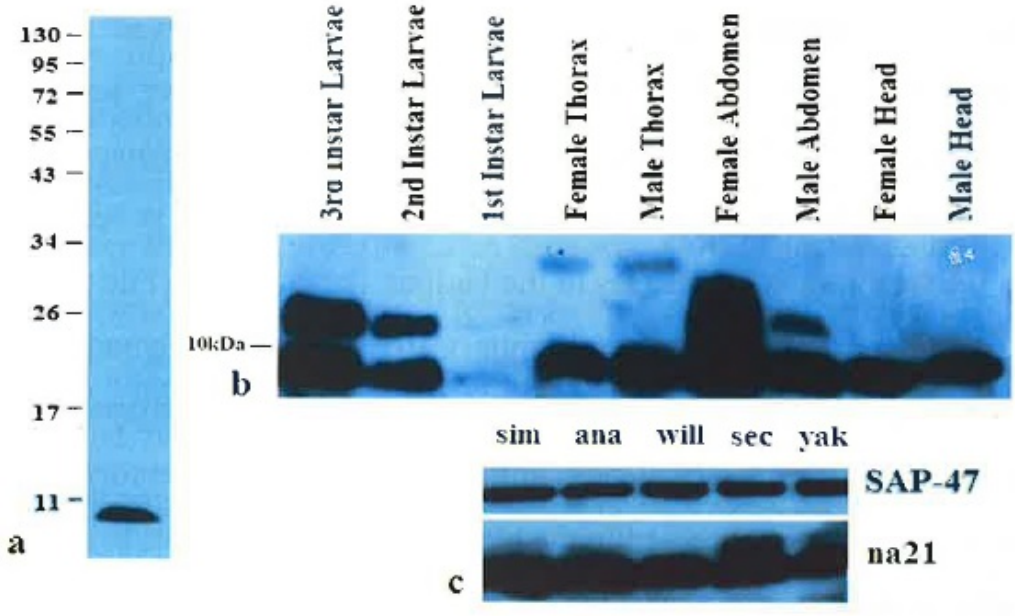


Fig. 18. (a) Western blot of 1 freshly homogenized CS fly head loaded, showing a single distinct signal for the mAb na21 (1:10). (b) Western blot of different life stages and body parts, showing presence of the na21 antigen in the various samples. (c) Western blot of various fruitfly species showing the na21 antigen to be conserved in all, like the loading control SAP-47. sim = *D. simulans*, ana = *D. ananassae*, will = *D. willistoni*, sec = *D. sechellia*, yak = *D. yakuba*.

Staining pattern of the mAbs na21: On cryosections of adult heads, na21 gave a highly specific and interesting staining pattern. It stains all synaptic neuropil in the brain (Fig. 19a), with particularly strong signals in specific layers within the optic lobe, like layers 1, 4/5 and 10 of the medulla, layer 1 and 2 of the lobula (Fischbach and Dittrich, 1989) and slightly fainter signal in the lobula plate as shown below in Fig. 19b. It is noteworthy that one of the optic neuropils, the lamina is not stained at all. Because a signal of similar M_r was detected in Western blot of various species of fruit flies by na21 and the mAb also gave a faint signal in

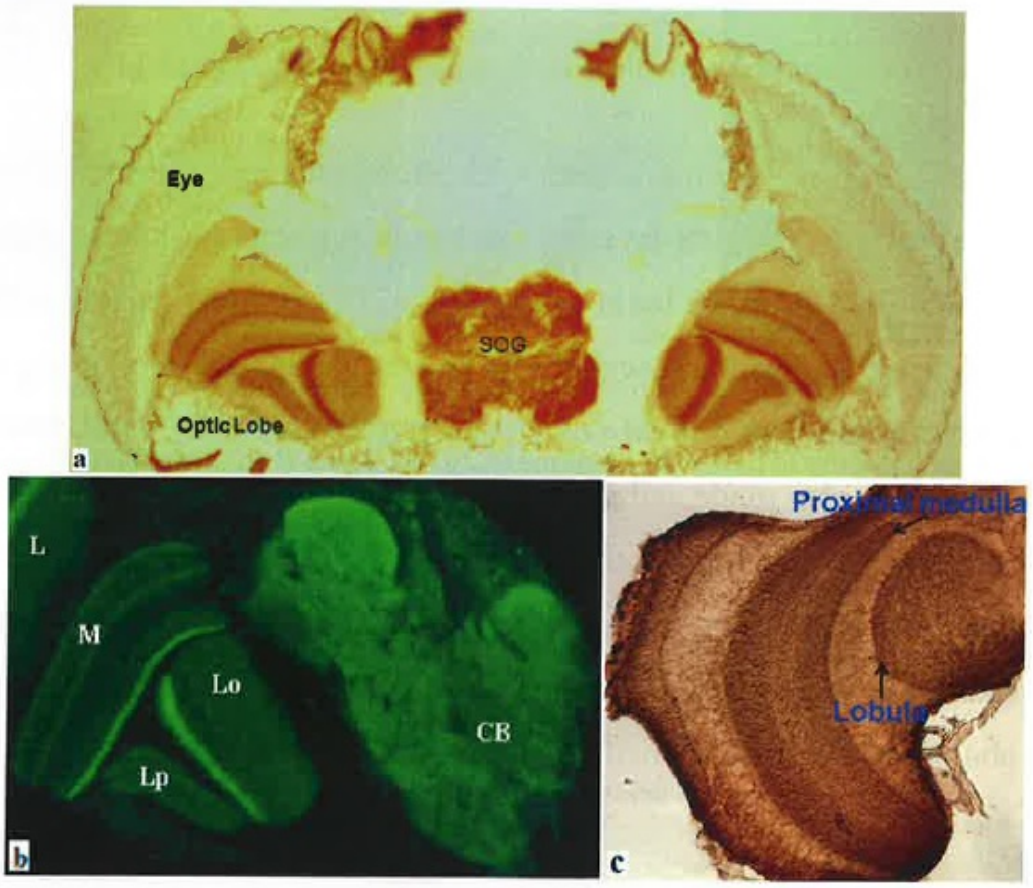


Fig. 19. (a) Cryosection of an adult head stained with na21 (1:3) and DAB showing strong labeling of all central brain neuropils and specific synaptic layers in the optic neuropil. (b) Staining of the same synaptic layers of the optic neuropil reproduced with fluorescent secondary antibody. (c) A cryosection of honey bee brain stained with na21 also showing a similar staining pattern. SOG =sub-oesophageal ganglion, L = lamina, M = medulla, Lo = Lobula, Lp = lobula plate, CB = central brain.

Western blot of honeybee brain, we checked for the staining pattern in honeybee brain cryosections (Fig. 19c).

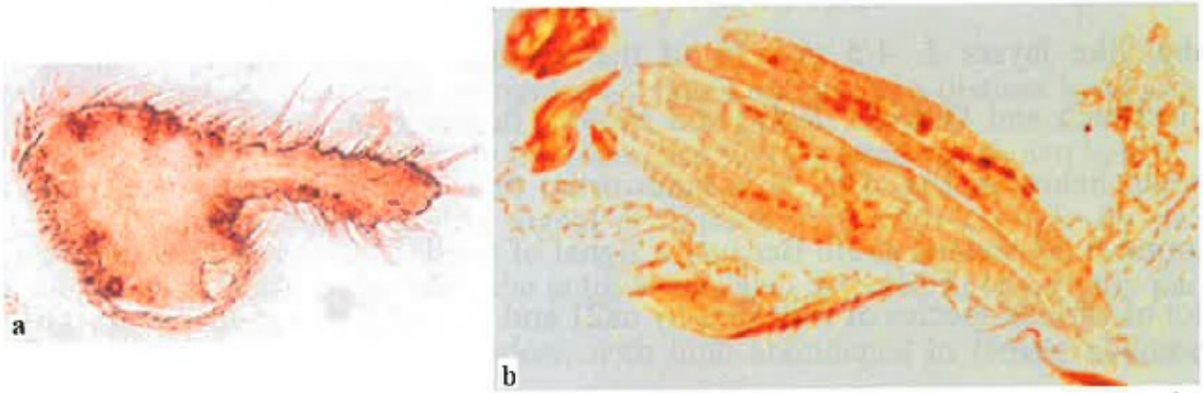


Fig. 20. Whole body cryosections, stained by na21 showing staining in sensory cells of the antennae (a) and specific synapses of the indirect flight muscle in the thorax (b).

na21 showed a similar staining pattern with strong staining of the most proximal layer of the medulla and first layer of the lobula (there is no lobula plate in the honey bee brain). Besides the brain neuropils, na21 also stains sensory cells in the antennae in adult fly head cryosections as shown in Fig. 20a. Following up with the Western blot showing presence of the antigen in other body parts, whole body cryosections were also made and stained with na21. It was found to stain specific synapses in the indirect flight muscles in the thorax as shown in Fig. 20b. The thoracic ganglion was also strongly stained (Fig. 21a), corresponding to the mAb's strong staining of the central brain neuropils. A pair of organs at the tip of the female abdomen, were also stained (Fig. 21b), which probably are the muscles for

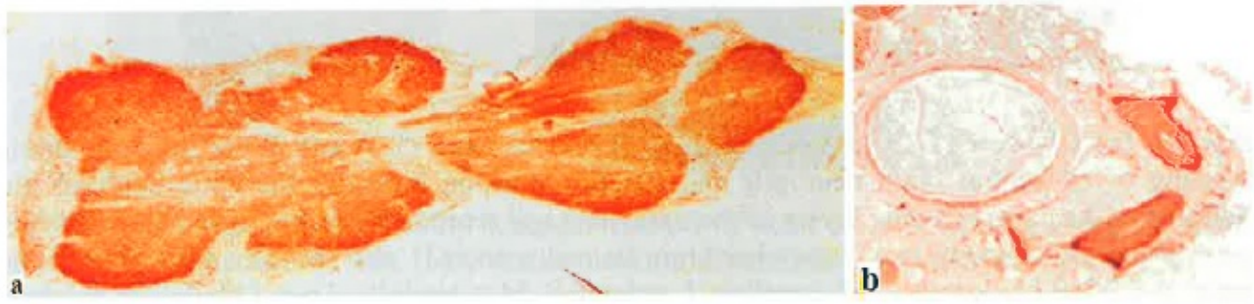


Fig. 21. (a) Cryosection of the thoracic ganglion showing strong staining of the synaptic neuropil (b) A pair of organs at the tip of the female abdomen was also stained strongly by the antibody.

copulation and egg laying. Cryosection of female abdomen also revealed conspicuous na21 staining in the oocytes. The mAb strongly stained the posterior apical cell in each oogonia, which is the egg cell indicating abundant expression of the antigen in egg cell, while the adjacent nurse cells were devoid of staining as shown below in Fig. 22. The strong signal from eggs was also confirmed on blots

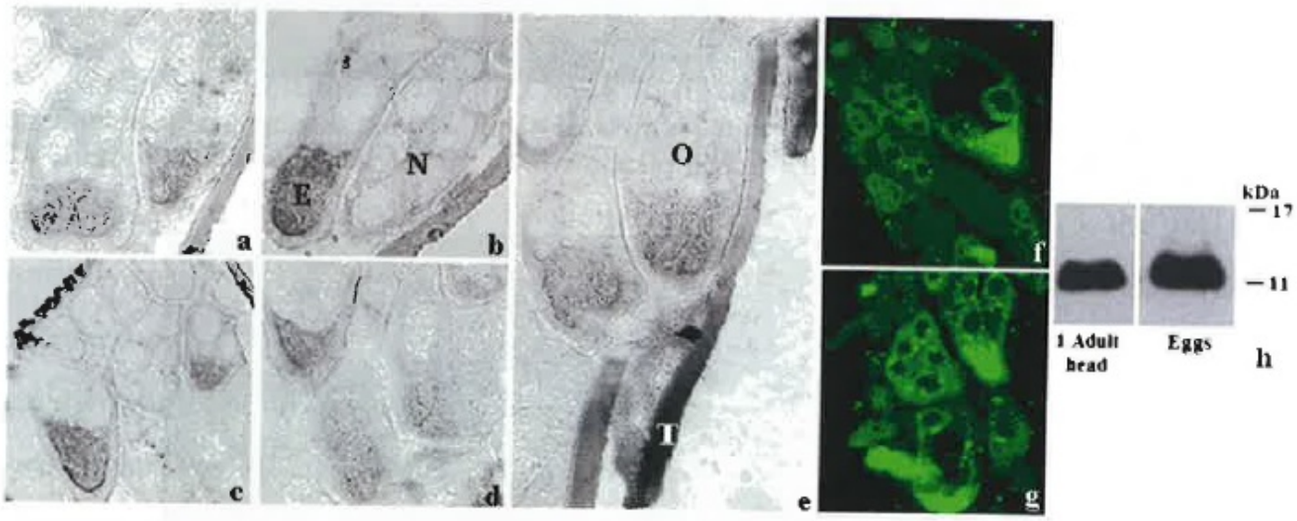


Fig. 22. Different sections of female abdomen with strong staining for the apical egg cell in oogonia with DAB (a-e) and fluorescence staining (f, g). E = egg cell, N = nurse cells, O = oogonium, T = tegument. (h) Western blot of 20 embryos compared to one adult head.

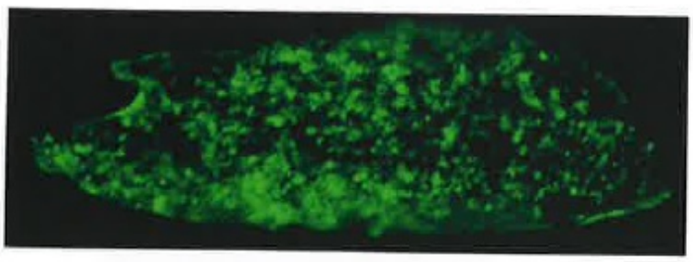


Fig. 23. Cryosection of embryo showing punctate staining with na21.

as shown below in Fig. 22h. Furthermore, cryosections of early embryos when stained with na21 also showed punctate staining as shown below in Fig. 23. Immunostainings of adult brain whole mounts also stained the specific synaptic layers (Fig. 24) as seen earlier in cryosections, thus confirming the specific staining pattern of na21. Staining of larval brain whole mounts and NMJs did not

show any strong staining that could be interpreted (Fig. 25).

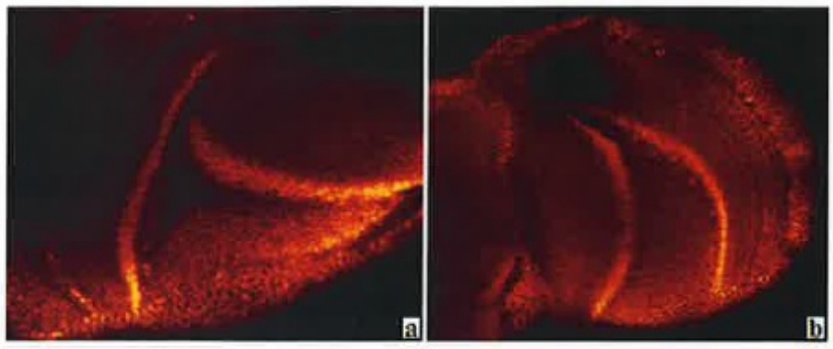


Fig. 24. (a) Confocal slice imaged in the horizontal plane showing a staining pattern similar to Fig 18b. (b) A slice imaged in the frontal plane showing the specific layers in the medulla and lobula.

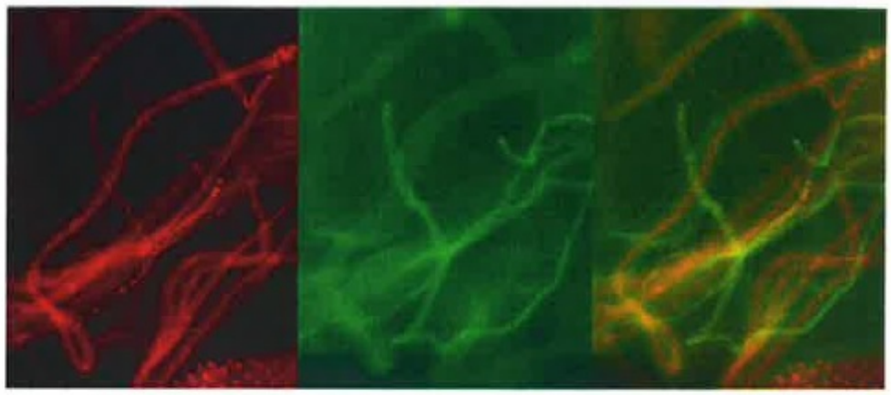


Fig. 25. na21 staining layer around neurolemma in larval motor neurons.

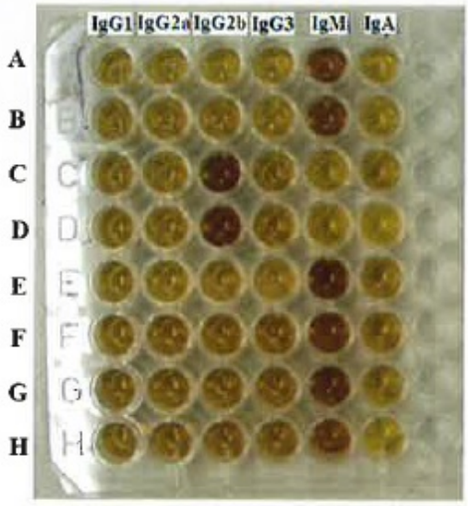


Fig. 26. ELISA plate showing stronger signals for IgM (5th column) for 3 different aliquots of na21 (duplicate rows A-B, E-F and G-H), while mAb 3C11 (anti-SYN) which is known to be an IgG2b was used as a positive control (duplicate row C-D).

Characterization of the mAb isotype: Upon isotyping with capture ELISA and immunoreactive strips (section 3.6), na21 was found to be an IgM with kappa (κ) type light chain (Fig. 26).

Biochemical properties of the na21 antigen: To find out more about the antigen's properties, biochemical studies were carried out. Its subcellular localization, conditions for optimal solubility etc., were studied.

Sub-cellular fractionation of the na21 antigen: Upon sub-cellular fractionation (section 3.9), the protein was found to exclusively remain in the pellet which represents the total membrane fraction (Fig. 27). To determine if it the na21 antigen could be a synaptic vesicle protein, the post-nuclear supernatant (S1) was resolved by (isopycnic) equilibrium density gradient centrifugation. 5-25% sucrose gradient was used as described in section 3.10. 9 fractions of 1 ml each were precipitated by TCA and analyzed by SDS-PAGE. A Western blot (Fig. 28) showed, that while cytosolic and peripherally attached proteins like Synapsins and

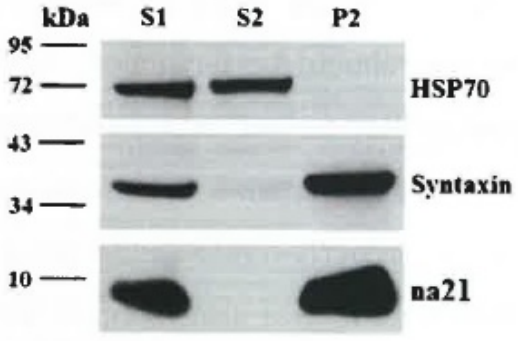


Fig. 27. Subcellular fractionation of the na21 antigen. S1 = post-nuclear supernatant, S2 = cytosolic supernatant, P2 = total membrane pellet. HSP70 was used as a cytosolic marker, while Syntaxin was used as a membrane marker.

SAP-47 stayed on top of the gradient, the synaptic vesicle protein CSP could enter the gradient and had a peak around fractions 3-5. The na21 antigen also had a similar distribution indicating that it could be a vesicle associated protein. To further confirm its integral membrane and vesicle associated nature, the membrane

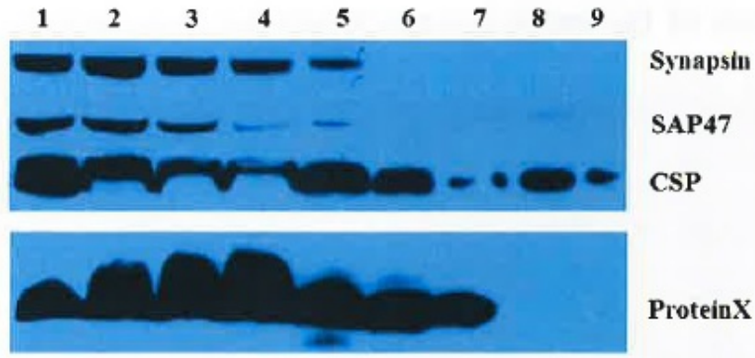


Fig. 28. Western blot showing na21 antigen to be distributed similar to CSP along the sucrose gradient.

(mostly integral) proteome was partially enriched by generation of proteoliposomes (section 3.11), in collaboration with Reinhard Jahn at the Max Planck Institute for Biophysical Chemistry in Göttingen. To first check for the possibility of formation of proteoliposomes, the total membrane pellet was dissolved by 1% (w/v) CHAPS, followed by ultracentrifugation to see if the na21 antigen is solubilized into the supernatant (S3). S3 was then diluted 10 times to bring the CHAPS concentration to 0.1% (w/v) which is below its critical micelle concentration (CMC) such that the micelles would be disrupted while the formation of proteoliposomes should be facilitated. Any proteoliposome formed were pelleted as P4. Western blot showed that the antigen was not found to be in the pellet but remained in the supernatant S4 (data not shown) apparently because the proteoliposomes were not properly formed under these conditions since most of the Synaptobrevin (control protein) was also found in the S4. So we attempted to generate proteoliposomes once again (section 3.11.2). The 9 fractions eluted around the Rhodamine-PE peak were TCA precipitated and tested on Western blots. It was found that the na21 antigen was dissolved from the total membrane pellet (P2) by the 3% CHAPS like Synaptobrevin, as shown in Fig. 29a. The dissolved antigen was used to form the proteoliposomes with Soybean phospholipids and resolved through the gel filtration column. The elution profile of

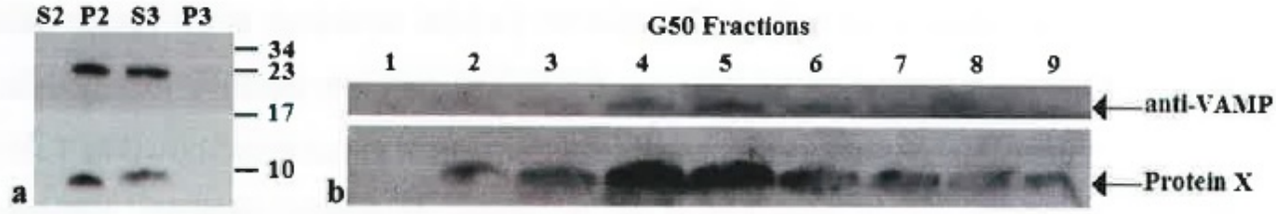


Fig. 29. (a) The na21 antigen was dissolved by 3% CHAPS from the P2 like Synaptobrevin. (b) The na21 antigen has a similar elution profile as Synaptobrevin.

the antigen was similar to that of Synaptobrevin, as seen in their peaks in similar fractions (Fig. 29b). Mass spectrometric analysis (by the group of Henning Urlaub, MPI-BPC, Goettingen) of fractions 4 and 5 led to more than 91 hits. The list of hits generated when arranged as per Mr (using the software Scaffold, ver 3.0, Proteome

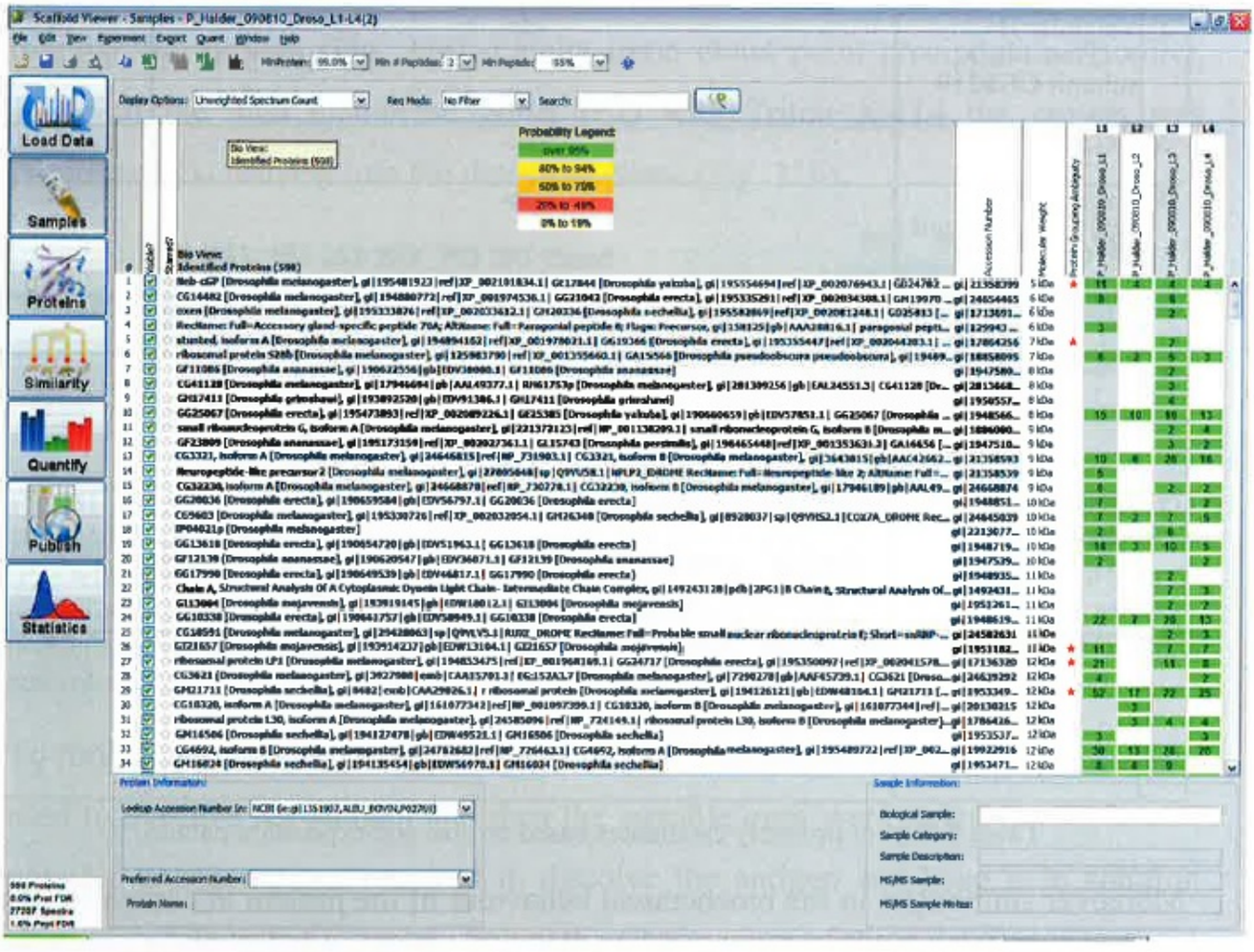


Fig. 30. Screen shot showing hits from MS analysis arranged as per M_r by Scaffold.

Software, Inc.) showed 26 hits which were <= 11 kDa as shown in the screen shot in Fig. 30. Upon careful analysis of the hits based on size, putative localization, level of expression, etc a list of 8 probable candidates was made as shown in Table 8. Interestingly most of these hits were ATPases, especially vacuolar ATPases which were putative proton pumps. Synaptic vesicles are also known to contain at least one V-ATPase (Takamori et al., 2006).

No.	Protein/ Gene	Information available on Flybase	Size (kDa)
1	Oligomycin sensitivity-conferring protein **CG4307**	***molecular function****:* hydrogen-exporting ATPase activity, phosphorylative mechanism, ***biological process****:* proton transport	13.5
2	Vacuolar H+ ATPase 14kD subunit **CG8210**	***-do-***	14
3	**CG3321**	***-do-***	9
4	Vacuolar H+ ATPase G-subunit **CG6213**	***-do-***	14
5	**CG4692**	***-do-***	12
6	**IP04021p CG1268**	***molecular function: -do-*** ***biological process:*** ATP hydrolysis coupled proton transport; ATP synthesis coupled proton transport	10
7	RE19842p VhaM9.7-2 **CG7625**	***molecular function: -do-*** ***biological process:*** ATP hydrolysis coupled proton transport; ATP synthesis coupled proton transport	10
8	**CG12400**	***molecular function:*** NADH dehydrogenase activity; NADH dehydrogenase (ubiquinone) activity, ***biological process:*** mitochondrial electron transport, NADH to ubiquinone	13

Table 8. List of probably candidates based on size and expression pattern.

Moreover similarities in the biochemical behaviour of the protein in comparison to other synaptical vesicle proteins like CSP and Synaptobrevin (VAMP2) also indicate that the protein can be a synaptic vesicle protein. Due to time limitations,

these candidates could not be individually tested. The candidates maybe tested by checking their corresponding deficiency and RNAi lines for lack and/or reduction of signal in Western blots in comparison to equal amount of wild-type heads loaded. Gradient loading of sample would be recommendable for facilitating ease in detecting half gene dosage in such cases.

To study the membrane association of the protein, alkaline extraction of the pellet (Fujiki et al., 1982) was done using 100 mM Na_2CO_3 pH 10, which could not release the protein from the pellet. However, at a higher pH of 11.5 the protein was partially extracted, thus indicating it to be a protein strongly associated to the membrane. The zwitterionic detergent Triton X-114 was able to completely dissolve the protein (Fig. 31a). Finally upon cloud point precipitation (Bordier, 1981) of the total membrane pellet (P2) with Triton X-114 the protein was partitioned exclusively into the detergent phase (Fig. 31b).

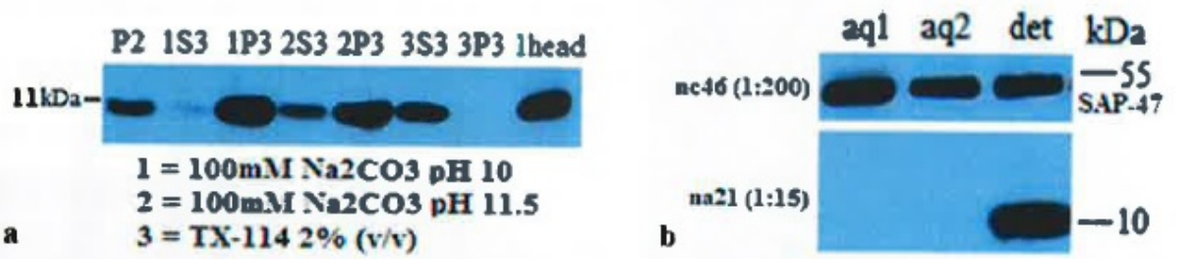


Fig. 31. (a) Alkaline extraction of the membrane pellet (P2) with 100 mM Na_2CO_3 pH 10 (1), 100 mM Na_2CO_3 pH 11.5 (2) and 2% Triton-X 114 (3). (b) Dissolution of the membrane pellet (P2) with 2% Triton X-114 followed by cloud point precipitation. aq1 = aqueous phase after 1st precipitation, aq2 = aqueous phase after 2nd precipitation, det = detergent phase after 2nd precipitation. SAP-47, a protein peripherally associated to the membrane was used as loading control.

To further find conditions for solubilization of the protein, various detergents were used to dissolve the antigen and then the suitable ones were tested for the lowest possible concentrations required to dissolve the antigen and keep it in solution (Fig. 32) while trying to remain below the CMC values of these detergents to avoid the formation of micelles, which are not suitable for downstream processes.

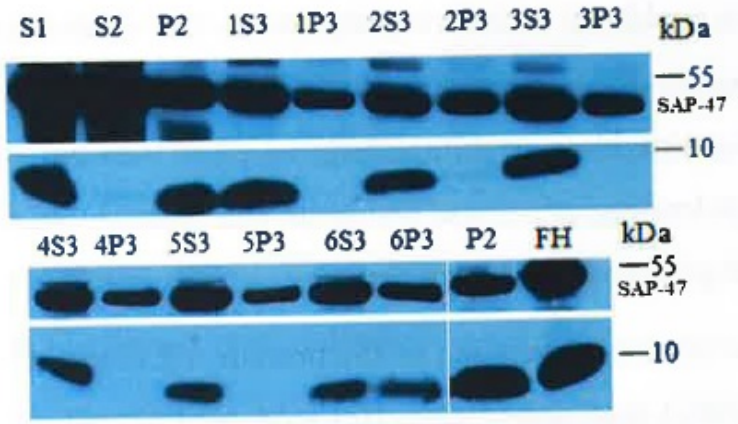


Fig. 32. Effectiveness of various detergents at low concentrations to dissolve na21 antigen. S1 = Supernatant after 1st round of spin at 13,000 rpm (Post Nuclear Supernatant), S2 = Supernatant after 2nd round of spin at 100,000x g (Soluble/Cytosolic fraction), P2 = Pellet after 2nd round of spin at 100,000x g (Membrane fraction). SAP-47 was used as a loading control.

1 = TX-114 0.5% (v/v) 2 = TX-114 0.25% (v/v) 3 = TX-100 0.5% (v/v)
4 = TX-100 0.25% (v/v) 5 = CHAPS 0.5% (w/v) 6 = CHAPS 0.25% (w/v)

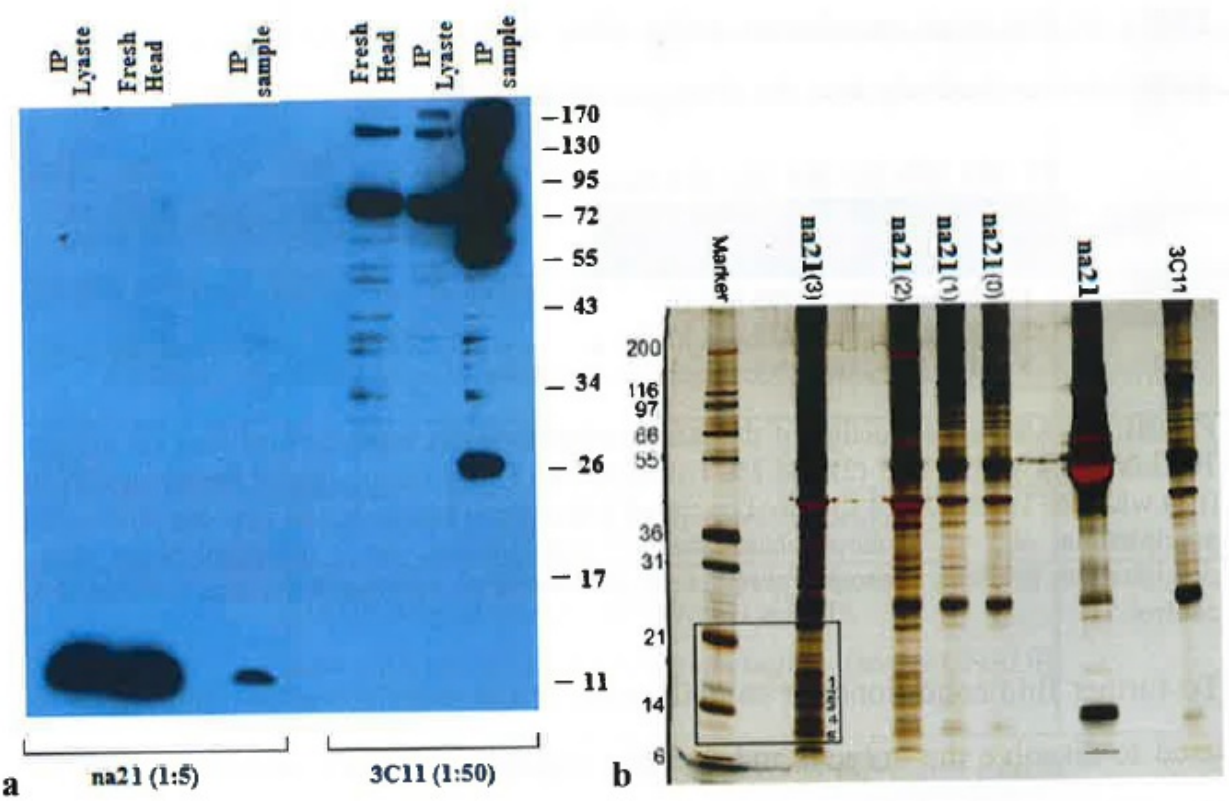


Fig. 33. (a) IP with na21 showing no enrichment of the antigen with respect to the input, while IP with the control mAb 3C11 shows successful enrichment of the antigen Synapsin. (b) Silver stained gel of the IP samples showing no major band, but multiple bands near the expected M_r.

Using the low detergent concentrations (0.25% TX-114, 0.25% TX-100 and 0.5% CHAPS) it was attempted to bring the antigen into solution and then pull down the protein by immunoprecipitations using protein-G beads. These experiments did not succeed in enriching the antigen with respect to the input as shown in Fig. 33a. Besides the poor binding of protein-G to IgMs, presence of detergents may also prevent the binding of the Ab to the antigen. When samples from such IPs that did show a signal for the antigen in Western blots, were silver stained, the presence of many bands in the same size range as the na21 antigen made it impossible to pinpoint the exact band corresponding to the IP signal (Fig. 33b).

Protein-L reported to bind to murine IgMs if they have the kappa-I (κ-I) type of light chains (Nilson *et al.* 1993). Since a method or kit for rapid determination of the light chain type of an Ab is not available, it was decided to purify the mAb from serum free supernatant to use it for an IP by covalent coupling to protein-L. If the mAb would be of the κ-I type, then it would bind to the protein-L beads and upon covalent coupling, we would have a bead-mAb complex ready for use in IP based immunoenrichment of the antigen. Hydroxyapatite based column chromatography was used as a simple, one-step method to purify the IgM from serum-free hybridoma culture supernatants (section 3.21). This work was done in collaboration with Clemens Grimm, at the department of Biochemistry, Biocenter, Uni Wuerzburg. As can be seen from the elution profile in Fig. 34A the mAb was eluted as a distinct peak (Peak 1) around 55% buffer B (320 mM sodium phosphate). A second peak was eluted (Peak 2) around 73% buffer B (392 mM sodium phosphate). The fractions were tested for the presence of the monoclonal antibody by Ponceau-S staining (Fig. 34B) and Western blot (Fig. 34C). Fractions 30-35 corresponding to peak 1, gave strong signals for the mAb light and heavy chains in both Ponceau-S stainings and Westerns blots. The protein concentration

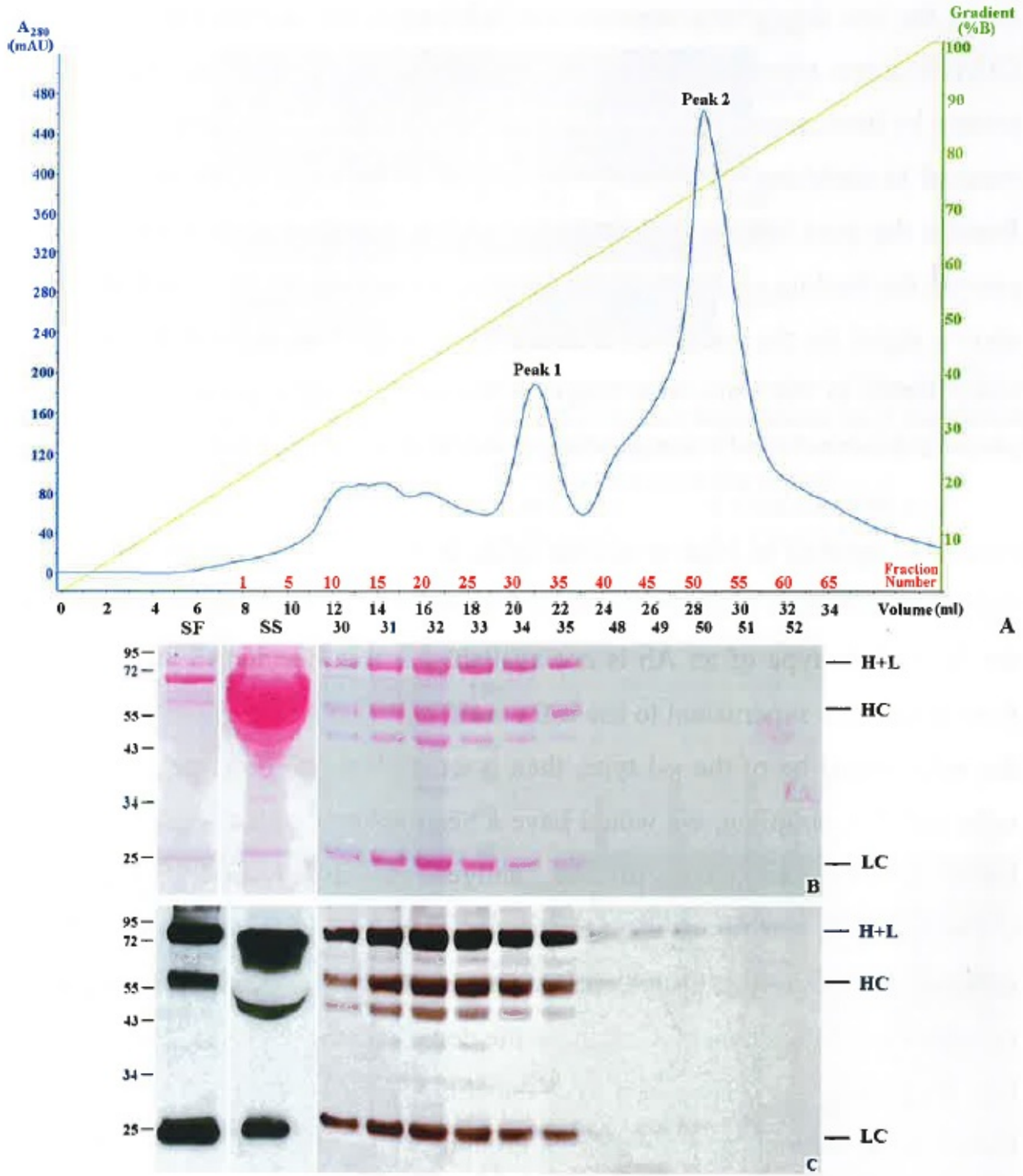


Fig. 34. (A) Elution profile of serum-free hybridoma supernatant applied to a CHT column using a linear sodium phosphate gradient. The A_{280} absorption is shown in blue, the salt gradient as fraction of buffer B in green. Peak 1 at 320 mM phosphate contained the IgM mAb, na21. (B) Ponceau-S stained blot showing protein profiles of serum-free (SF) and serum-supplemented (SS) supernatant as well as the eluted fractions. (C) Western blot of the same membrane. The used antibody mixture detects the heavy chain (HC), light chain (LC) and a band that can be ascribed to a heavy chain/light chain heterodimer (H+L). Fractions 30-35 correspond to peak 1, fractions 48-52 correspond to peak 2.

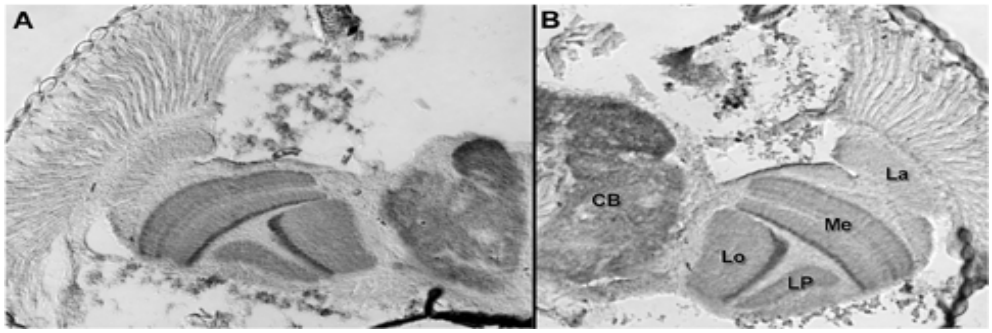


Fig 36. Frontal cryosections of adult fly heads immunostained with mAb na21 serum-free supernatant at dilution 1:3 (A) and purified fraction C15 at dilution 1:100 (B). The mAb in both cases stains most of the neuropil of the central brain (CB) seen here at the level of the esophageal canal. In the optic lobe neuropil na21 highlights specific layers in medulla (Me), and lobula (Lo) while the lamina (La), LP=lobula plate.

Thus the mAb was purified to a high degree and eluted as a distinct peak. This purified mAb was then used in an attempt to immunoenrich the target antigen by covalent coupling to protein-L beads, but it was not able to pull down the antigen. In fact the failed elution of the Ab chains in the IP sample indicated that the IgM had not bound to the beads. This could be a hint that the IgM may not have the κ-I type of light chains. Hence mAb based immunopurification of the antigen did not seem to be the right approach to purify the antigen.

So we tried to use Tricine SDS-PAGE (for better resolution of the proteins in the lower M_r range. Thereafter the proteins resolved on a 15% Tricine gel were partially blotted (i.e. for a shorter period of time so that most of the proteins still remained in the gel) and then the gel was stained with colloidal Commassie, while the membrane was developed to get a Western blot signal. By superimposing the images of the Western blot and the stained gel, the part of the gel corresponding to the Western signal which was then cut out and subjected to MS analysis to identify the protein (Fig. 37). The major candidate among the hits was the protein coded by the gene CG11051 called Neuropeptide like protein precursor-2 (Nplp2). But a deficiency line for the gene did not show any half-gene dosage effect when the

for fraction number 31 from the center of peak 1 was determined to be 0.14 mg/ml by BCA assay. Jugded from the Ponceau S stained blot (Fig 34B), peak 1 consists of approximately 90% pure mAb na21. Fractions 48-52, corresponding to peak 2, neither showed any protein bands on the Ponceau-S stained blot (Fig. 34B) nor gave any signals for mAb light or heavy chains in the Western blot (Fig. 34C). Protein concentration for fraction number 50, from the center of peak 2, was found to be 0.04 mg/ml. Therefore, peak 2 likely contained significant amounts of certain non-protein cell culture medium components with a high absorbance at 280nm.

To test the functionality of the purified mAb, we resolved proteins from 2 freshly homogenized, CS fly heads per lane by SDS-PAGE, followed by blotting and detection of the target antigen with the purified mAB na21 in different lanes at dilutions 1:200, 1:500, 1:1000 and 1:2000, respectively. At a dilution of 1:2000, a faint signal was visible. A dilution of 1:1000 produced a satisfactory signal indicating a 100-fold activity increase per volume compared to crude supernatant, which yields a comparable signal only at a dilution of 1:10 (Fig 35).

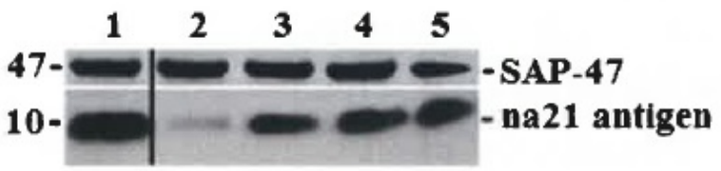


Fig. 35. Western blot of wildtype fly heads (2 heads per lane) showing the target antigen recognized by the unpurified supernatant at 1:10 dilution (lane 1) in comparison to the purified fraction C15 at a dilution 1:2000 (lane 2), 1:1000 (lane 3), 1:500 (lane 4), and 1:200 (lane 5), demonstrating that the eluted mAb was functional on Western blots at more than 100 fold higher dilution. SAP47 detected by mAb nc46 (1:200) was used as loading control.

In immunohistochemical preparations of adult fly-head cryosections un-purified na21 supernatant (Fig. 36A) shows the characteristic staining pattern (cf. Fig. 19) at a dilution of 1:3, while the purified mAb stains with similar intensity at a dilution of 1:100 (Fig. 36B).

same amount of wild type and deficient samples were compared by a Western blot (data not shown). To definitely exclude Nplp-2 as the antigen for na21, the Nplp-2 cDNA

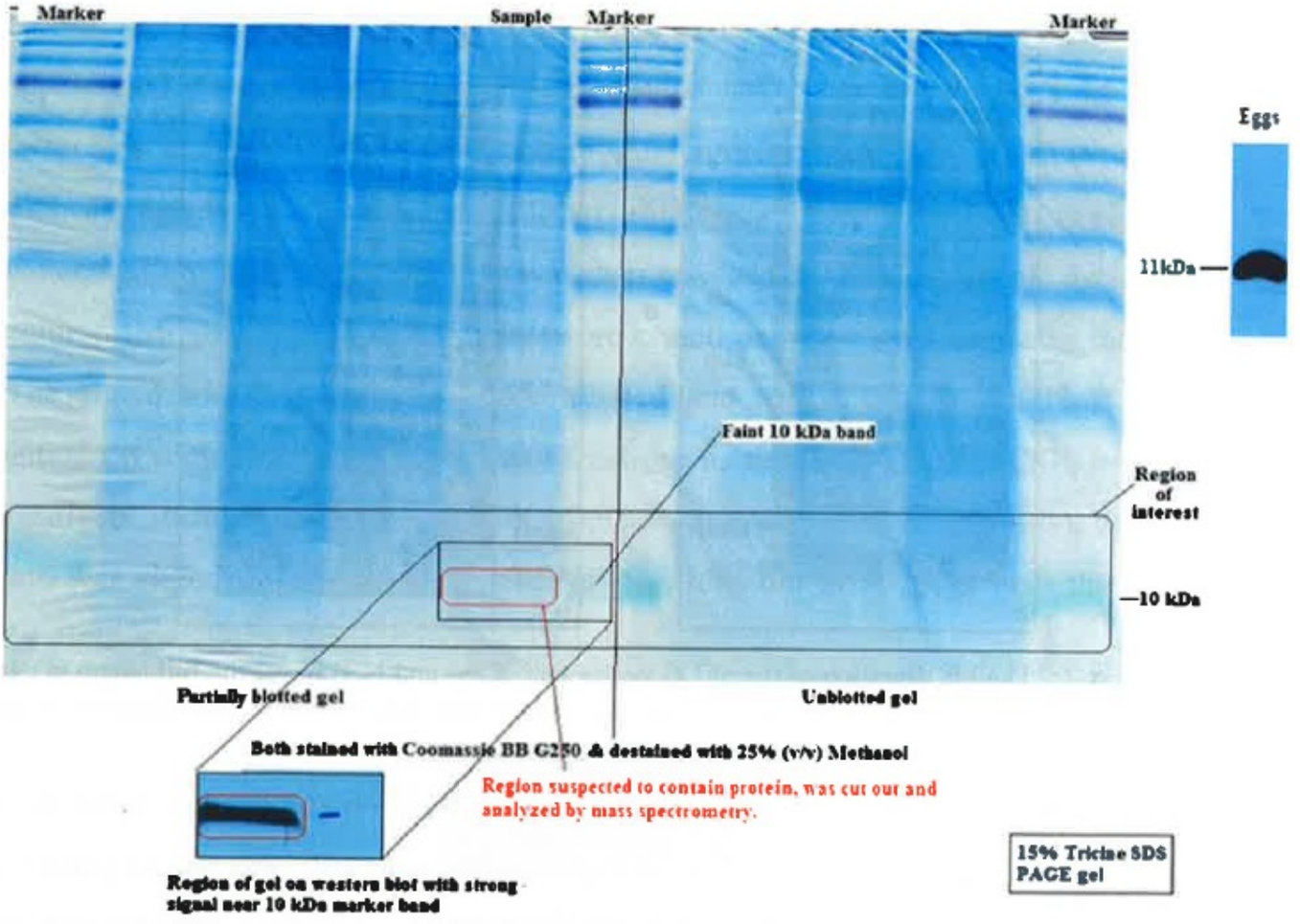


Fig. 37. Coomassie stained Tricine-SDS-PAGE gel with left half partially blotted and stained and right half directly stained, and used to compare the separation profile with the blot to pinpoint the region of the gel (red) corresponding to the Western blot signal.

(RH08410) was obtained from the Drosophila Genomic Resource Center (DGRC, Indiana University, USA) as a pFLC plasmid, which was then double digested at the KpnI and NotI sites to get the full length cDNA out of the vector (Fig. 38a) followed by cloning into the pUAST vector. Success of cloning was cross-checked by double digestion of the vector to release the insert (Fig. 38b), which was confirmed by sequencing (MWG Operon). This pUAST vector was used for

generation of transgenic, UAS-Nplp-2 lines by P-element mediated germ-line transformation (Best Gene Inc.). Cryosections of flies with ectopic expression of the Nplp-2 transgene in the retina obtained by crossing the UAS-Nplp-2 lines with

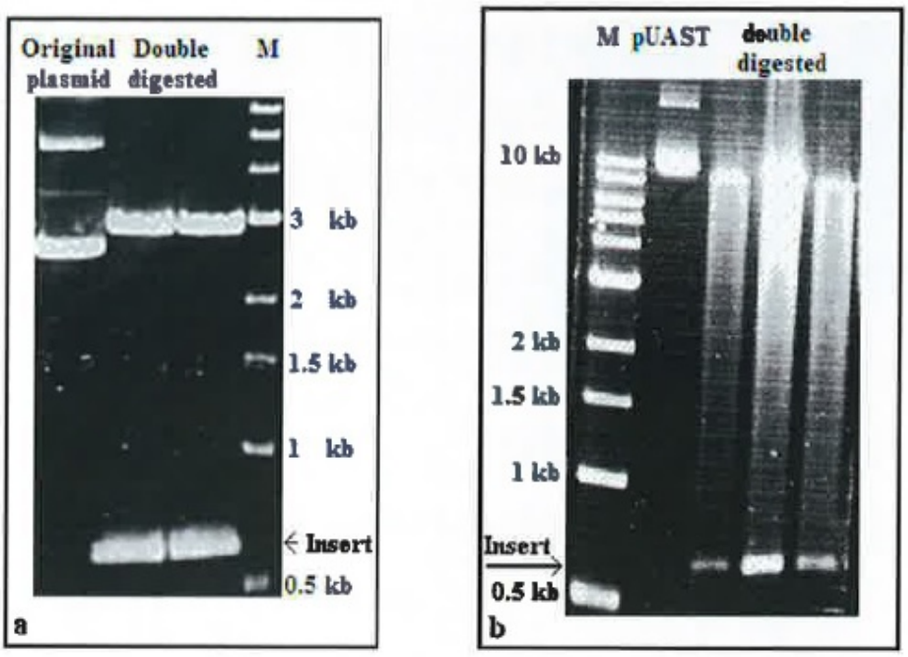


Fig. 38. (a) Double digestion of the pFLC vector with KpnI and NotI to get the full length cDNA insert. (b) Cloning of the insert into the pUAST vector followed by the double digestion to get the insert, which was checked by sequencing.

the GMR-Gal4 driver line did not show any staining in the eyes with the mAb na21, though the normal staining of the synaptic neuropils was found (data not shown). As a result we could concluded that Nplp-2 was only a false hit and not antigen of mAb na21.

In order to reduce the number of candidates prior to MS analysis biochemical purification of the antigen from homogenized heads was attempted in collaboration with Clemens Grimm, at the department of Biochemistry in the Biocenter at the University of Wuerzburg. Since the antigen was expressed endogenously and could not be overexpressed, a very large amount of starting material had to be used

keeping in mind the dramatic losses to be expected in various fractionation steps like centrifugations, chromatography etc.

Total membrane pellet P2 (~ 30000 heads) was dissolved in 0.4% (w/v) CHAPS (below its CMC to avoid micelle formation), ultracentrifuged at 100000 g and the supernatant containing the protein was diluted to 7.5 volumes with 4 M Urea (which is a chaotrope and keeps membrane proteins in solution) followed by dialysis against 4 M Urea to further dilute the detergent to a negligible concentration. The dialyzed sample was then resolved through an anionic exchange column (MonoQ™, GE) and proteins were eluted using a 0.1 - 1 M salt gradient. The eluted fractions were TCA precipitated and tested on Western blots. The antigen was eluted as a peak over two fractions as shown in Fig. 39. CSP, being a relatively sticky protein also present in membrane was used as a positive control and was eluted in a broad range over all fractions but had a peak near the na21 fractions.

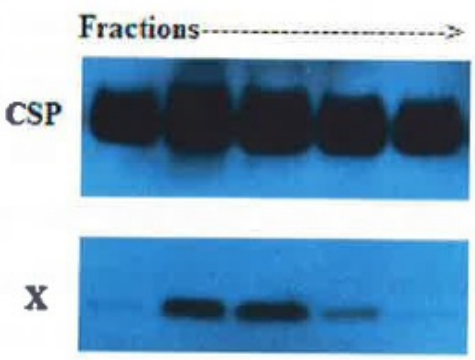


Fig. 39. Western blot of fractions eluted from anionic (MonoQ™) column showing the antigen eluted as a peak in two distinct fractions. CSP was used as a positive control.

4.2.1 Discussion

The signal around 9 kDa showed that the protein was a rather small one which could be around 87 amino acids. The antigen was present in all life stages and could be detected already in early embryos indicated that it was present as a

maternal protein and may also be expressed at a very early stage in development. This could indicate its possible role in development or a fundamental role in cellular system which requires its presence from such an early stage. The proteins presence in 5 other fruitfly species and a similar staining pattern of the mAb na21 in honey bee brains also indicates towards the conserved nature of the protein, which is often an indication of the degree of necessity of a protein in the biological system.

The ubiquitous staining of the central brain neuropils (which was continued via the sub-esophageal ganglion to the thoracic ganglion) but selectively stronger staining in the layers of the synaptic neuropils of the optic lobe, illustrates an interesting localization pattern for the protein. Hence it seems to be necessary in most central neuropils while being needed only in specific synapses of the optic neuropils. Similarly its presence in other body parts like sensory cells of the antennae, specific synapses of the indirect flight muscles, etc also indicated specialized role of the protein in various body parts. The 2^{nd} and 3^{rd} larval stages showed a second band in Western blots, which could be a splice variant of the gene (which would mean that the epitope is likely to be present in the common fragment). The presence of multiple isoforms also supports the possibility of differential localization due to differential splicing of the mRNA and possibly different roles of the isoforms in different tissues.

Hence we could infer that the antigen is widely expressed in different body parts as indicated by the stainings. Though this was in agreement to the Western blot signals, but specificity of these staining could only be confirmed with absence of staining in a mutant for the antigen. This is however not possible until the target antigen recognized by na21 is identified.

The subcelular fractionation seemed to work well with the cytosolic marker HSP70 and membrane marker Syntaxin being present exclusively in the supernatant and pellet fractions respectively. The partial extraction of the protein from the membrane pellet upon highly alkaline treatment indicated that the protein could be strongly associated to the membrane. Finally upon cloud point precipitation, the protein was partitioned exclusively into the detergent phase which indicating that it is probably an integral membrane protein. In the proteoliposome enrichment experiment, the elution profile of our protein of interest was similar to that of Synaptobrevin. This also indicated that the antigen behaves like an integral membrane protein and could be a vesicle associated protein. na21 was found to be an IgM, so its application in IP for enrichment of its target antigen was unsuitable. IgMs are pentameric and show weak or no binding to protein-A, -G and A/G beads (Kronvall et al., 1974; Björck and Kronvall, 1984; Sikkema, 1989). However IP was still attempted but, as expected, it was not successful. This could also be due to the low antibody concentration of the antibody in the supernatant, evident from the fact that a strong Western blot signal is achieved only when the supernatant is used at dilution 1:10 or less.

To try immunopurification with protein-L beads the IgM was purified by HA column chromaptgraphy. The eluted fractions from the CHA column when tested on gel showed the expected bands for the light and heavy chains. These fractions also gave a strong signal at M_r ~85 kDa in the Western blots. The molecular weight of this band can be explained by the presence of a covalently linked heterodimer of a heavy and a light chain. An additional band at around 45 kDa was also observed. A possible explanation for the existence of this band could be a non-reduced intramolecular disulphide bridge within an antibody peptide chain or partial proteolytic degradation of the antibody.

Finally the purified IgM when used to immunoenrich the target antigen by covalent coupling to protein-L beads, but was not able to pull down the antigen. Infact elution of the Ab chains in the IP sample also indicated non-binding of the mAb to the beads. This could be possible if the IgM did not have the κ-I type of light chain. Hence mAb based immunopurification of the antigen seemed to be a difficult approach due to the IgM nature of the mAb and the membrane associated nature of the target antigen.

This also explained the failure in initial attempts to purify the antigen by immunoprecipitation, as lack of sufficient detergents in the buffers would not allow solubilization of the membranes to release the protein into solution for the possibility of interaction with the mAb. Integral membrane proteins are also highly hydrophobic hence highly prone to aggregation and precipitation at pH values near their pI. This could probably explain the lack of success in trying to resolve the protein by conventional 2DE (IEF-PAGE).

Failure of IPs, 2DE and Tricine SDS-PAGE to enrich the protein dentification, along with the membrane associated nature, small size of the protein and IgM nature of the mAb clearly indicated biochemical purification to be one of only possible methods for purification of the antigen sufficient enough to give a single specific band in 1DE which would have a corresponding Western signal with the mAb so that the protein could be identified by MS analysis.

Elution of the protein through an anionic exchange column (MonoQ™, GE) as peak over two fractions, indicated that the protein is probably anionic in nature. These preliminary experiments on biochemical purification gave promising results and showed that using biochemical methods the na21 antigen could be purified by a series of fractionation, ultracentifugation, dialysis and chromatographic steps.

However due to lack of time, the project could not be completed within this doctoral thesis, but could very well be pursued in the future based on the available data.

4.3 Other mAbs:

Besides the already mentioned mAbs, the Würzburg Hybridoma Library contains about 200 mAbs, which selectively stain specific structures in the brain of *Drosophila melanogaster*. Frontal z-projections of confocal stacks of the staining patterns of some of these mAbs are described below.

4.3.1 ab47: The mAb ab47 was found to be an IgM with κ type of light chain. It stained the fan-shaped body and few cells in the central brain, few cells in the *pars intercerebralis* (PI), few cells in the auxillary medulla, with widespread arborizations all over the medulla (Fig. 40).

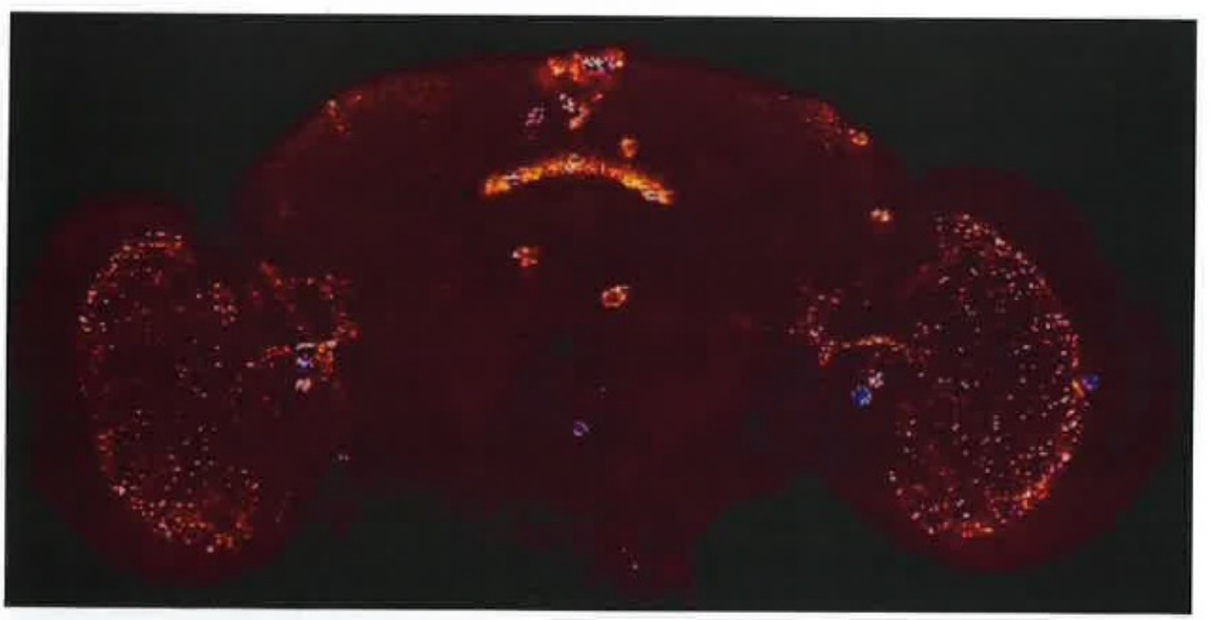


Fig. 40. Staining pattern of ab47 (provided by B. Mühlbauer)

4.3.2 ab158: The mAb ab158 stained large cells in the *pars intercerebralis* (PI) with some arborizations in the dorsal part of the brain and innervations to the fan-

shaped body. Few cells in the auxillary medulla were also stained with arborizations in the medulla (Fig. 41).

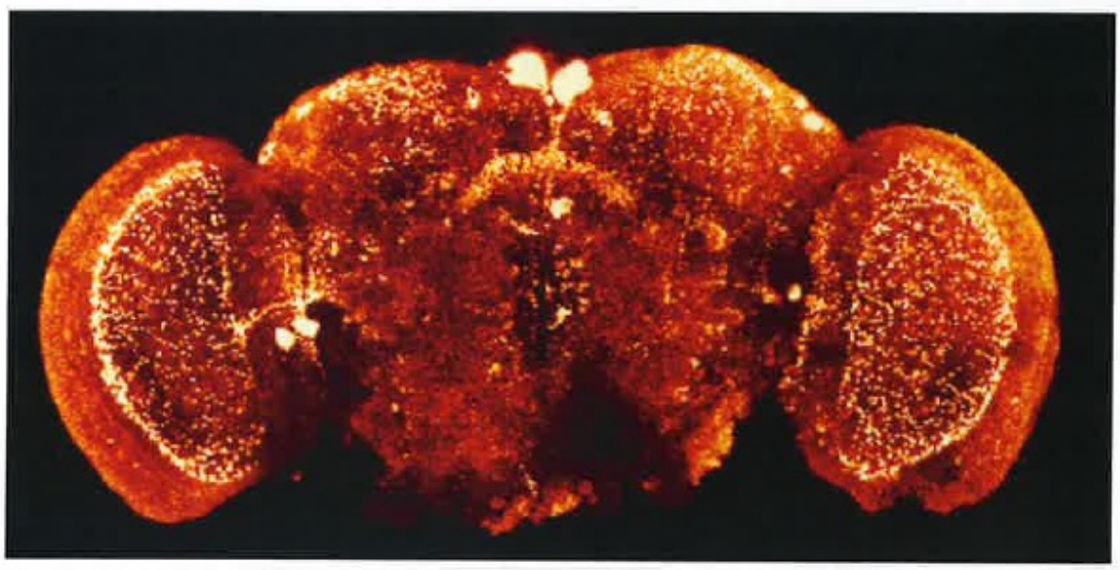


Fig. 41. Staining pattern of ab158 (provided by B. Mühlbauer)

4.3.3 fb20: The mAb fb20 was found to be an IgM with κ type of light chain. It stained a pair of dorsal cells on adjacent to the PI with arborizations in the dorsal part of the brain and in a layer of the fan-shaped body. A few cells near the auxillary medulla were also stained with innervations into the medulla (Fig. 42).

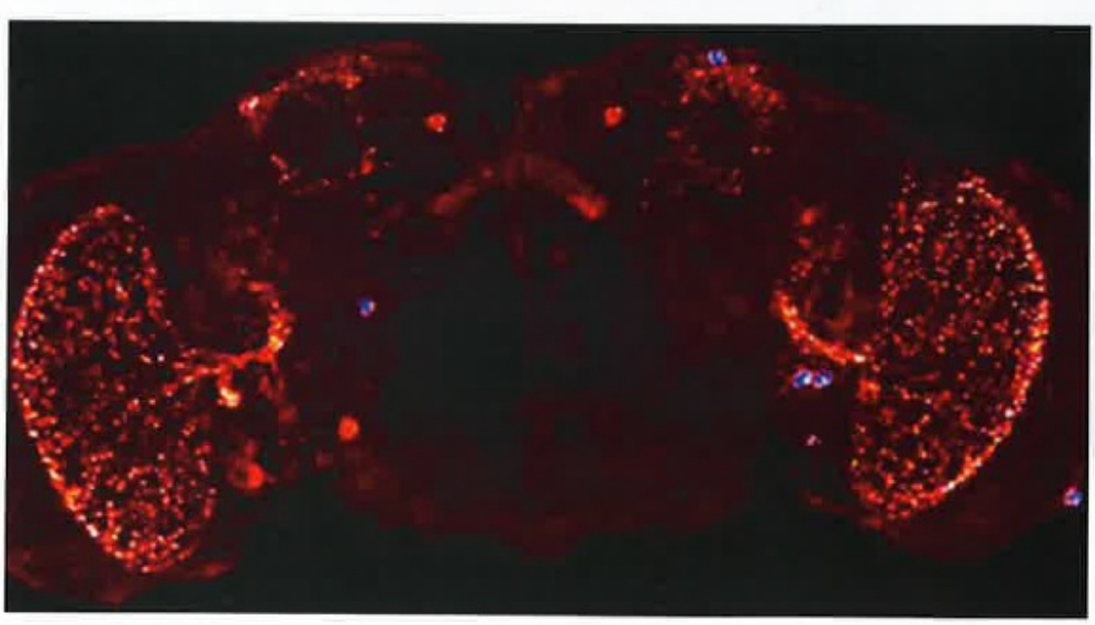


Fig. 42. Staining pattern of fb20 (provided by B. Mühlbauer)

4.3.4 nb168: The mAb nb168 was found to stain the adult brain in a pattern (Fig. 43) similar to the mAb nb33. nb33 is another mAb of the Wuerzburg Hybridoma Library which was already known to recognize the PDF precursor peptide and hence stained PDF positive neurons (Hofbauer, 2009). To test whether nb168 and

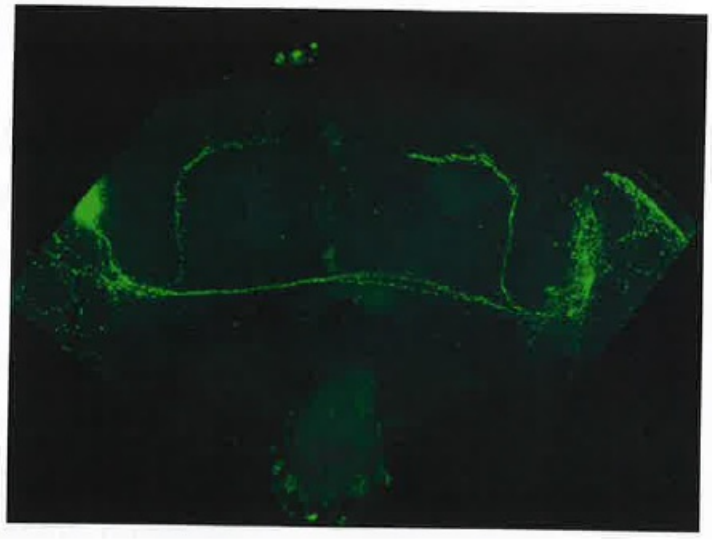


Fig. 43. Staining pattern of nb168 (done with B. Blanco)

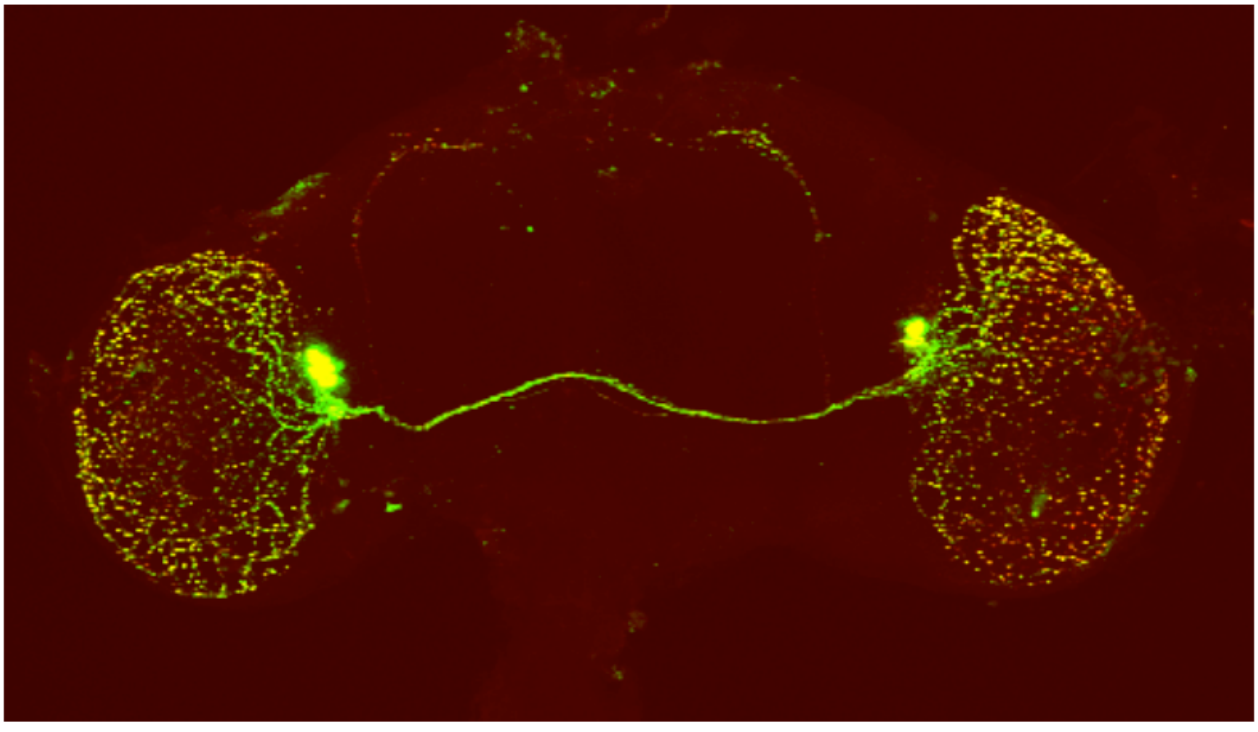


Fig. 44. Merge of double staining using **anti-PDF** and **nb168** (done with B. Blanco)

nb33 stain the same cells double labeling using nb168 and rabbit anti-PDF antibody was done, which showed almost complete merge of the two signals, shown below in Fig. 44. Thus it was confirmed that nb168 also recognized PDF positive neurons and hence could also be used as a marker antibody for these cells, just like nb33.

4.3.5 nb169: The mAb nb169 was found to be an IgG_1. It stained a pair of large neurons in the central brain, on either side of the esophageal canal, which apparently send out a widespread network of arborizations throughout rest of the central brain. Besides the two large neurons, there are also 3-4 smaller neurons near the auxillary medulla in the region between the optic neuropils and the central brain. In some preparations a thin neurite is seen to connect the few cells near the auxillary medulla, passing through the medulla all the way to its outer edge as marked by the white arrows in Fig. 45. When observed under higher magnification, as shown below in Fig. 46, it is suggested that the stained structure is actually a single large neuron with a single nucleus, and not a collection of more than one neurons close to one another. The smaller neurons near the auxillary medulla are also clearly visible.

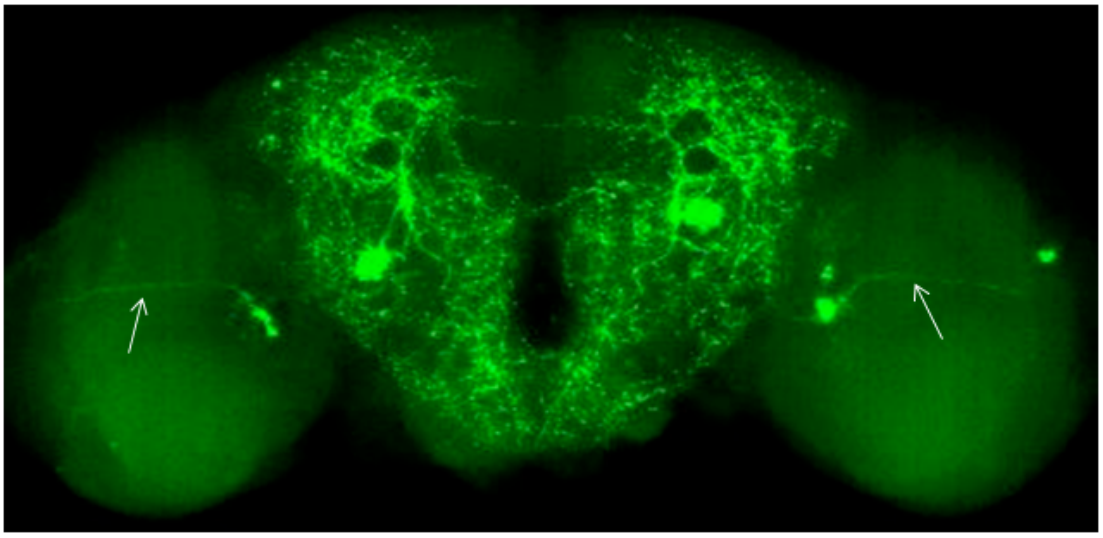


Fig. 45. Staining pattern of nb169 (provided by B. Mühlbauer)

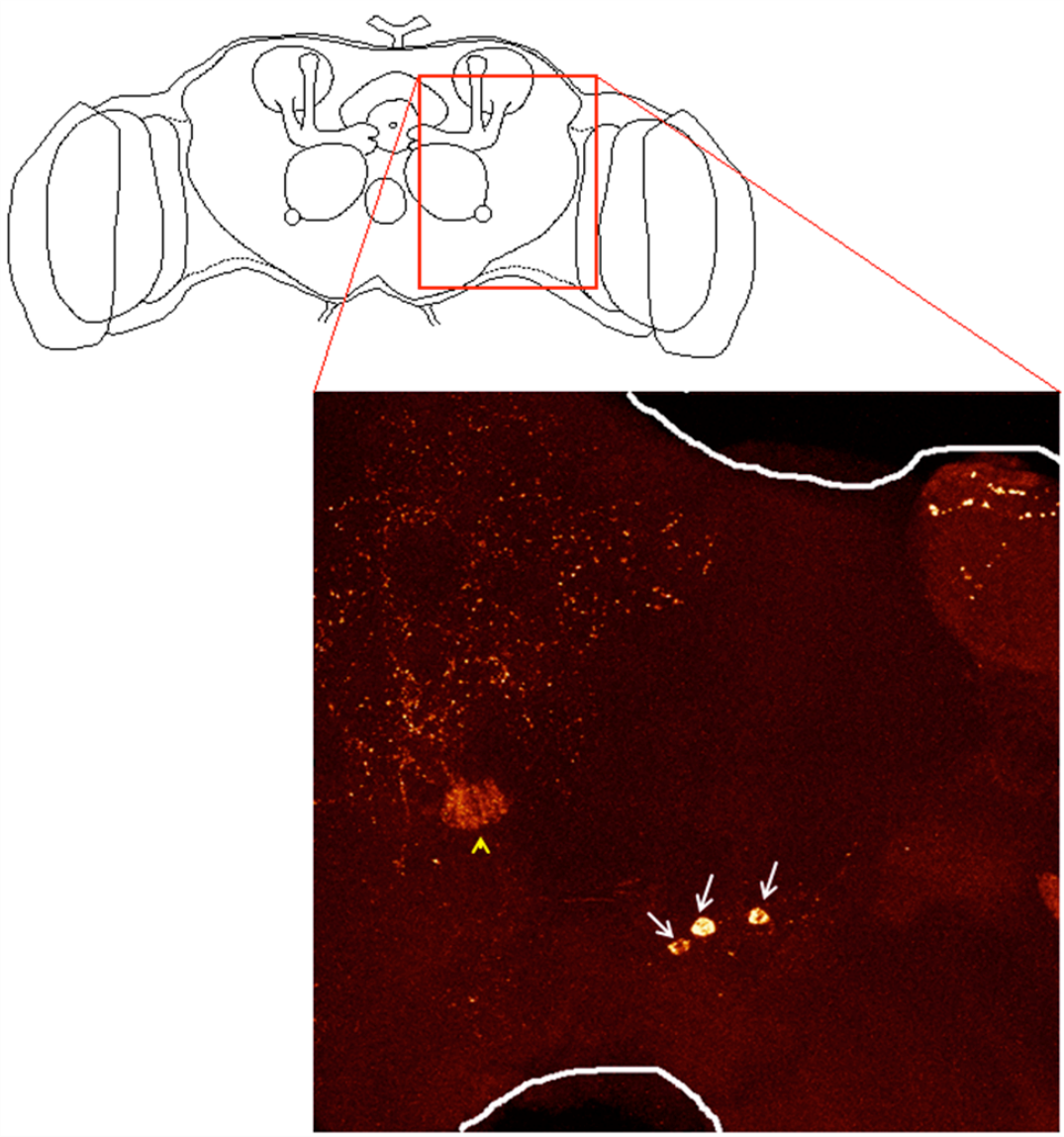


Fig. 46. 63x magnified confocal image showing large neuron (yellow arrow head) and smaller neurons (white arrows) stained by nb169. (Whole brain schematic adapted from JFly http://jfly.iam.u-tokyo.ac.jp/html/figures/Head&Brain_Outline/Head&Brain_Outline.html).

Analysis of the 3D structure (using Fiji) created by the stack of confocal images revealed that the pair of large neurons in the central brain are located in the posterior cellular cortex of the brain while their arborizations are directed to the anterior side as shown in Fig. 47. Interestingly the nb169 was found to also stain a pair of cells on the exterior edges of 5 consecutive segments in larval ventral ganglion as shown below in Fig 48.

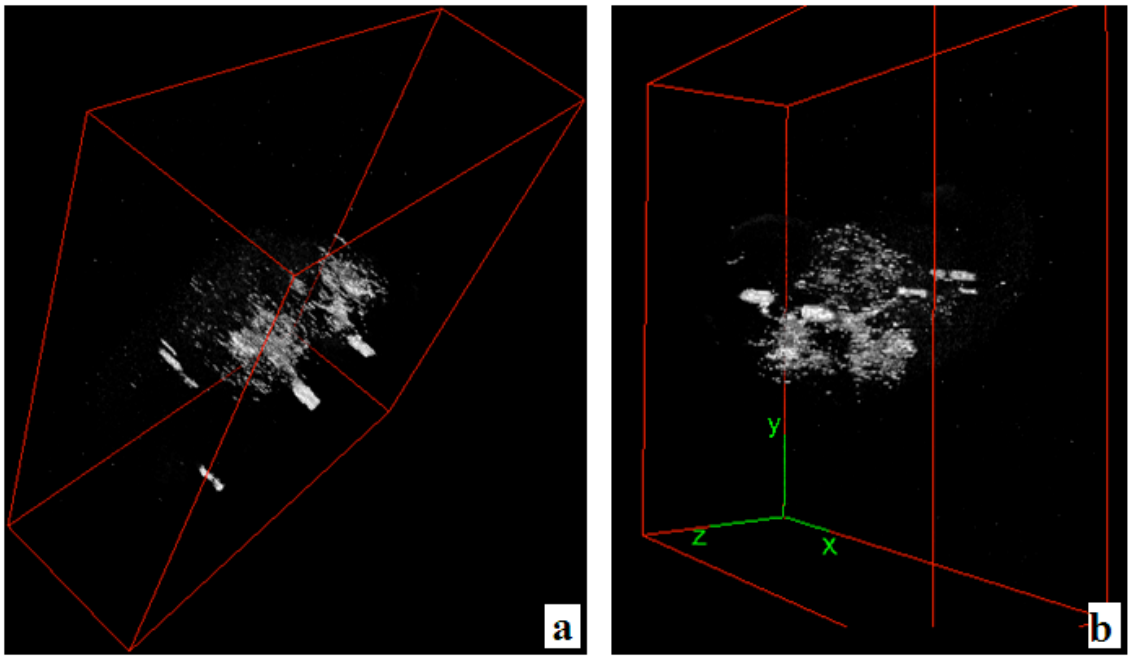


Fig. 47. Two views of 3D reconstruction of the staining pattern of nb169.

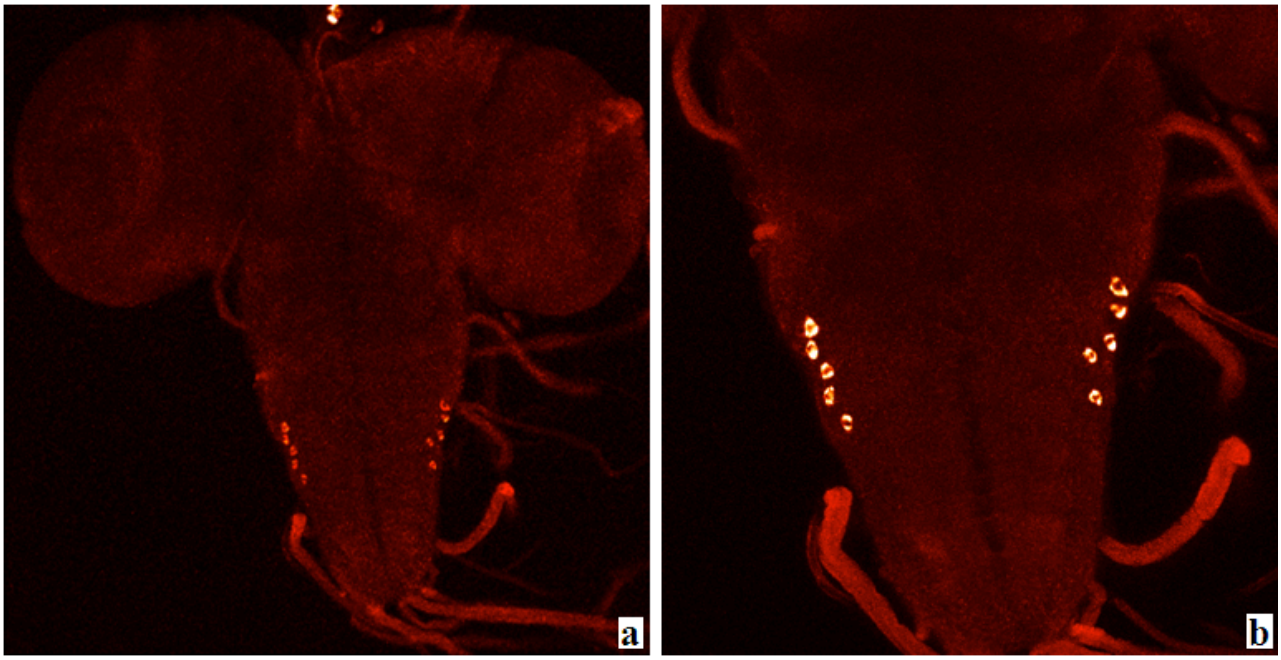


Fig. 48. Pairs of cells in 5 consecutive segments in larval whole brain stained by nb169.

4.3.6 nc7: The mAb nc7 was found to specifically stain the trachea and thus beautifully revealed the majority of the tracheal network within the adult brain as shown below in Fig. 49.

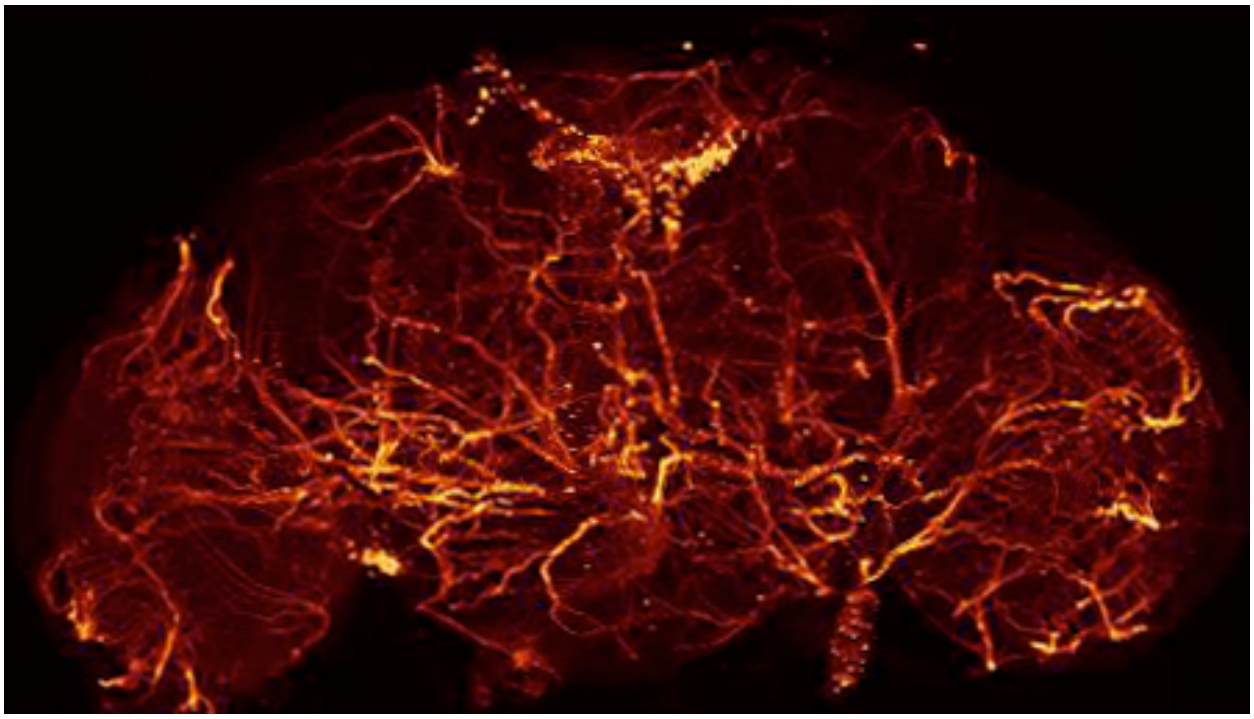


Fig. 49. Staining pattern of nc7 (provided by B. Mühlbauer)

4.4 Discussion

Besides these mAbs, there are some which stain structures (like eyes, occelli, lamina, etc) which are usually removed in whole mount and hence can not be stained in such preparations. They are being pursued by immunohistochemistry on cryosections. Upon looking at the 5 pairs of cells labeled in larval brains ventral ganglion with the mAb nb169 (Fig. 48) and the large pair of cells in the adult with the 3-4 pair of smaller cells in the adult brain (Fig. 46) a question that comes to my mind is that, could these be the same cells, such that these pairs of cells in the larval stage migrates to the central brain during metamorphosis and one of them gives rise to the large pair of neuron while the rest migrate near the auxillary medulla to become the 3-4 smaller pairs of cells. This can be confirmed by staining the adult thoracic ganglion (which develops from the larval ventral ganglion) to see if any cells there are stained, similar to the ones stained in the larval ventral ganglion. Staining of the pupal CNS could also give more insights into this aspect.

The Würzburg Hybridoma Library contains about 200 mAbs, which selectively stain specific structures in the brain of *Drosophila melanogaster*. Unfortunately most of these mAbs are still uncharacterized. In many cases these mAbs stain very few cells or limited structures, as seen in examples above, thus indicating a very low abundance of the endogenously expressed target antigen in the brain. Thus no signal in Western blots of homogenized heads are obtained, making it difficult to follow the target protein through a purification process. Besides many of the mAbs are also IgMs making the enrichment of the target antigen by antibody based immopurification strategies difficult. Identification of such mAb antigens would unconventional strategies like immunostaining of the specific cells by the mAb without fixation in large number of brains, followed by mild proteolytic digestion of the tissue, to dissolve the matrix and release cells into a suspension which could be then sorted by FACS to enrich for the stained cells, which could then be used for IP or other purification methods, to end up with substantial amount of the antigen which would probably give a signal in WB and a protein band which could be stained by Coomassie, thus ensuring sufficient amount of the protein to be identified by peptide mass fingerprinting using mass spectrometry.

Since immunostaining without fixation and dissolution of brains by mild proteolysis are not trivial, there are no established protocols to do this. Due to lack of insight and time, such attempts could not be made within the scope of this thesis. As a first step to more detailed analysis of such antigens, immunostainings were done to characterize the staining patterns, which indicate the localization of the target antigens.

These and other mAbs with specific and interesting staining pattern are currently being studied by a colleague Beatriz Blanco and further characterization of such mAbs and their target antigens would need more time and work.

Chapter 5 SUMMARY

For a large fraction of the proteins expressed in the human brain only the primary structure is known from the genome project. Proteins conserved in evolution can be studied in genetic models such as Drosophila. In this doctoral thesis monoclonal antibodies (mAbs) from the Wuerzburg Hybridoma library are produced and characterized with the aim to identify the target antigen. The mAb ab52 was found to be an IgM which recognized a cytosolic protein of Mr ~110 kDa on Western blots. The antigen was resolved by two-dimensional gel electrophoresis (2DE) as a single distinct spot. Mass spectrometric analysis of this spot revealed EPS-15 (epidermal growth factor receptor pathway substrate clone 15) to be a strong candidate. Another mAb from the library, aa2, was already found to recognize EPS-15, and comparison of the signal of both mAbs on Western blots of 1D and 2D electrophoretic separations revealed similar patterns, hence indicating that both antigens could represent the same protein. Finally absence of the wild-type signal in homozygous *Eps15* mutants in a Western blot with ab52 confirmed the ab52 antigen to be EPS-15. Thus both the mAbs aa2 and ab52 recognize the Drosophila homologue of EPS-15. The mAb aa2, being an IgG, is more suitable for applications like immunoprecipitation (IP). It has already been submitted to the Developmental Studies Hybridoma Bank (DSHB) to be easily available for the entire research community.

The mAb na21 was also found to be an IgM. It recognizes a membrane associated antigen of Mr ~10 kDa on Western blots. Due to the membrane associated nature of the protein, it was not possible to resolve it by 2DE and due to the IgM nature of the mAb it was not possible to enrich the antigen by IP. Preliminary attempts to biochemically purify the endogenously expressed protein from the tissue, gave

promising results but could not be completed due to lack of time. Thus biochemical purification of the protein seems possible in order to facilitate its identification by mass spectrometry. Several other mAbs were studied for their staining pattern on cryosections and whole mounts of Drosophila brains. However, many of these mAbs stained very few structures in the brain, which indicated that only a very limited amount of protein would be available as starting material. Because these antibodies did not produce signals on Western blots, which made it impossible to enrich the antigens by electrophoretic methods, we did not attempt their purification. However, the specific localization of these proteins makes them highly interesting and calls for their further characterization, as they may play a highly specialized role in the development and/or function of the neural circuits they are present in. The purification and identification of such low expression proteins would need novel methods of enrichment of the stained structures.

Chapter 6 ZUSAMMENFASSUNG

Für einen Großteil der Proteine, die im menschlichen Gehirn exprimiert werden, ist lediglich die Primärstruktur aus dem Genomprojekt bekannt. Proteine, die in der Evolution konserviert wurden, können in genetischen Modellsystemen wie Drosophila untersucht werden. In dieser Doktorarbeit werden monoklonale Antikörper (mAk) aus der Würzburger Hybridoma Bibliothek produziert und charakterisiert, mit dem Ziel, die erkannten Proteine zu identifizieren. Der mAk ab52 wurde als IgM typisiert, das auf Western Blots ein zytosolisches Protein von Mr ~110 kDa erkennt. Das Antigen wurde durch zwei-dimensionale Gelelektrophorese (2DE) als einzelner Fleck aufgelöst. Massenspektrometrische Analyse dieses Flecks identifizierte dass EPS-15 (epidermal growth factor receptor pathway substrate clone 15) als viel versprechenden Kandidaten. Da für einen anderen mAk aus der Bibliothek, aa2, bereits bekannt war, dass er EPS-15 erkennt, wurden die Western-Blot-Signale der beiden Antikörper nach 1D und 2D Trennungen von Kopfhomogenat verglichen. Die Ähnlichkeit der beiden Muster deuteten darauf hin, dass beide Antigene dasselbe Protein erkennen. Das Fehlen des Wildtyp-Signals in homozygoten *Eps15* Mutanten in einem Western Blot mit mAk ab52 bestätigten schließlich, dass EPS-15 das Antigen zu mAk ab52 darstellt. Demnach erkennen beide mAk, aa2 und ab52, das Drosophila Homolog zu EPS-15. Da mAk aa2 ein IgG ist, dürfte er für Anwendungen wie Immunpräzipitation (IP) besser geeignet sein. Er wurde daher bereits bei der Developmental Studies Hybridoma Bank (DSHB) eingereicht, um ihn der ganzen Forschergemeinde leicht zugänglich zu machen.

Der mAk na21 wurde ebenfalls als IgM typisiert. Er erkennt ein Membran assoziiertes Antigen von Mr ~10 kDa auf Western Blots. Aufgrund der Membranassoziierung des Proteins war es nicht möglich, es in 2DE aufzulösen und

da es sich um ein IgM handelt, war eine Anreicherung des Antigens mittels IP nicht erfolgreich. Vorversuche zur biochemischen Reinigung des endogenen Proteins aus Gewebe waren Erfolg versprechend, konnten aber aus Zeitmangel nicht abgeschlossen werden. Daher erscheint eine biochemische Reinigung des Proteins für eine Identifikation durch Massenspektrometrie möglich.

Eine Reihe weiterer mAk wurden hinsichtlich ihrer Färbemuster auf Gefrierschnitten und in Ganzpräparaten von Drosophila Gehirnen untersucht. Allerdings färbten viele dieser mAk sehr wenige Strukturen im Gehirn, so dass nur eine sehr begrenzte Menge an Protein als Startmaterial verfügbar wäre. Da diese Antikörper keine Signale auf Western Blots produzierten und daher eine Anreicherung des Antigens durch elektrophoretische Methoden ausschlossen, wurde keine Reinigung versucht. Andererseits macht die spezifische Lokalisation dieser Proteine sie hoch interessant für eine weitere Charakterisierung, da sie eine besonders spezialisierte Rolle in der Entwicklung oder für die Funktion von neuralen Schaltkreisen, in denen sie vorkommen, spielen könnten. Die Reinigung und Identifikation solcher Proteine mit niedrigem Expressionsniveau würde neue Methoden der Anreicherung der gefärbten Strukturen erfordern.

Chapter 7 REFERENCES

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Chapter 8 APPENDIX

8.1 Publications relevant to the thesis:

8.1.1 Published papers:

Hofbauer A, Ebel T, Waltenspiel B, Oswald P, Chen YC, Halder P, Biskup S, Lewandrowski U, Winkler C, Sickmann A, Buchner S, Buchner E - The Wuerzburg Hybridoma Library against Drosophila brain. *Journal of Neurogenetics* (2009) 23(1-2): 78-91 (Review article in special issue on Martin Heisenberg).

Halder P, Chen Y-C, Brauckhoff J, Hofbauer A, Dabauvalle M-C, Lewandrowski U, Winkler C, Sickmann A, Buchner E - Identification of Eps15 as the Antigen Recognized by the Monoclonal Antibodies aa2 and ab52 of the Würzburg Hybridoma Library Against *Drosophila* Brain. *PLoS ONE (2011).*

8.1.2 Published abstracts:

Halder P, Brauckhoff J, Hofbauer A, Buchner E (2009) 'Identification and characterization of proteins of brain proteins in Drosophila melanogaster', presented at the 12th European Drosophila Neurobiology Conference (Neurofly), University of Wuerzburg 6-10 Sep, 2008. Published as collection of abstracts in *Journal of Neurogenetics* Vol. 23 Suppl 1:1-84.

Halder P, Brauckhoff J, Hofbauer A, Buchner E (2009) 'Identification of the antigens for the MABs na21 and ab52 from the Würzburg Hybridoma library of Drosophila melanogaster' presented at the 8th Göttingen Meeting of the German Neuroscience Society, Göttingen. Published as proceedings of the meeting.

Halder P, Hofbauer A, Buchner E (2010) ‘Monoclonal antibodies of the Wuerzburg hybridoma library: pearls from the sea’ presented at the 13th European Drosophila Neurobiology Conference (Neurofly), University of Manchester. Published as collection of abstracts in *Journal of Neurogenetics* Vol. 24 Suppl 1:1-95.

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The Wuerzburg Hybridoma Library against *Drosophila* Brain

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Abstract: This review describes the present state of a project to identify and characterize novel nervous system proteins by using monoclonal antibodies (mAbs) against the *Drosophila* brain. Some 1,000 hybridoma clones were generated by injection of homogenized *Drosophila* brains or heads into mice and fusion of their spleen cells with myeloma cells. Testing the mAbs secreted by these clones identified a library of about 200 mAbs, which selectively stain specific structures of the *Drosophila* brain. Using the approach ''from antibody to gene'', several genes coding for novel proteins of the presynaptic terminal were cloned and characterized. These include the ''cysteine string protein'' gene (*Csp*, mAb ab49), the ''synapse-associated protein of 47 kDa'' gene (*Sap47*, mAbs nc46 and nb200), and the ''Bruchpilot'' gene (*brp*, mAb nc82). By a ''candidate'' approach, mAb nb33 was shown to recognize the pigment dispersing factor precursor protein. mAbs 3C11 and pok13 were raised against bacterially expressed *Drosophila* synapsin and calbindin-32, respectively, after the corresponding cDNAs had been isolated from an expression library by using antisera against mammalian proteins. Recently, it was shown that mAb aa2 binds the *Drosophila* homolog of ''epidermal growth factor receptor pathway substrate clone 15'' (Eps15). Identification of the targets of mAbs na21, ab52, and nb181 is presently attempted. Here, we review the available information on the function of these proteins and present staining patterns in the *Drosophila* brain for classes of mAbs that either bind differentially in the eye, in neuropil, in the cell-body layer, or in small subsets of neurons. The prospects of identifying the corresponding antigens by various approaches, including protein purification and mass spectrometry, are discussed.

Keywords: brain proteins, CSP, synapsin, SAP47, Bruchpilot, calbindin, PDF, Eps15

INTRODUCTION

The brain is distinguished from other organs by its enormous complexity, both at the structural and the molecular levels. In order to understand the development of neuronal networks and how the various cell types operate and interact to initiate, maintain, modulate, and terminate behavior, it is necessary to identify and localize the proteins that are specifically expressed in the brain as a prerequisite for the study of their molecular, cellular, and systemic function. Of particular interest are those proteins that are cell-specifically expressed and are responsible for the differentiation of neurons and glial cells into thousands of different cell types with their individual molecular, structural, and functional characteristics. One approach to the identification and localization of brain proteins makes use of the exquisite ability of the mammalian adaptive immune system to recognize foreign proteins, which can be exploited by the generation of monoclonal antibodies (mAbs) against brain homogenates. This approach has been used successfully with various organisms, and in *Drosophila*, it has been pioneered many years ago by the group of the late Seymour Benzer (Fujita et al., 1982; Fujita, 1988). The present review summarizes the information on the genes and proteins identified by Abs of the Wuerzburg hybridoma library (Hofbauer, 1991) (Table 1). In this project, we intended to find Abs that bind to specific cells or compartments of the *Drosophila* brain and use them to identify the genes coding for the corresponding antigens.

MATERIALS AND METHODS

Fly Strains

Wild-type Berlin and Canton S were used as wild-type control and for the staining on cryostat sections. The

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Pdf^{01} mutant (Renn et al., 1999), as well as lines with a modified expression of the *Pdf* gene, were obtaind from Paul Taghert: *y w;; P{w*$^{+}$ *mF6-2} Pdf*^{01} expresses AA 1-82 of the pigment dispersing factor (PDF) precursor with the PDF peptide replaced by *Locusta* myosupressin. In *y w; P{w*$^{+}$ *mE10-2A}; Pdf*^{01} PDF is expressed, whereas the rest of the precursor is replaced by preproFMRFamide sequences.

Immunizations

Inbred BALBc mice (Charles River WIGA, Sulzfeld, Germany) were used for immunizations throughout. The injections were done mostly intraperitoneally, sometimes in combination with subcutaneous injections (aa, ab, and ca). Adjuvans was used only when purified proteins were injected. Homogenates were prepared by using a glass homogenator (Dounce, 2 mL). We tried different immunization strategies by using either brains or heads, combinations of mutants and wild type, and immunizations of newborns with mutants missing parts of the visual system in combination with later immunizations, using wild type, to provoke Abs against the differences between mutant and wild-type heads. There might have been some effects, but we could not find any mutant-specific differences; therefore, no further details are presented here. mAbs Pok13 and 3C11 were generated by injecting calbindin32 and synapsin, respectively, that had been cleaved from bacterially expressed glutathione S-transferase (GST) fusion protein purified by glutathion-agarose from bacterial lysate. The general pattern of immunizations is presented in Table 2.

Cell Culture

Hybridoma medium consisted of 0.7 RPMI 1640 (7 parts), 0.2 medium 199 (2 parts), and 0.1 fetal calf serum (FCS) (1 part) and was hybridoma tested (Invitrogen, Paisley, UK). Selection medium (HAT medium) consisted of hybridoma medium with a hypoxanthin-aminopterin-thymidin cocktail added. Polyethylene glycol (PEG) solution consisted of PEG 4000 (Merck, Darmstadt, Germany), 1 g/mL in RPMI, plus 10% (vol) dimethyl sulfoxide (DMSO).

The fusion procedure was based on the methods described in Zola and Brooks (1982). Immunized mice were sacrificed, then the spleen was removed and mazerated between the frosted ends of sterile glass slides. To the cell suspension, about 2 x 10^7 myeloma cells were added. The cells were washed in RPMI medium and centrifuged. The pellet was slowly and gently resuspended by adding 1 mL of PEG solution over 1 minute. After another 90 seconds, the suspension was gradually diluted by adding 10 mL of RPMI in increasing amounts over the next 2 minutes. After pelleting the cells, the supernatant was replaced by cell-culture medium and after 3 minutes by pure hybridoma tested FCS. The resuspended cells were individually seeded in cell-culture plates (96 wells) containing equilibrated HAT medium. Cells were grown to cultures, and aliquots were frozen as well as samples of supernatants. Cell lines producing Abs as confirmed by enzyme-linked immunosorbent assay (ELISA) were subcloned. Subclones were accepted as the origin for cell lines if microscopic examination showed only one clone per well.

ELISA

To test cell lines for the production of Abs, we used the TSP system (Nunc, Langenselbold, Germany). With this technique, Abs were adsorbed to plastic knobs inserted into the culture wells. The Abs were then detected by using the ABC technique (Vector, Burlingame, California, USA) with horseradish peroxidase (HRP) as enzyme and catechol/phenylendiamine as chromogen (Hanker et al., 1977).

Immunohistochemistry

Phosphate-buffered paraformaldehyde (4%) was used for fixation. Flies were cold anesthetized, in ice-cold fixative the proboscis and the air sacks of the head were removed, and the flies were fixed for 2 hours. The fixative was replaced by 25% sucrose overnight. Heads were embedded in methyl cellulose and frozen rapidly in liquid nitrogen. Cryostat sections (10 or 20 μm) were used for immunohistochemical staining. The hybridoma supernatant was used at a dilution of 1+1 in phosphate-buffered saline (PBS) for the first try and adjusted to optimal dilution in later experiments. The primary Ab was detected by using the ABC elite kit (Vector Laboratories, Burlingame, CA, USA) with HRP as the detecting enzyme and diaminobenzidine (Sigma-Aldrich, Taufkirchen, Germany) as chromogen. All reaction solutions contained 0.1% TritonX100.

Mass Spectrometry (MS)

Subsequent to gel electrophoretic separations, gel spots/bands of interest were excised and proteins reduced, carbamidomethylated, and tryptically digested, as described previously (Schindler et al., 2008). The resulting peptide mixtures were subjected to nanoscale liquid chromatographic separation coupled online to a LTQ-XL mass spectrometer (Thermo-Fischer Scientific, Dreieich,

Germany). An UltiMate 3000 high-performance liquid chromatography (HPLC) system (Dionex, Idstein, Germany) was used in combination with a preconcentration setup comprising a 100-μm inner diameter × 2 cm length precolumn and a 75-μm inner diameter × 15 cm length separation column, both with Synergy Hydro-RP material (Phenomenex, Aschaffenburg, Germany). Peptides were separated by using a binary gradient system (solvent A: 0.1% formic acid in water; solvent B: 84% acetonitrile, 0.1% formic acid). Online mass spectrometric detection by LTQ-XL was achieved by using a duty cycle consisting of a single survey scan, followed by five tandem-MS scans of selected precursors.

Mass spectrometric data interpretation was performed by using the Mascot Algorithm (version 2.1; Matrix Science, London, UK) and Mascot Daemon (version 2.1.6). Searches were conducted against Flybase (http://flybase.bio.indiana.edu/) with the following settings. Carbamidomethylation (C) was set as fixed and oxidation (M) as variable modification. Trypsin, with one miscleavage site, was chosen as protease, while precursor and fragment-ion tolerance was set to 1.5 Da. Identified peptides were validated manually after identification by the algorithm.

RESULTS

The number of genes expressed in the developing, or the adult, nervous system is of the order of several thousands, and probably more than half of them are still unknown. We, therefore, injected heterogeneous cocktails of possible antigens obtained by homogenizing native or chemically fixed isolated brains or whole heads into mice and fused their spleen cells with myeloma cells to obtain about 1,000 hybridoma cell lines. The supernatants of these were screened for immunoglobulin content by ELISA and for staining of brain structures by immunohistochemistry on frozen head sections. This approach resulted in a collection of about 200 mAbs directed against diverse antigens of the *Drosophila* brain. This library was supplemented by a few hybridoma clones, which were obtained after immunization of mice with affinity-purified tagged proteins of bacterially expressed *Drosophila* cDNAs, which had been isolated from a cDNA expression library due to cross-reactions with antisera against mammalian proteins.

Histological Staining Patterns

After immunizations with a complex mixture of antigens, as contained in the crude homogenates of brains or heads, one has to expect Abs directed against a wide variety of different types of antigens. Because we characterized the Abs in a first screen according to their staining pattern on cryostat sections after formaldehyde fixation, the general patterns we describe here refer to anatomical and histological aspects of the nervous system. They are not exclusive, as some Abs show overlapping patterns.

A minority of Abs label different body tissues or cellular compartments, such as the trachea, cell-body layers due to specific nuclear staining, muscle, cuticula, or the perineureum (Figure 1).

In the retina, several Abs recognize specific cell types, such as receptor cells or pigment cell types, staining whole cells or cell compartments. Some are

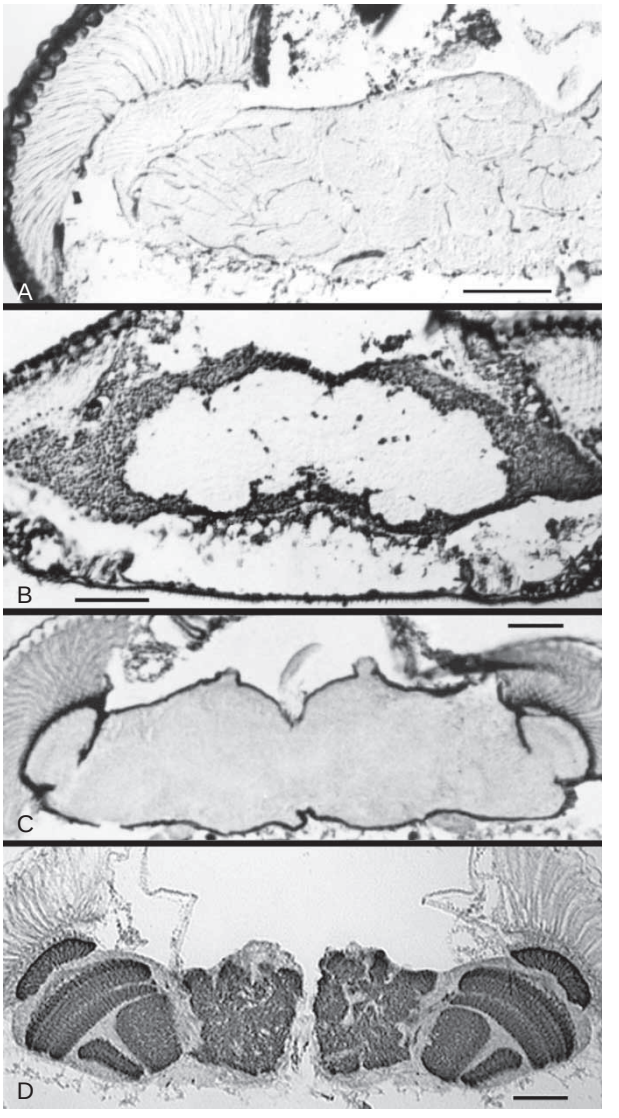

Figure 1. Tissue-specific staining patterns in the *Drosophila* brain. (**A**) Antibody nc7 is specific for chitin and, therefore, demonstrates the tracheal system in the brain. (**B**) Selective staining of all cell nuclei (ga21). (**C**) nc120 binds exclusively to the perineureal sheeth. (**D**) ab49 binds to cysteine string protein and stains all neuropil. Horizontal cryostat sections, scale bar: 50 μm.

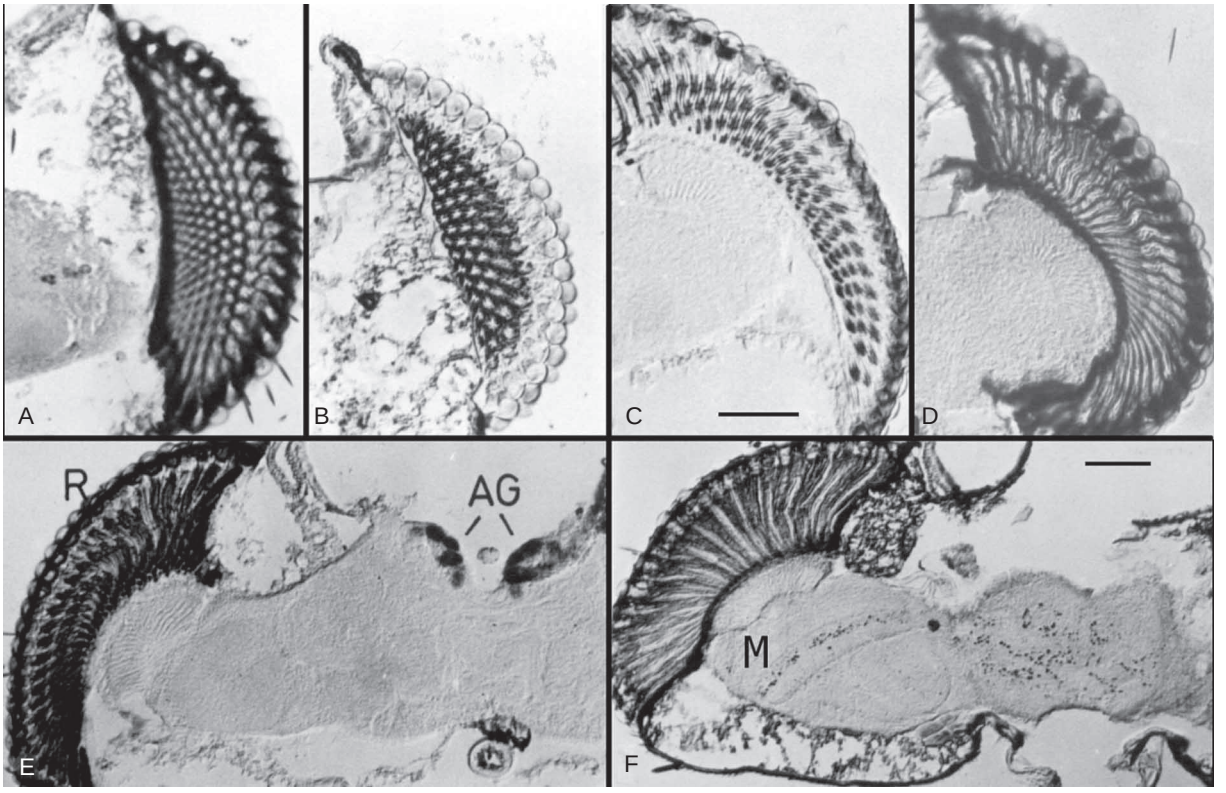

Figure 2. Different staining patterns in the *Drosophila* retina indicating selective staining of cell types or cell compartments. (**A**) antibody nc133, (**B**) ab160, (**C**) ab79, and (**D**) nc42. (**E**) Antibody nc10 is selective for retinal and antennal cells innervating a subpopulation of antennal glomeruli. (**F**) ab47 stains tangential cells in the medulla and further cells in the central brain, in addition to strong retina staining. AG, antennal glomeruli; M, medulla; R, retina. Horizontal cryostat sections, scale bars: 50 μm.

restricted to the retina, whereas some also stain other areas of the brain (Figure 2). One of these Abs, nb181, is specific to all photoreceptor cells staining their full cell morphology. It was used to describe the eyelet, a group of four extraretinal photoreceptor cells at the posterior rim of the compound eye (Hofbauer and Buchner, 1989). These receptor cells originate from the Bolwig organ, the larval photoreceptor organ (Helfrich-Förster et al., 2002). Recently, it was shown that during metamorphosis, the surviving photoreceptor cells of the eyelet switch from rhodopsin-5 to -6 gene expression (Sprecher and Desplan, 2008).

One class of Abs appeared to stain many cells, but differentiated between various components of the cells. Most conspicuous among these are Abs selective for synaptic neuropil regions (Figure 3A–3H). Some of them do not bind to perikarya, but stain neuropil regions rather uniformly, presumably representing the density of synapses because the antigen is contained in all synapses, while others clearly differentiate between different neuropil areas. This was most obvious in the neuropil areas of the optic lobes, there revealing characteristic patterns of layers mainly in the medulla (Figure 3). One of these Abs, nb236, shows staining exclusively in the lamina neuropil (Figure 3F). It might be specific for a type of lamina glia.

The largest class of Abs, also the class most difficult to characterize, comprises Abs that clearly bind to a few individual elements in the nervous system. Typically, these Abs stain a small number of cell bodies and only parts of their arborizations or even only substructures of their terminals (Figure 4A–4E). Again, this can be demonstrated most easily in the optic lobes, where many Abs reveal individual cell bodies in the area between the anterior medulla neuropil and the central brain, as well as a pattern of dots in one or few distinct layers of the medulla neuropil, possibly representing terminal boutons of tangential cells (Figure 4D 4E). We found no morphologically distinct staining of repetitive cells in the optic lobes, such as constituents of medulla columns or lamina cartridges.

Abs staining small cell populations in the optic lobes usually also stained cells in the central brain. These cell bodies were scattered in the cell-body layers, most frequently in the posterior cortex and in the dorsal area in or near the pars intercerebralis (Figure 4A and 4C). Terminal staining was most conspicuous in the central

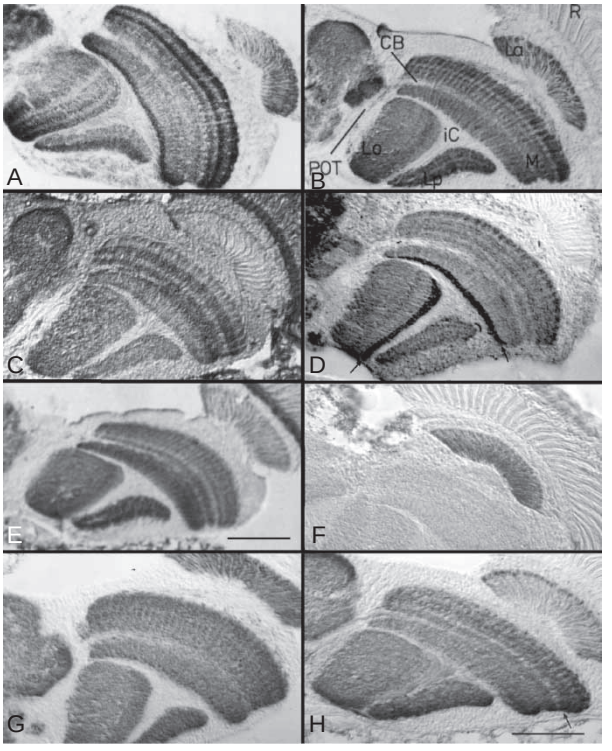

Figure 3. Staining patterns of neuropil structures, as demonstrated in the optic lobes. Selective specificities to cell types or subcellular structures result in characteristic layering patterns. (**A**) ca51, (**B**) ab49, (**C**) nb43, (**D**) na21, (**E**) nc82, (**F**) nb236, (**G**) aa2, and (**H**) nc46. CB, Cucatti's bundle of the medulla; iC, inner chiasm; La, lamina; Lo, lobula; Lp, lobula plate; M, medulla; R, retina. Horizontal cryostat sections, scale bars in **E** for **A**–**E**, in **H** for **F**–**H**: 50 μm.

complex, especially the fan-shaped body, but was found also in other areas (Figure 4C–4E).

Only in a small number of cases did the Ab bind to all parts of a cell, dendrites, axons, neurite, and cell body, such that the complete morphology of the targeted cells was revealed. An obvious example for such a fortunate case is nb33, an Ab that selectively stains cells expressing the *pigment dispersing factor* gene (*Pdf*) (Figure 4B).

Antigen Analysis

We have used several of our Abs to identify the corresponding antigen and its encoding gene. Three approaches have been used: 1) screening a cDNA expression library with the antibody and verification of positive clones by generating new mouse polyclonal antisera against the bacterially expressed cDNA-encoded protein; whenever the Western signal and the staining pattern on brain sections of the mAb matched with the signals obtained by the new polyclonal antisera, one could be reasonably certain that the correct gene was identified; 2) a second approach uses the mAb to affinity purify the antigen from head homogenates by immunoprecipitation. Alternatively, in favorable cases, the antigen can be purified by two-dimensional (2-D) gel electrophoresis. The purified protein can then be subjected to MS analysis and identified by comparison of the observed peptide fingerprint with the *Drosophila* proteome inferred from the genome sequence; and 3) the third—candidate—approach can be applied only if the cells that are stained by the mAb are known such that one can speculate which proteins might specifically be expressed in these cells. Final proof for the correct association of mAb and gene is obtained in all three situations when the staining is eliminated in a null mutant for the identified gene. Table 1 lists the mAbs of the Wuerzburg hybridoma library, for which the antigen has been identified or for which some information has been published. The present knowledge about the identified proteins is briefly reviewed in the following paragraphs.

mAb ab49: Cysteine String Protein (CSP)

The mAb, ab49, was selected for the first large screens of a cDNA expression library that had been generated by K.E.Z. in our group. mAb ab49 stains all neuropil (Figure 1D) and synaptic boutons on adult and larval muscles. A number of clones from the same gene were isolated with mAb ab49. To verify that these clones indeed code for the ab49 antigen, bacterially expressed GST fusion protein was purified and injected into mice whose antisera showed a staining pattern essentially identical to mAb ab49. By dot blots of sequential decapeptides, it was later shown that mAb ab49 recognizes the amino-acid epitope, FTGA (Arnold et al., 2004). Cysteine string proteins are conserved cysteine-rich molecular cochaperones for Hsp70 family chaperones and are attached to vesicles of synapses and secretory organs by a string of palmitoylated cysteine residues (Buchner and Gundersen, 1997; Chamberlain and Burgoyne, 2000; Zinsmaier and Bronk, 2001; Evans et al., 2003). Deletion of the *Csp* gene in *Drosophila* impairs synaptic transmission, especially at high temperatures (Zinsmaier et al., 1994; Umbach et al., 1994; Eberle et al., 1998; Bronk et al., 2005). CSP is also required for nerve terminal growth and prevention of neurodegeneration in *Drosophila* and mice (Fernández-Chacón et al., 2004; Chandra et al., 2005; Schmitz et al., 2006; Dawson-Scully et al., 2007). Transgenic pan-neural expression of wild-type cDNAs in the *Csp* null mutants rescues the temperature-sensitive paralytic phenotype. However, if cDNA constructs lacking the codons for the cysteine string are used, targeting of CSP to vesicular membranes fails and no phenotypic rescue is observed (Arnold et al., 2004). A similar defect of CSP targeting is observed when palmitoylation of the cysteine string is prevented by mutation of the palmitoyl transferase

huntingtin-interacting protein 14 (HIP14) (Ohyama et al., 2007; Stowers and Isacoff, 2007). In vertebrates, CSPs have been shown to form a trimeric complex with HSC70 (70-kDa heat-shock cognate protein) and SGT (small glutamine-rich tetratricopeptide repeat domain protein) that can function as a guanine nucleotide exchange factor (GEF) for Gαs (Swayne et al., 2006). Various interaction partners of CSPs have been described, such as G-proteins, cystic fibrosis transmembrane conductance regulators, α-synuclein, huntingtin, HIP14, and myosin 1e, but the molecular function of CSP that prevents the temperature-sensitive failure of synaptic exocytosis observed in *Drosophila Csp* null mutants remains to be elucidated.

mAbs nc46 and nb200: *Synapse-associated* Protein of 47 kDa (SAP47)

These two antibodies stained all neuropil similar to Figure 1D. By screening the Zinsmaier cDNA expression library with MAB nc46, five independent cDNAs were isolated, but protein expressed from only one clone generated antisera with a similar staining pattern as nc46. This clone identified the *Sap47* gene of *Drosophila* (Reichmuth et al., 1995) and was shown to recognize an epitope in the N-terminal decapeptide, FSGLTNQFTS. A second Ab, mAb nb200, showed very similar staining, both on sections and in Western blots, and later was shown to recognize the same protein (epitope QQAKHF in a central domain). Very little is known about the protein encoded by the *Sap47* gene. Homologs can be found in sea anemones, sea urchins, insects, frogs, fish, and mammals, but no information is available on their function. The encoded polypeptides belong to a novel superfamily of proteins containing the ''BSD'' domain, which is found in conserved transcription factors (e.g., BTF2 of mammals), synapse-associated proteins (e.g., SAP47 of *Drosophila*), and DOS2-like proteins (e.g., DOS2 of yeast) (Doerks et al., 2002). *Drosophila Sap47* null mutants are viable and fertile (Funk et al., 2004) but show complex behavioral phenotypes, which are presently investigated. SAP47 largely colocalizes with synapsins to synaptic terminals but is also found in axons and in some perikarya (Reichmuth et al., 1995). Interestingly, preliminary experiments indicate an interaction between *Drosophila* SAP47 and synapsin. Intrigued by the fact that mAb nc46 also stains synaptic regions in fish and mouse brain (Reichmuth et al., 1995), we used immunoprecipitation and MS (Protana, Denmark) to identify the proteins recognized in vertebrate brains. It turned out that mAb nc46, which in *Drosophila* specifically detects the synapse-associated protein, SAP47, does not recognize the mouse homolog of SAP47, but cross-reacts with mouse synapsin I and immunoglobulin heavy-chain binding protein (BiP). In mouse-brain sections, nc46 shows staining similar to Abs against the synaptic protein, synaptophysin (Figure 5). The epitope recognized by mAb nc46 in *Drosophila* SAP47 (FSGLTNQFT) is not found in the mammalian synapsin I or the BiP sequence. Thus, the cause of this cross-reaction remains unknown.

Table 1. List of antibodies which have been published or for which the antigen is known

Antibody	Stains	Figure	Antigen (approach)	Reference
aa2	All neuropil	3G	EPS15 (2)	
ab43	Few cells			Buchner et al. (1988)
ab47	Few cells	4D, E		Buchner et al. (1988)
ab49	All neuropil	1D, 3B	CSP (1)	Buchner et al. (1988), Zinsmaier et al. (1990)
ca8	Few cells			Buchner et al. (1988)
ca51	Antennae, layers in visual system	3A		Buchner et al. (1988), Störtkuhl et al. (1994)
fb45	Mushroom bodies, visual system			Bicker et al (1993)
nc82	All neuropil, presynaptic active zone	3E	Bruchpilot (2)	Wagh et al. (2006), Kittel et al. (2006)
nb33	PDF-neurons	4B	PDF precursor (3)	
nb181	Photoreceptors			Hofbauer and Buchner, 1989
nb230	Antennae, layers in visual system			Störtkuhl et al. (1994)
na21	Antennae, layers in visual system	3D		Störtkuhl et al. (1994)
nc10	Retina, antennae, olfactory glomeruli	2E		Störtkuhl et al. (1994)
nc46 nb200	All neuropil	5, 3H	(mouse synapsin), SAP47 (1)	Reichmuth et al. (1995), Funk et al. (2004)
pok13	Numerous cells, selected neuropil	6	calbindin-32 (1)	Reifegerste et al. (1993)
3C11	All neuropil		synapsin (1)	Klagges et al. (1996), Godenschwege et al. (2004)

Approaches: 1, screen of cDNA expression libraries; 2, protein isolation and mass spectrometry; 3, candidate approach.

mAb 3C11 (anti-SYNORF1): *Synapsin*

The *Synapsin* gene of *Drosophila* was cloned by chance due to the cross-reaction of an unrelated peptide antiserum at a time when unsuccessful homology screens with vertebrate synapsin cDNA sequences had led to the speculation that invertebrates might not have synapsin homologs. The isolated cDNA clone was tagged and expressed in *Escherichia Coli*, and the purified protein was injected in mice for the generation of mAbs. The clone, 3C11, produced the strongest staining of most or all synaptic terminals (similar to Figure 3H) and was selected for further analysis. The recognized epitope, LFGGMEVCGL, lies in the conserved C domain, such that mAb 3C11 binds to synapsins of many invertebrate species. The synapsin gene (*Syn*) has several unusual features. On its opposite strand lies the *Timp* gene overlapping with intron 9 and exons 8 and 9 of *Syn*. This nested organization of *Syn* and *Timp* genes has been conserved in evolution (Pohar et al., 1999; Yu et al., 2003). Translation of the *Syn* mRNA apparently starts at a noncanonical CTG codon, as no ATG is found between an upstream in-frame stop codon and the codons for the conserved A-domain (Klagges et al., 1996). This conjecture is supported by N-terminal peptide sequencing by Edman degradation (Godenschwege et al., 2004). Another striking feature is the TAG stop codon that separates two large open-reading frames and is found in all known cDNAs of *Syn*. Translation termination at this stop codon leads to isoforms of 70–80 kDa, while isoform(s) of about 140 kDa are generated presumably by TAG read-through (Godenschwege et al., 2004). Alternative splicing, as a mechanism to remove the stop codon, seems less likely, because transgenic expression of the largest cDNA in *Syn* null mutants produces both the large and the small isoforms, and no high-score splice sites are detected in the cDNA sequence that could explain the removal of the stop codon as part of an alternatively spliced intron. Yet another intriguing feature of the *Drosophila* synapsin gene is the fact that its pre-mRNA is very efficiently edited by the enzyme, adenosine deaminase acting on RNA (ADAR) (Diegelmann et al., 2006). The encoding of the consensus sequence, RRXS, for phosphorylation by protein kinase A (PKA) and calcium/calmodulin dependent kinases I and IV (CamK-I and -IV) in the conserved ''A'' domain of vertebrate synapsins is conserved in the genomic DNA of *Drosophila* but modified to RGXS in most or all mature transcripts of late larvae, pupae, and adults. The N-terminal decapeptide, containing the edited sequence, is not significantly phosphorylated by bovine PKA *in vitro*, while the genomically encoded decapeptide is an excellent substrate (Diegelmann et al., 2006). The *in-vivo* consequences of *Syn* mRNA editing are presently investigated.

Vertebrate synapsins are believed to regulate the trafficking of synaptic vesicles between reserve and cycling pools, but also may have other roles, as they show structural similarity to ATPases and interact with sarcoma (Src) kinase to enhance their activity in synaptic vesicle fractions (Hilfiker et al., 1999, 2005; Baldelli

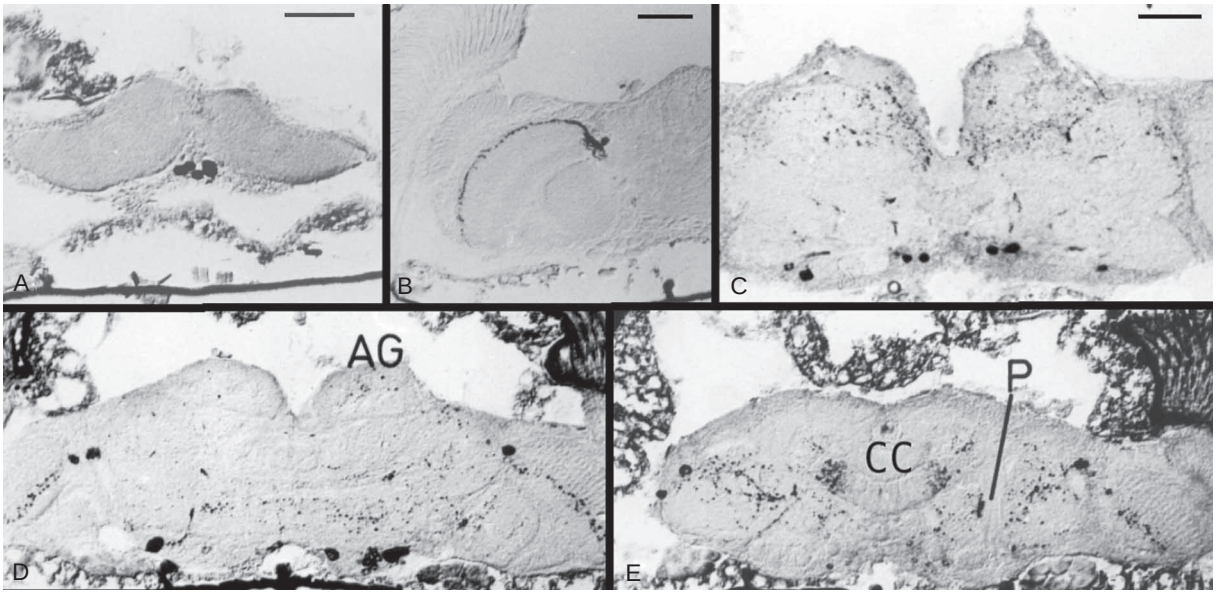

Figure 4. Selective staining of small neuronal subpopulations in the *Drosophila* brain. (**A**) A cluster of neurons stained in the pars intercerebralis (ab135). (**B**) nb33 is specific for *Pdf*-expressing neurons. Here, one cell body and arborizations in the most distal medulla neuropil are shown. (**C**–**E**) Scattered cell bodies and traces of arborizations in the central brain. (**C**) nc53, (**D**), and (**E**) ab47 (two sections out of a series). AG, antennal glomeruli; CC, central complex; P, peduncule of the mushroom bodies. Horizontal cryostat sections, scale bars (in **C** for **C**–**E**): 50 μm.

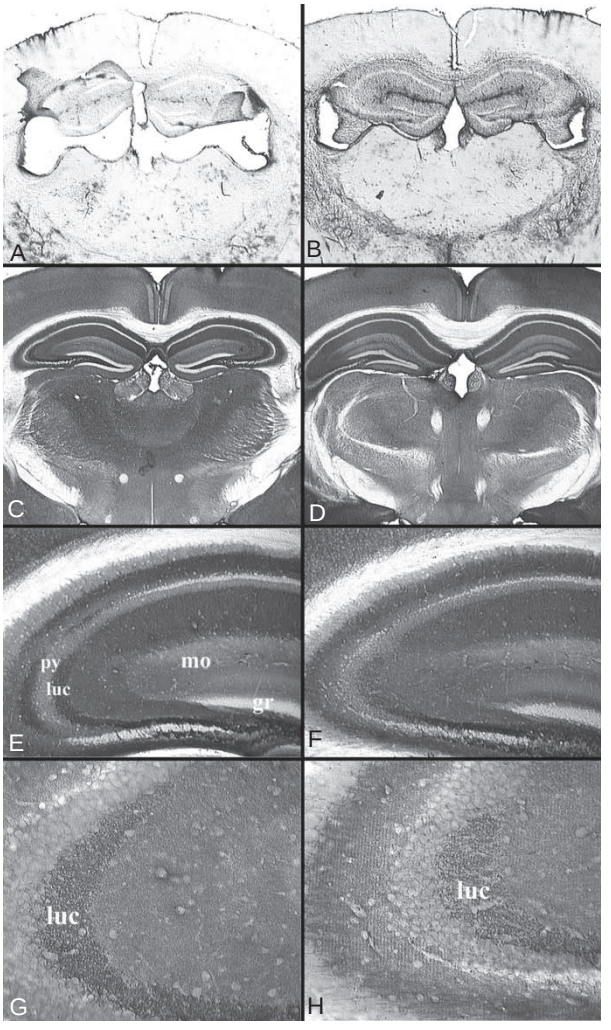

Figure 5. Immunohistochemical localization of the monoclonal antibody (mAb) nc46 antigen in mouse brain. (**A**) negative control without first antibody. (**B**) Positive control with a polyclonal antibody against GFAP, a marker of astrocytes, dilution 1:1,000. (**C**, **E**, and **G**) Section stained with mAb nc46, dilution 1:100, at 10, 20, and 40 times magnification. (**D**) Section treated with a polyclonal antiserum against the synaptic protein synaptophysin, dilution 1:2,000. CA, cornu ammonis; mo, stratum moleculare; gr, stratum granulare; py, stratum pyramidale; luc, stratum lucidum.

et al., 2005; Onofri et al., 2007). They are substrates for various kinases, including PKA and CamK-I, which phosphorylate the conserved N-terminal ''A'' domain. Extensive attempts have been made to elucidate the cellular and systemic function of *Drosophila* synapsins. Null mutants with large or small deletions in the *Syn* gene are viable and fertile but show altered responses in various behavioral paradigms, including several learning assays (Godenschwege et al., 2004; Michels et al., 2005). Electron microscopy and basic electrophysiology of larval neuromuscular junctions failed to reveal clear differences between wild-type and *Syn* null mutants, but recent FM1-43 staining demonstrated altered distributions of recycling and reserve pools of synaptic vesicles in type-Ib boutons of the Syn^{97} null mutant (Akbergenova & Bykhovskaia, 2007). The role that the editing of synapsin pre-mRNA plays for learning and memory and interaction with SAP47 are major unsolved problems.

mAb nc82: Bruchpilot Protein (BRP)

During a sabbatical of SB and EB in the laboratory of Veronica Rodrigues in 2000–2001, it was noted that mAb nc82, which in adults, stains all neuropil (Figure 3E), binds in larval nerve-muscle preparations selectively to small spots within each synaptic bouton. The obvious speculation that these spots might represent active zones was first verified by Wucherpfennig et al. (2003) by demonstrating a close association of nc82 staining with the distribution of postsynaptic glutamate receptors. By 2D-electrophoresis and Western blotting, two protein spots could be isolated and analyzed by MALDI-TOF, which identified a protein encoded by the annotated gene, CG30337. Reverse-transcriptase polymerase chain reaction (RT-PCR) experiments and sequencing in collaboration with the Sigrist group connected the three annotated ''genes,'' CG12933, CG30336, and CG30337, to a single large gene, which received the name ''bruchpilot'' (*brp*) (German for crash pilot), based on the unstable flight of animals panneurally expressing a *brp*-RNAi construct. The largest known BRP isoform of 190 kDa contains domains with high homology to the mammalian active zone protein, ELKS/CAST/ERC, and a large C-terminal coiled-coil domain. Other isoforms are yet to be characterized. The *brp* knock-down animals also showed almost no spontaneous walking activity, and in larval neuromuscular preparations, the quantal content of excitatory junction currents (EJCs) was reduced to about 50%. At the ultrastructural level, presynaptic electron dense projections (T-bars) were absent from adult photoreceptor terminals and drastically reduced in larval neuromuscular boutons (Wagh et al., 2006). In sub-diffraction resolution STED (stimulated emission depletion), fluorescence microscopy BRP is arranged in donut-shaped rings at the center of active zones. Null mutants are lethal, but synaptic boutons of larval escapers can be investigated. They entirely lack T-bars, display presynaptic membrane ''rufflings,'' show increased miniature EJC amplitude, a 70% reduction in EJC quantal content, and transient short-term facilitation. Also, the expression of GFP-labeled presynaptic voltage-sensitive calcium channels was severely reduced at larval synaptic boutons lacking BRP, suggesting that in wild-type synapses, BRP is involved in concentrating calcium channels near docking sites for synaptic vesicles (Kittel et al., 2006a). Obvious questions on the function of BRP,

such as its interactions with other proteins and its role during active zone assembly, remain to be solved (Kittel et al., 2006b).

mAb aa2: Epidermal Growth Factor Receptor Pathway Substrate Clone 15 (EPS-15)

This Ab (Figure 3G) recognized a protein present in all synapses, both in adults and in larvae. Confocal double-labeling experiments, during a sabbatical of EB and SB in the laboratory of K. Zinsmaier on larval neuromuscular boutons with mAb aa2 and rabbit antidynamin serum (kindly provided by Mani Ramaswami), indicated colocalization of the two antigens. Initial attempts to purify the aa2 antigen by immunoprecipitation failed, but after raising the hybridoma cells in serum-free medium (to eliminate bovine immunoglobulins, which compete with aa2 for binding to the protein-G agarose), two gel pieces, containing a 100-kDa Coomassie-stained band, were subjected to analysis by a linear ion-trap mass spectrometer (LTQ LC/MS). Among 25 proteins of the *Drosophila* proteome with peptide patterns similar to proteins in the gel pieces (scores above 150), four were known components of the presynaptic endocytosis complex of *Drosophila* (dynamin, EPS-15, α-adaptin, dynamin-associated protein 160; DAP160). Since mAb aa2 did not recognize GFP-labeled dynamin (flies kindly provided by Richard Ordway), we tested wild-type and homozygous EPS-15 null mutant larvae (escapers) with mAb aa2 and anti-EPS-15 antiserum (mutants and antiserum kindly provided by Hugo Bellen) and found that aa2, like anti-EPS-15 antiserum, failed to label synaptic terminals of the mutant. We, thus, conclude that mAb aa2 most likely binds to EPS-15.

mAb na21

The staining of this Ab in the antennae of *Drosophila* has briefly been described previously (Störtkuhl et al., 1994). Here, we include the staining pattern of na21 in the adult optic lobes (Figure 3D), which closely resembles that of an Ab against choline acetyl transferase (Chat) (Buchner et al., 1986). The identification of the na21 antigen is presently attempted.

mAb pok13: Calbindin

Vertebrate parvalbumin, calbindin D-28k, and calretinin are calcium-binding proteins with four to six EF-hand calcium-binding domains. In the brain, they are expressed at high levels in various cell types; parvalbumin is often colocalized with the neurotransmitter, gamma aminobutyric acid (GABA). These proteins presumably function both as calcium buffers and calcium sensors and are important for calcium homeostasis. Human calbindin could play a role in nitric oxide regulation, as 80% of its cysteines can be S-nitrosylated (Tao et al., 2002). It has been shown that calbindin interacts with caspase-3 and protects the cells against apoptosis (Bellido et al., 2000). Knock-out mice lacking calretinin and/or calbindin suffer from ataxic movement disorders, which are accompanied by fast network oscillations in the cerebellar cortex (Cheron et al., 2004), while parvalbumin deficiency leads to a propensity to epileptic seizures (Schwaller et al., 2004). In flies, only a single gene codes for a protein that shows high homology to these related calcium-binding proteins. It has been identified by screening the Zinsmaier λ-gt11 head cDNA expression library with an antiserum against carp parvalbumin. It codes for six EF-hand domains and has been named the calbindin-32 gene (*Cbn*) due to the apparent size of the encoded protein (Reifegerste et al., 1993) (synonyms: calbindin 53E; *Cbp53E*). Spleen cells of a mouse injected with purified cDNA-encoded protein were fused with myeloma cells, the hybridoma cells were screened to isolate the mAb, pok13, which binds to calbindin both on Western blots and in histochemical preparations (Figure 6). mAb pok13 stains certain muscles and a large number of neurons, such as in the distal lamina (probably monopolar cells L2), a group of cell bodies caudal to the medulla (probably T2 and/or T3) (Fischbach and Dittrich, 1989), and cells in the third antennal segment. While most neuropil stains weakly, several regions of the central-brain neuropil are conspicuously devoid of staining, such as the ellipsoid body, the fan-shaped body, and the mushroom bodies. Attempts to isolate mutants or *P*-insertions in the *Cbn* gene have failed so far, and apart from its calcium-binding property, as indicated by a shift in apparent molecular weight in the presence of calcium (Figure 6C), at present no functional information on calbindin in *Drosophila* is available.

mAb nb33: Pigment Dispersing Factor (PDF) Precursor

nb33 stains, very selectively, both the ''large ventral lateral neurons'' and the ''small ventral lateral neurons'' in *Drosophila* (lLNv, sLNv), in addition to a few cells in the abdominal ganglia. The lateral neurons are known to be clock neurons containing the peptide PDF, the *Drosophila* homolog of the crustacean peptide pigment dispersing hormone (PDH) (Helfrich-Förster & Homberg, 1993). Indeed, the nb33 staining pattern is essentially identical to the pattern observed with an antiserum against crab PDH, and the antibody has been used as an alternative to anti-PDH serum (Veleri et al., 2003;

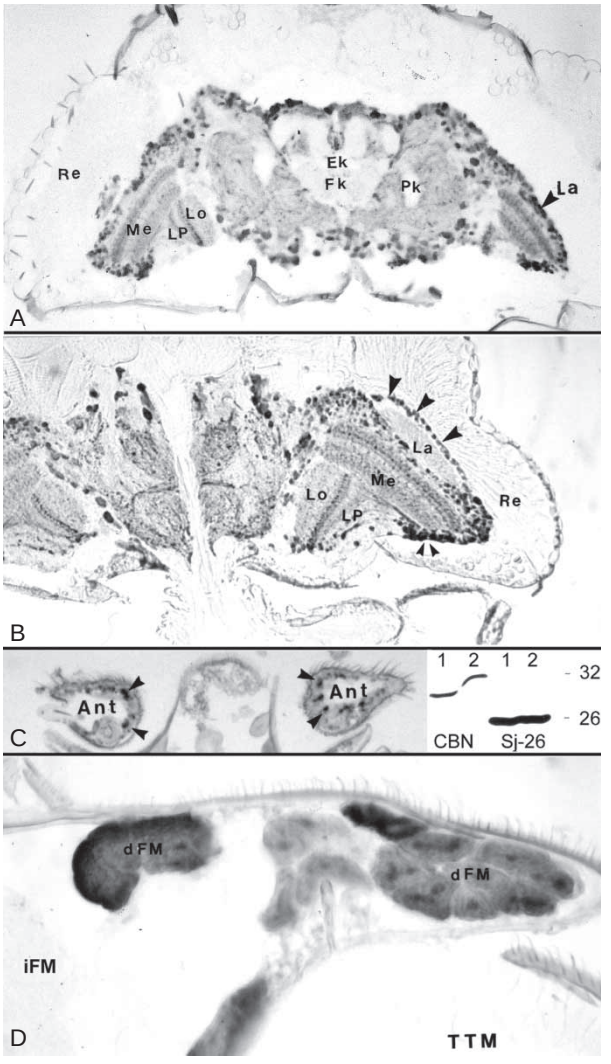

Figure 6. Staining patterns of pok13 directed against *Drosophila* calbindin. (**A**) In the dorsal protocerebrum, numerous cell bodies and the ''unstructured'' neuropil are labeled, while the mushroom bodies, the fan-shaped body, and the ellipsoid body show very low staining. (**B**) The horizontal section at the level of the esophagus canal illustrates the distribution of CBN immunopositive cell bodies, specifically in the lamina cortex and posterior to the medulla (possibly of L2 and T2/T3 neurons, respectively). (**C**) Cells in the third antennal segment are labeled. Inset: Coomassie staining of 7.5% SDS gel loaded with affinity-purified bacterially expressed *Drosophila* calbindin (lanes 1 and 2) or the GST-tag Sj-26 (lanes 3 and 4) in the presence of 1 mM CaCl2 (lanes 1 and 3) or 5 mM EGTA (lanes 2 and 4). (**D**) Direct flight muscles (dFMs) show strong staining, while indirect flight muscles (iFMs) and tergotrochanter muscle (TTM) lack CBN. Ant, antennae; Re, retina; La, lamina; Me, medulla; Lo, lobula; LP, lobula plate; Pk, mushroom body; Ek, ellipsoid body; Fk, fan-shaped body.

Helfrich-Förster et al., 2007). There might be subtle differences in the circadian distribution of the antigen and in the staining intensity of cell compartments, but the morphology of stained cells is identical.

The *Drosophila Pdf* gene codes for a putative 102-amino-acid precursor protein; only the last 18 amino acids represent the final PDF peptide. This peptide shows high homology to the PDH of crustaceans and to the PDF of other Drosophilids and other insects. In contrast, the remaining precursor sequence appears much more variable (Park & Hall, 1998). In experiments on Pdf^{01}, a null mutant for PDF, we obtained no staining with nb33 nor with anti-PDH. Transgenic flies expressing a modified PDF precursor without the final PDF sequence on a Pdf^{01} background showed normal nb33 staining, but no staining with anti-PDH serum. Flies expressing a precursor truncated to the final PDF sequence produced no staining with nb33, but we obtained normal staining with the serum against PDH (fly strains kindly provided by P. Taghert). Thus, nb33 recognizes an epitope in the precursor region, which is not contained in the final PDF peptide.

We compared the nb33 staining pattern of *D. melanogaster* with several other *Drosophila* species. There is virtually no difference in the staining among the species of the melanogaster species subgroup tested (*D. melanogaster, D. teissieri, D. yakuba, D. simulans*). Of the other species subgroups combined in the melanogaster species group, we found weak staining in *D. eugracilis, D. ananassae*, and *D. saltans*. The staining in this species was less distinct, often reduced to the staining of cell bodies only. In addition, cross-reactions of the Ab with antigens in additional cell nuclei and cell-body layers became evident. No staining was obtained in *D. pseudoobscura*. (subgenus *Sophophora*) and *D. virilis* (subgenus *Drosophila*).

Considering that the epitope of nb33 is somewhere on the precursor outside the PDF peptide domain, it is not surprising that the species range of nb33 is much more restricted, compared to the anti-PDH serum (Nässel et al., 1993, Helfrich-Förster et al., 1998).

DISCUSSION

This review summarizes the information available for several novel brain proteins identified by the approach from antibody to gene and, for the first time, presents the immunohistochemical staining patterns for a number of unpublished Abs from the Wuerzburg hybridoma library in a generally accessible form. In the original screen of the library, these staining patterns served as the selection criterion. By this procedure, we primarily obtained a collection of Abs that reveal anatomical and morphological details of the *Drosophila* brain. A subgroup of Abs was then used to identify the corresponding antigens and their encoding genes. Even without any knowledge about the antigens or genes, the library of Abs is useful for descriptive neuroanatomical work. Details revealed by

Table 2. Series of immunizations

Code	Antigen	Immunization newborns	Immunization (juveniles, adults)
aa ab	Fixed brains, freeze-dried	–	Wild-type
ca	Brains, freeze-dried	sol (+s)	sol (+s) wild-type
fa fb	Heads	mnb (+s)	mnb (+s) wild-type
ga	Heads	rol sol (+s)	rol sol (+s) wild-type
na nb nc	Heads	mnb sol	mnb sol (+s) wild-type
po	Dmel\calbindin-32	–	Purified protein (+F)
3C	Dmel\synapsin	–	Purified protein (+F)

Code refers to the first two characters of the name of the hybridoma clone, with the first letter referring to the immunization regime, the second letter distinguishing individual animals. +s, immunization followed by immunsuppression (cycloheximide); F, Freund's adjuvant.

such Abs can be compared between different species or between wild-type und morphological mutants affecting the nervous system. In addition, one of the synapse-specific antibodies, nc82, is widely used for convenient staining of all neuropil to obtain a framework for cell-specific labeling by other Abs or green flourescent protein (GFP) expression. This Ab was actually used for establishing a standard reference tool for neuroanatomical work on *Drosophila* (Rein et al., 2002). Since nc82 exclusively binds to a small spot in each synapse (the active zone), the brain remains transparent in confocal immunofluorescence microscopy of whole-mount preparations.

The route from a mAb to the identification of its antigen and the corresponding gene is not necessarily straightforward. Screening of cDNA expression libraries with the mAb cannot be successful if the epitope recognized by the Ab is generated by post-translational modification. Also, conformational epitopes that require chaperones for correct folding may not form in the bacterial environment of the expression clones. Another problem of most cDNA expression libraries is the fact that the cDNAs are inserted into the phage with random orientation and random reading frame. Thus, only one of six clones can, in principle, contain the correct coding sequence, and five of six clones may generate new epitopes that are not present in the fly but might cross-react with the Ab and produce false-positive signals. It is, therefore, not surprising that in our screens, we always had false-positive clones necessitating considerable effort to identify the correct clone. Oligo-dT primed cDNA libraries, in addition, are likely to contain many 5' incomplete cDNAs and thus may lack epitopes near the N-termini of the encoded proteins. Attempts with this approach to identify the antigens of various mAbs that only bind to few brain cells, including mAbs nb33, ca8, nb236, and fa56, produced only false-positive clones, indicating that in addition to the mentioned limitations, this approach can be expected to be successful only if the mRNA encoding the antigen is relatively abundant in the head poly-A^+ mRNA fraction.

MS of trypsin-digested proteins, and comparison of the observed peptide spectra with the predicted peptides of all hypothetical proteins encoded by the whole genome of the species under study, has become a powerful technique for the identification of isolated proteins. For *Drosophila*, this approach became feasible in 2000, when the nonrepetitive genome sequence became available (Adams et al., 2000).

From Table 1, it is apparent that it has been possible to identify the antigens for several mAbs. However, both molecular approaches worked, so far, only for antigens, which are present in most or all neurons. The only antigen that is expressed exclusively in a small number of neurons was identified by the candidate approach, making use of knowledge on cells with a similar gestalt in other species. Apparently, the number of cDNA copies in head libraries and the amount of antigen in head homogenates are too low for a successful molecular approach. Thus, it seems necessary to enrich mRNAs or proteins only from cells positive for the mAb. This could, perhaps, be achieved by dissociating the cells from immature brains and subjecting them to fluorescence-activated cell sorting.

Alternative approaches to identify cell-specifically expressed genes have been developed in recent decades. Embryonic expression patterns were determined for 44% of the protein-coding genes of *Drosophila* in a large-scale approach using *in-situ* hybridization and quantitative microarray time-course analysis (Tomancak et al., 2007). For adult flies, the enhancer trap system (O'Kane & Gehring, 1987; Brand & Perrimon, 1993) has received the highest attention. The most extensive collection of some 4,000 enhancer trap lines has been generated by a Japanese consortium headed by K. Ito (available from the Bloomington stock center). However, many enhancer trap lines suffer from limited specificity of the expression drivers, since not all regulatory elements of a gene can be expected to interact with the promotor used in the enhancer trap construct in exactly the same fashion as with its endogenous promotor. In addition, the expression patterns may depend on the genomic insertion site of the transposon relative to the promotor, and even variability

of expression, depending on the UAS-reporter used, has been described for various Gal4 lines (Ito et al., 2003; A. Jenett, personal communication). Thus, it is not surprising that the cell-specific all-or-none staining observed with the majority of our mAbs is only rarely seen in enhancer-trap-reporter stainings. Attempts to overcome these limitations of the enhancer trap system use the ''gene trap'' (or fly trap) technique which requires a P transposon construct containing an artificial exon coding for the reporter to insert into an intron, thus generating a fusion protein with the endogenous expression pattern (Kelso et al., 2004; Buszczak et al., 2007). The important advantage of these techniques is the possibility of a straightforward identification of the gene coding for a protein of the observed expression pattern.

CONCLUSION

In conclusion, we predict that the mAbs of our library, which selectively stain only a few neurons, will be useful for reproducible display of cell morphology, but that the systematic identification of the protein recognized by the Ab will require new, sophisticated approaches.

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PLoS one

Identification of Eps15 as Antigen Recognized by the Monoclonal Antibodies aa2 and ab52 of the Wuerzburg Hybridoma Library against *Drosophila* Brain

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Abstract

The Wuerzburg Hybridoma Library against the *Drosophila* brain represents a collection of around 200 monoclonal antibodies that bind to specific structures in the *Drosophila* brain. Here we describe the immunohistochemical staining patterns, the Western blot signals of one- and two-dimensional electrophoretic separation, and the mass spectrometric characterization of the target protein candidates recognized by the monoclonal antibodies aa2 and ab52 from the library. Analysis of a mutant of a candidate gene identified the *Drosophila* homolog of the Epidermal growth factor receptor Pathway Substrate clone 15 (Eps15) as the antigen for these two antibodies.

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Introduction

The generation of hybridomas was one of the milestones in modern biology [1,2] leading to the production of monoclonal antibodies (mAbs), one of the most important tools in biology. Instead of remaining as mere tools for research, mAbs have become indispensible in therapeutics as well as diagnostics [3]. Today mAbs are important therapeutic agents for a wide variety of diseases like cancers [4], autoimmunity [5], respiratory diseases [6], infectious diseases [7] and AIDS [8]. Currently mAbs represent over 30% of all biological proteins undergoing clinical trials and are the second largest class of biodrugs after vaccines [9–11]. With the advent of more efficient, genetically engineered antibodies [12] this trend is expected to grow [13,14]. The advancement of technology and the widespread applications of mAbs led to the development of alternate methods of production, like nonrodent hybridomas [15], plants [16], ascites [17] and bioreactors [18]. Soon after the production of mAbs against specific proteins, mAbs were randomly generated as 'hybridoma libraries' against complex protein mixtures from tissues or subcellular compartments [19–21]. The production of such libraries against the *Drosophila* nervous system was pioneered by the group of the late Seymour Benzer [22,23]. One such extensive hybridoma library, generated against *Drosophila* brain homogenate is the Wuerzburg Hybridoma Library [24,25]. MAbs from this library can be used as tools for cell-specific neuroanatomical staining [24] and, in favorable cases, for the identification of novel brain proteins by either a "candidate" or "from antibody to gene" approach. By the candidate approach the antigen of mAb nb33 which binds to pigment dispersing factor (PDF) containing neurons was identified as the PDF precursor protein (but not the PDF itself) [25]. The approach "from antibody to gene" has led to the discovery of several novel synaptic proteins. Initially, target proteins were identified by screening of cDNA expression libraries for clones expressing proteins with an epitope recognized by a given mAb. The cysteine string protein (CSP) and its gene (*Csp*) was discovered by mAb ab49 [26], the synapse-associated protein of 47 kD (SAP-47) and its gene (*Sap47*) was discovered by mAb nc46 and later shown to be also recognized at a different epitope by nb200 [25,27]. More recently, protein purification and mass spectrometry was used for the identification of the protein Bruchpilot (BRP) and its gene (*brp*) recognized by the mAb nc82 [28,29]. In addition to the mAbs generated against brain homogenate our hybridoma library contains mAbs 3C11 and pok13 which were generated against bacterially expressed *Drosophila* synapsin and calbindin-32, respectively [30,31]. Besides these mAbs with known targets the library contains a large collection of mAbs which recognize different structures like body

tissues (eyes, muscles, cuticula, perineureum, trachea) or cellular compartments (cell-body layers, nucleus, membranes) or small subsets of neurons, but whose target proteins are unknown [25]. Only few antibodies with unknown targets bind to synaptic neuropil. In line with our long standing research focus on synaptic proteins [26–31] we here describe the mAbs aa2 and ab52, their binding to synaptic neuropil of the adult brain, their immunohistochemical staining of the synaptic boutons of larval motor neurons, and the experiments leading to the identification of the homologue of Epidermal growth factor receptor Pathway Substrate clone 15 (Eps15) as the antigen they recognize in the *Drosophila* brain. Eps15 is a substrate for the tyrosine kinase activity [32] of the Epidermal Growth Factor Receptor (EGFR) and contains Ca^{2+} binding EF hands, which comprise the Eps15 homology (EH) domain [33]. Proteins containing EH domains interact with partner proteins containing the Asn-Pro-Phe (NPF) motif and play an important role in synaptic vesicle recycling and receptor endocytosis [34].

Materials and Methods

Fly strains

Unless indicated otherwise, Canton S (CS) was used as the wild type for all experiments. *eps15*$^{\Delta 29}$ mutant flies [35] were kindly provided by H. Bellen.

Hybridoma cell culture, monoclonal antibody production

Hybridoma clones were generated as described earlier [24,25]. For mAb production, cryopreserved cell lines were thawed and cultured, initially in 24 well Nunclon$^{TM}\Delta$ plates (Nunc) with HT medium as described earlier [25]. 50 µl of fetal bovine serum was added per well to facilitate the initial growth of the thawed cells. Growth of cells was monitored daily under an inverted microscope (Zeiss). Upon proliferation after 2–3 days, 1 ml of actively growing cell suspension was used to inoculate 5 ml HT medium in 50 ml T flasks (Greiner Bio) and further cultured for 2 days. Thereafter fresh medium was provided and after 2 more days cell density was determined by Trypan Blue (Sigma) exclusion staining of cell suspension using a Neubauer-counting chamber (GLW). When the supernatant appeared yellowish (cell density $\sim 10^6$ cells/ml), supernatant medium was withdrawn and centrifuged at 2000 rpm for 5 min to pellet all cells and the cell-free supernatant was tested for the presence of antibodies to find the optimal dilution for a reliable signal in western blots and/or immunohistochemistry. Upon detection of an antibody signal, the antibody producing cells were further cultured for continued antibody production until the cell density reached $\sim 10^6$ cells/ml. At this stage they could be either split into more flasks or used to inoculate larger flasks (250 ml, 75 cm^2, Grenier Bio). Supernatant from larger flasks was withdrawn every 3 days.

Characterization of the monoclonal antibodies

For the characterization of monoclonal antibodies their isotype was determined by capture ELISA using the ISO2-KT (Sigma) mouse monoclonal isotyping kit following the manufacturer's instructions. 5-Aminosalicylic acid (Sigma) was used as substrate (1 mg/ml) in 0.02 M sodium phosphate buffer (pH 6.8) with 0.01% H_2O_2 (v/v). Isotype of a given mAb was visually evident as development of color by the chromogenic substrate with its corresponding anti-isotype antibody. The isotype was further confirmed by immunoassay based Isoquick Strips (Envirologix) following the manufacturer's instructions. For storage of the monoclonal antibodies, suitably sized aliquots of the culture supernatant were snap frozen in liquid nitrogen and stored at -20°C. However in case of IgM antibodies, which (like IgG3) tend to aggregate upon freezing and thawing, they were stored at 4°C by adding 0.02% NaN_3 (w/v) as antimicrobial agent.

Immunostaining of fly heads

Cryosections of adult fly heads were made essentially as previously described [36]. Series of consecutive sections were collected on subbed glass slides (Menzel Gläser), thawed on the slide and air-dried. The slides were blocked for 2 hr at RT in a humid chamber with normal serum from the species in which the secondary antibody was generated (Vector Labs) diluted 1:20 in 1x PBST (phosphate buffered saline, pH 7.6 with 0.1% Triton-X100). Thereafter the sections were incubated with primary mAbs (aa2 1:2, ab52 1:5) in PBST at 4°C overnight in a humid chamber. Excess primary mAb was removed and the slides were washed three times for 20 min each with PBST. The slides were incubated with Cy2 labeled α-mouse secondary Ab (diluted 1:500 in PBST) and either DAPI (0.2 µg/ml) or Cy3 labeled anti-HRP (1:500) (Jackson Immuno Res. Inc.) at room temperature for 2 hrs (anti-HRP cross-reacts with a carbohydrate epitope on *Drosophila* neuronal membranes). After washing in PBST the sections were permanently mounted with Vectashield® (Vector Labs). Optical sections were obtained by a confocal scanning microscope (Leica TCS-SP2).

Immunostaining of motorneuron terminals

The procedure for obtaining larval nerve-muscle preparations for immuno-labeling has recently been described [37]. The 'filets' were blocked in 5% normal goat serum (Vector Labs) in 1x PBST for 1 hour at RT with gentle shaking. Thereafter the preparations were incubated overnight in the primary antibody (mAB aa2 diluted 1:2, mAb ab52 (1:2), guinea pig anti-Eps15 antiserum (kindly provide by H. Bellen) (1:300) in PBST) at 4°C. Next day the preparations were washed in 1x PBST', once for 30 min and 4 times for 1 h each at room temperature with gentle shaking. Then they were incubated overnight in the goat-α-mouse-Alexa488 and goat-α-guinea pig Cy3 secondary antibodies (Invitrogen), both diluted 1:500 in the blocking solution at 4°C. Next, the preparations were washed 3 times in PBST for 20 min each at room temperature with gentle shaking. The preparations were mounted in Vectashield®. Images were acquired with a confocal scanning microscope. The confocal stacks were analyzed using the Fiji package [38] based on ImageJ [39,40].

SDS-PAGE and Western blot

Samples were prepared in 1x LDS sample buffer (Invitrogen) and resolved using the NuPAGE® precast gel system (Invitrogen) by SDS-PAGE. In brief, samples were run on Novex® Bis-Tris 12% gels with 1x MOPS SDS running buffer (Invitrogen). Gels were transferred onto 0.45 µm nitrocellulose membrane (Protran®, Whatman) with 3 mm Chr paper (Whatman) sandwiches in a Mini Trans-Blot® (Bio-Rad) apparatus using the Towbin buffer system [41] at 100 V for 1 hr. Thereafter the membranes were stained with Ponceau S (0.1% w/v) solution (Sigma) and blocked in 5% (w/v) non-fat dry milk (Roth) in 1x TBST (10 mM Tris pH 7.6, 150 mM NaCl, 0.05% v/v Tween-20) for 2 hours at room temperature. Thereafter blots were incubated overnight in primary antibodies at suitable dilutions in 1x TBST at 4°C. The mAb aa2 was used at dilution of 1:2 while ab52 was used at a dilution of 1:10. Next morning, blots were washed in 1x TBST three times for 5 minutes each and then incubated for one hour with the goat-α-mouse-HRP secondary antibody (Bio-Rad) diluted at 1:7500 in 1x TBST at room temperature. Thereafter blots were washed as earlier and developed with ECL^{TM} (Amersham, GE)

and signals were obtained as exposures on X-Ray films in the dark and developed them using developing and fixing solutions (Kodak).

Subcellular fractionation

Adult CS flies were anesthetized with CO_2, collected in 50 ml falcon tubes and snap frozen by immersing the tubes in liquid N_2. Frozen flies were vigorously vortexed to separate all jointed body parts and passed through a stack of two sieves. The upper sieve with 800 µm mesh size retained thorax and abdomen while the lower sieve with 500 µm mesh size retained the heads, and smaller body parts passed through. Frozen heads from the lower sieve were collected and pulverized in a mortar-pestle, which was prechilled to −80°C. The powder was dissolved in homogenization buffer A (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA final pH 7.3) supplemented with 1 tablet (per 10 ml buffer per 1 gm of fly heads) of protease inhibitors mix (Complete Mini^{TM}, Roche). The sample was thoroughly mixed to get a uniform homogenate, which was then incubated on ice for 5 minutes. Thereafter it was spun twice at 13000 rpm for 15 min each, at 4°C to pellet the exoskeleton, cell debris, nuclei (P1). The post-nuclear supernatant (S1) was re-spun in an ultracentrifuge (L8 Beckman, 60Ti rotor) at 100000 g for 1 hr at 4°C to get the cytosolic fraction as the supernatant (S2) and the total membrane fraction as the pellet (P2) which were then tested on Western blots.

Two-dimensional electrophoresis (2DE), IEF/SDS-PAGE

Proteins from fly head homogenate were resolved by 2D electrophoresis using the Zoom® 2D (Invitrogen) setup. In brief, 100 freshly isolated CS fly heads were homogenized in 100 µl of Zoom® 2D Protein Solubilizer1 (Invitrogen) containing 1x protease inhibitors (Complete-Mini^{TM}, Roche). The homogenate was then centrifuged at 13000 rpm for 15 min at 4°C, to remove the exoskeleton, cell debris, and nuclei. 1 µl of 99% N,N-Dimethylacrylamide (DMA, Sigma) was added to the post-nuclear supernatant and incubated on a rotary shaker at room temperature for 15 min to alkylate the proteins. Thereafter 1 µl of 2 M DTT was added to quench any excess DMA and the sample was ready for loading. 25 µl of this homogenate, equivalent to 25 fly heads was mixed with strip rehydration buffer (Zoom® 2D Protein Solubilizer1, 20 mM DTT, traces of Bromophenol-blue) supplemented with 0.01% (v/v) 3–10 pH ampholyte (Serva) to get a final volume of 165 µl. Immobilized pH gradient (IPG) (Zoom®, Invitrogen) strips for the range 3–10 pH were rehydrated with this sample as per the manufacturer's instructions in the Zoom® IPG Runner^{TM} cassette overnight at 18°C. Next day the sample in the rehydrated strips was resolved by isoelectric focusing with a Zoom® Dual power supply unit (Invitrogen), while keeping the power limited to 0.1 W per strip and using the voltage regime shown in Table 1.

Table 1. Voltage regime for isoelectric focusing.

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

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Thereafter the strips were incubated in the equilibration solution (1x NuPAGE^{TM} LDS sample buffer with 1x NuPAGE^{TM} Reducing Agent, both Invitrogen) and alkylation solutions (1x NuPAGE^{TM} LDS sample buffer) with 125 mM Iodoacetamide (Sigma) for 15 minutes respectively, with gentle shaking. Thereafter the strips were loaded into the IPG well of 4–12% Bis-Tris NuPAGE^{TM} (Invitrogen) 2D PAA gel and overlaid with agarose (0.5% w/v in 1x MOPS running buffer). The second dimension was run at 100V after which proteins were blotted from the gel on two separate membranes consecutively to get duplicate blots of the same 2D separation profile. These membranes were then incubated with the mAb aa2 and ab52 separately and developed to compare their signal profiles.

Two-dimensional electrophoresis NEPHGE/SDS-PAGE

For separation of larger amounts of proteins, Non-Equilibrium pH Gradient gel Electrophoresis (NEPHGE) was performed according to the method of O'Farrell *et al.* [42] with some modifications. In brief, tube gels [9 M Urea, 4% acrylamide, 2.5% NP40, 5% ampholytes (Servalyte^{TM}, Serva) pH 2–11, 0.03% APS, 0.2% TEMED] of length 11 cm and diameter 3 mm, were casted overnight. Soluble (cytosolic) fraction S2 was obtained as described above and 100 µl (100 head equivalents) were precipitated with 900 µl of chilled acetone for 3 hours at −20°C. The sample was centrifuged at 10000 g for 10 min at 4°C to pellet the precipitated proteins. The supernatant was discarded, the pellet was air-dried and resuspended in 50 µl sample loading buffer 1 (9.5 M Urea, 0.5% SDS, 5% β-mercaptoethanol, 2% ampholytes pH 2–11). Upon dissolution,

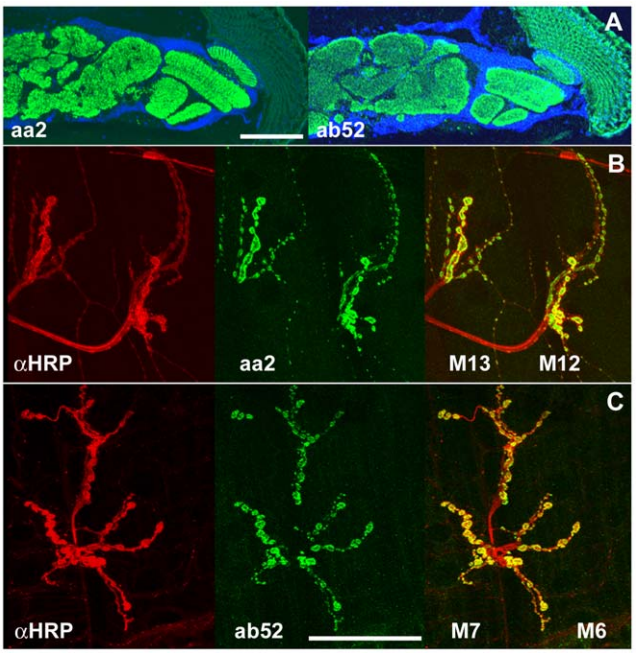

Figure 1. Staining patterns of mAbs aa2 and ab52. (A) Cryosections of an adult fly head were probed with mAb aa2 (left) and mAb ab52 (right). Both antibodies stain all synaptic neuropil (green) but not the surrounding the cell body layer whose nuclei are stained with DAPI (blue). (B, C) Synaptic boutons of larval motor neuron terminals stained with anti-HRP (left, red) and mAbs aa2 (B, middle, green), or ab52 (C, middle, green). The overlays in the right column demonstrate that the epitopes recognized by both mAbs are present in all boutons (here shown for muscles M12/13 (B) and muscles M6/7 (C)) but not in the axons. Scale bars in A: 100 µm; in C for B and C: 50 µm).
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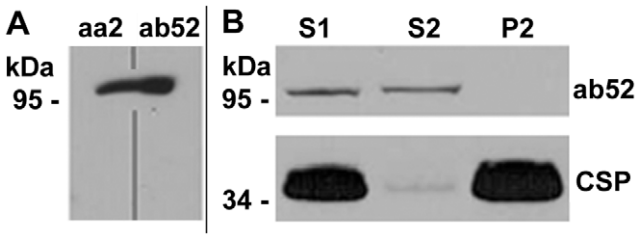

Figure 2. mAbs aa2 and ab52 recognize soluble proteins at identical M_r on 1D Western blot. (A) Blot of a single SDS gel electrophoresis lane loaded with homogenate from 2 wild-type (CS) heads. The blotted membrane was vertically cut in two halves; one was developed with mAb aa2 (left), the other with mAb ab52 (right). Signals at identical M_r suggests that both mAbs probably recognize the same antigen. (B) Western blot of subcellular fractions of wild-type (CS) fly heads showing that the protein detected by mAb ab52 is exclusively present in the cytosolic supernatant. S1 = postnuclear supernatant; S2 = cytosolic fraction; P2 = total membrane fraction (10 head equivalents loaded per lane). The synaptic vesicle protein CSP recognized by the mAb ab49 was used as a marker for the total membrane fraction.
doi:10.1371/journal.pone.0029352.g002

an equal volume of buffer 2 (9.5 M Urea, 5% NP-40, 5% β-mercaptoethanol, 2% ampholytes pH 2–11) was added. The sample was loaded on top of the tube gel and overlaid with 40 µl of overlaying solution (6 M Urea, 5% NP-40, 1% ampholytes pH 2–11). Electrophoresis was carried out in the Model 175 Tube Cell (Bio-Rad) setup at 200 V for 15 min, followed by 300 V for 30 min and finally at 400 V for 120 min. 10 mM H_3PO_4 and 20 mM NaOH were used as anode and cathode electrophoresis buffers respectively. As a marker for highly basic proteins, cytochrome C having a pI>11, was loaded on one of the tube gels as a control for the progress of the 1st dimension. At the end of the run, the NEPHGE gel with the sample was slowly withdrawn from the glass tube, equilibrated for 20 min with the SDS sample buffer (60 mM Tris-HCl, 2% SDS, 5% β-mercaptoethanol, 10% Glycerol, pH 6.8) and overlaid with 1% agarose in SDS sample buffer on a 12% PAA gel. 10 freshly homogenized fly heads were also loaded in an adjacent lane to serve as a 1D reference to the 2D profile. Electrophoresis was carried out at 15 mA for 16 hr. The tube gel with cytochrome C was cut into 0.5 cm pieces and each piece was incubated overnight with 3 ml dH_2O at 4°C such that the pH along the length of the gel could be measured next morning.

Partial blot and silver staining of NEPHGE gel

After 2DE, the gel was blotted for 20 minutes using the Towbin transfer buffer system at 2 mA/cm^2 of gel area in a PerfectBlue^{TM} (peqLAB) semi-dry blotting apparatus. By this procedure only part of the protein content of the gel was transferred onto the membrane, while the rest was retained in the gel, which was then silver stained for MS compatibility as described earlier [43]. The blot was blocked as described above and then incubated with primary antibody (ab52, diluted 1:10) and developed as described above. Overnight exposure was done to get a strong signal for the antigen along with weak non-specific signals on the blot, which would serve as landmarks for comparison with the silver-stained gel. Images of the over-exposed blot and the silver-stained gel were digitally superimposed with Photoshop (Adobe) to pinpoint the protein spot in the silver stained gel that corresponded to the signal in the Western blot. This spot was then excised and analyzed by mass spectrometry as described below.

Immunoprecipitation of the antigen for mAb aa2

3000 fly heads were homogenized in 1 ml of homogenization buffer (50 mM Tris, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 0.01% β-mercaptoethanol, protease inhibitors as in buffer-A, final pH 7.5), centrifuged at 14000 rpm, 4°C for 10 min to yield the supernatant S1. 600 µl of protein-G agarose beads (Roche) were washed in homogenization buffer and incubated in 8 ml of undiluted mAb aa2 supernatant (neutralized to pH 7.5 with 1 M Na_2HPO_4) for 3 h at 4°C with gentle mixing, followed by washing and incubation with supernatant S1 for 3 h at 4°C. The mixture was centrifuged (1500 rpm, 4°C, 2 min), the pellet was washed with 1 ml homogenization buffer, and 60 µl of 5x Lämmli buffer was added to the beads. The sample was heat denatured and loaded in two gels and resolved in parallel by SDS-PAGE. One gel was stained by Coomassie [44] while the other was blotted. The blot was blocked as described earlier and incubated with the primary antibody aa2 (1:2), followed by development as described above. Superimposition of the stained gel and blot images allowed the identification of the proteins bands in the stained gel that corresponded to the Western blot signal. The specific bands were excised and analyzed by mass spectrometry as described below.

Mass spectrometric (MS) analysis

The protein spot from the silver stained NEPHGE gel, corresponding to the mAb ab52 Western signal, and the

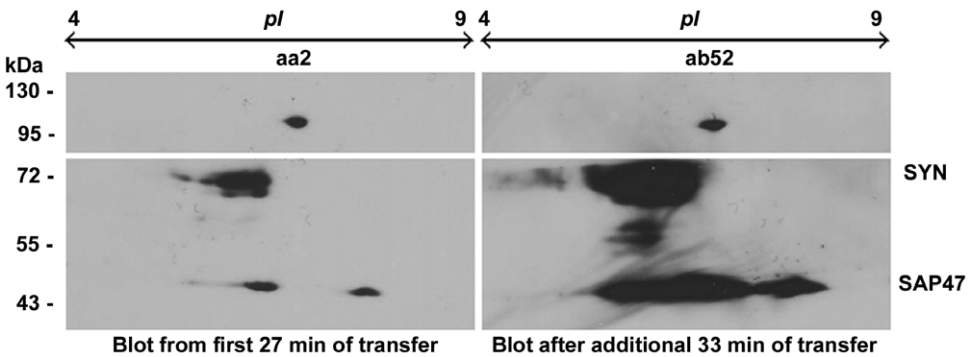

Figure 3. aa2 and ab52 produce signals at identical M_r and pI on 2D Western blot profile. Two sequential Western blots from a single 2DE gel loaded with sample equivalent to 25 fly heads. The two membranes were cut along the horizontal white line, the upper parts were developed with mAbs aa2 (left, dilution 1:2) or ab52 (right, dilution 1:10), the lower parts were stained with mAbs 3C11 (anti-SYN, 1:100) and nc46 (anti-SAP47, 1:200) as controls for both blots.
doi:10.1371/journal.pone.0029352.g003

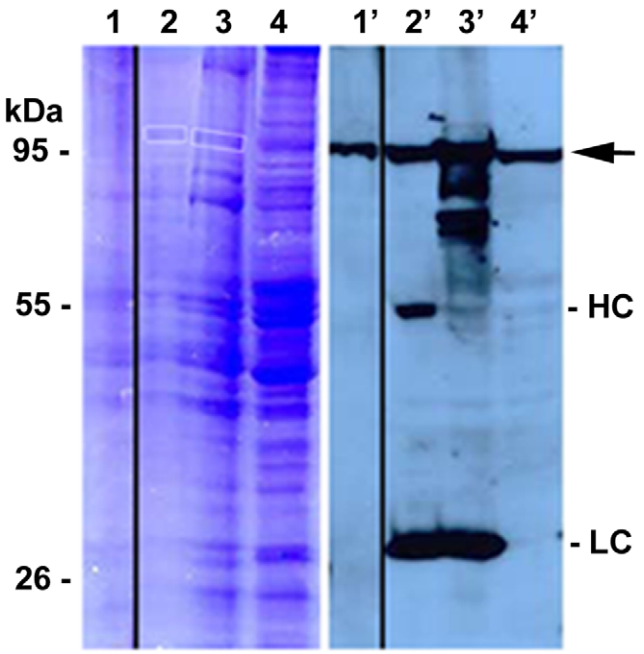

Figure 4. Identification of mAb aa2 antigen by comparison of a Coomassie stained gel and a Western blot. Coomassie-blue stained gel (left) and Western blot of a gel run in parallel (right). Loaded was: supernatant S1 (3 head equivalents, lanes 1, 1′), immunoprecipitation of S1 with mAb aa2 and protein-G beads (~80 head equivalents, lanes 2, 2′ or ~800 head equivalents, lanes 3, 3′), and 3 heads homogenized in Lämmli buffer (lanes 4, 4′). The blot was developed with mAb aa2. LC, light chain, HC, heavy chain of mAb aa2. The boxed bands of the gel were cut out and subjected to MS analysis.
doi:10.1371/journal.pone.0029352.g004

Coomassie-stained immunoprecipitated proteins in the 1-D SDS gel corresponding to the mAb aa2 Western signal were excised and the proteins were reduced, carbamidomethylated, and digested by trypsin followed by MS analysis as described earlier [25]. For data evaluation, raw-data was converted to Mascot-mgf files using ProteomeWizard (http://proteowizard.sourceforge.net/). Searches were conducted against a subset of the Swissprot database (www.uniprot.org) containing 35373 sequences (5th May 2011). Mascot (v2.2) was used as search engine with Mascot Daemon support (v2.2.0) with the following parameters: Trypsin was set as protease with one miscleavage site allowed, precursor and fragment ion tolerance was 0.5 Da, carbamidomethylation (C) was chosen as fixed and oxidation (M) as variable modification. Peptides with $p<0.01$ and scores above 38 were considered for subsequent manual validation.

Results and Discussion

Characterization of the mAb isotypes

aa2 was found to be an IgG1, while ab52 was found to be an IgM. The fact that aa2 and ab52 are of different isotypes indicates that they are produced by two distinct hybridoma cell lines and hence not subclones from a common parent hybridoma cell. Both mAbs had the kappa (κ) type of light chain.

Staining pattern of the mAbs aa2 and ab52

On cryosections of adult heads these two antibodies equally stain all synaptic neuropil as shown in Fig. 1A. In larval nerve-muscle preparations both antibodies stain all synaptic boutons (Fig. 1B,C).

Migration pattern of antigens recognized by aa2 and ab52 on 1DE

In Western blots of freshly homogenized CS fly heads, both antibodies produce a single signal around 100 kDa. To test for identical migration properties of the recognized antigens, proteins from 2 freshly homogenized CS fly heads were resolved by SDS-PAGE, followed by Western blotting and then the blot of a single lane was vertically cut into two halves. One half of the lane was incubated with mAb aa2 and the other with mAb ab52, both halves were separately washed, incubated with secondary antibody, washed again and then developed together (Fig. 2A). The developed blots suggest that the antigens recognized by the two mAbs have the same M_r and hence may be the same protein.

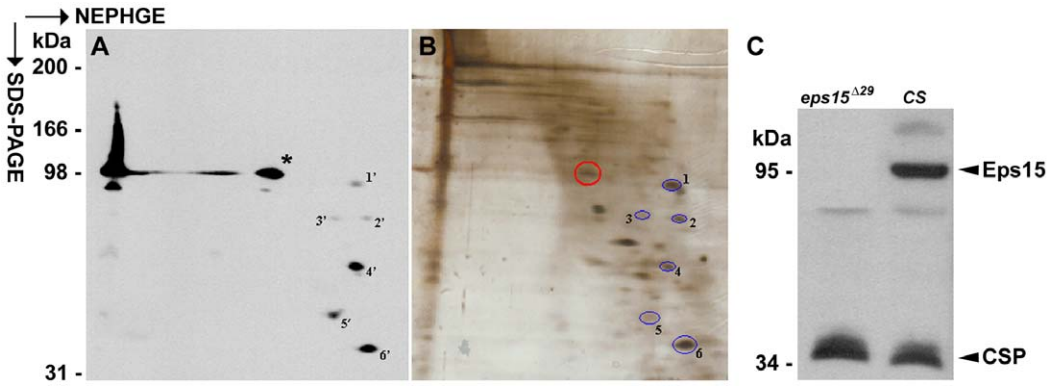

Figure 5. Enrichment of the ab52 antigen for MS by NEPHGE/SDS-PAGE and its final identification as Eps15. (A) Western blot developed with mAb ab52 after partial transfer of proteins from the gel shown in (B). The signal from the mAb ab52 can be clearly seen as a distinct spot ~100 kDa (asterisk), with a corresponding strong signal in the 1D lane loaded with 10 freshly homogenized fly heads. Non-specific signals were numbered as landmarks 1′–6′. (B) Silver stained NEPHGE gel with protein spots corresponding to the non-specific Western signals of (A) numbered 1–6 and the spot corresponding to the Western signal from mAb ab52 (encircled). (C) Western Blot developed with mAbs ab52 and ab49 showing that in the Eps15 null mutant *eps15*$^{\Delta 29}$ the ~100 kDa signal characteristic for ab52 in the wild type (CS) is absent. CSP recognized by mAb ab49 was used as a loading control.
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Table 2. Proteins with cumulative Mascot scores>300 identified in the gel pieces boxed in lane 2 and 3 of Fig. 4.

Accession	Protein	MW	Cumulative Mascot	Sequence coverage
			scoring lane 2/3	lane 2/3
Q9XTL9	Glycogen phosphorylase	97334	746/916	22/24
P27619	Dynamin *	98147	678/249	16/6
Q9W0E4	Puromycin sensitive aminopeptidase *	99891	612/−	11/−
A4V310	Cheerio, Filamin *	93171	594/364	11/8
Q7KN62	Transitional endoplasmic reticulum ATPase TER94	89545	423/704	11/13
P91926	AP-2 complex subunit alpha/alpha adaptin*	106352	411/−	9/−
Q8MMD3	Epidermal growth factor receptor pathway substrate clone 15*	119761	348/593	9/10
P13060	Elongation factor 2 *	95424	310/−	9/−
Q9VAY2	Glycoprotein 93	90296	258/371	8/10
Q9VUC1	Hsc70Cb, isoform A	89016	257/471	9/12
Q9V9U3	CG1910 *	51670	530/−	23/−
P41073	Zinc finger protein on ecdysone puffs	78570	136/386	6/11

Protein identifications marked with asterisks (*) featured several different accession entries which belonged to the same protein but isoforms were not resolvable. Accessions are given in Swiss-Prot format.

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Subcellular fractionation of ab52 antigen

Upon subcellular fractionation as described in Materials and Methods, the mAb ab52 antigen remained in the cytosolic supernatant (S2), indicating that it is soluble under the conditions of homogenization (Fig 2B). CSP (cysteine string protein) detected by mAb ab49 [26], remains in the pellet, which represents the total membrane fraction, thus demonstrating the effectiveness of the fractionation.

Migration pattern of antigens recognized by aa2 and ab52 on 2DE

Since aa2 and ab52 seemed to recognize the same antigen on 1DE and since the antigen recognized by ab52 was found to be a soluble, cytosolic protein, we used 2DE to resolve this protein as a distinct spot and compared the Western blots signals of the 2DE profile for both mAbs aa2 and ab52. CS fly head homogenates were resolved by 2DE (IEF/SDS-PAGE) followed by partial Western blot of the same gel consecutively on two separate membranes to get duplicate blots of the same 2DE separation profile. Development of the blots incubated with aa2 and ab52 separately, revealed signals for both antibodies as a single, distinct spot with identical patterns having M_r ~100 kDa in the pI range 6–7 as shown in Fig 3. This further indicated that both mAbs indeed recognize the same antigen.

Enrichment of the antigen of mAb aa2 by immunoprecipitation

mAb aa2 was used to enrich the target antigen it recognized by immunoprecipitation (IP) to facilitate its identification by MS. Homogenized fly heads were subjected to immunoprecipitation using serum-free supernatant and protein G beads as described in Materials and Methods. Identical aliquots of the S1 input to the IP, of the proteins eluted from the beads by SDS buffer, and of homogenate from 3 fresh heads were loaded in two gels, one was Coomassie stained and one was blotted and developed with mAb aa2. Among numerous bands in the Coomassie-stained gel, one band appeared to correspond to the mAb aa2 signal in the Western blot (boxed in Fig. 4). Note that in lane 3′ compared to lane 2′ of the gel a tenfold higher amount of protein was loaded, leading to the recognition of proteolytic degradation products of the aa2 antigen in the blot lane 3′.

Enrichment of the antigen of mAb ab52 by NEPHGE followed by SDS PAGE

mAb ab52 was found to be an IgM, making its application in IP for enrichment of its target antigen unsuitable. However the soluble nature of the antigen allowed us to resolve it as a distinct spot by 2DE. Thus the cytosolic supernatant (S2) was subjected to

Table 3. Proteins identified in the gel piece encircled in Fig. 5B.

Accession	Protein	MW	Cumulative Mascot scoring	Sequence Coverage
Q8MMD3	Epidermal growth factor receptor pathway substrate clone 15*	119761	1039	35
Q9VUC1	Hsc70Cb *	89016	483	13
P16568	Protein Bicaudal D	89127	115	3

doi:10.1371/journal.pone.0029352.t003

Non-Equilibrium pH Gradient gel Electrophoresis (NEPHGE) followed by SDS-PAGE as described in **Methods.** Western blotting (Fig. 5A) was done for only 20 min, to transfer only part of the total protein content of the gel onto the membrane, while retaining the rest of it in the gel, which was later visualized by MS-compatible silver staining (Fig 5B). The blot was incubated with mAb ab52 and developed with a long exposure of 2 hours to obtain the specific signal for ab52 and in addition, some non-specific spots (Fig. 5A). The non-specific spots on the Western blot were numbered 1′–6′ and their corresponding spots on the gel were numbered 1–6. These pairs of spots were used to align the blot to the silver-stained gel and thus pinpoint the silver-stained spot (circled in Fig. 5B) corresponding to the mAb ab52 antigen signal in the Western blot.

Mass spectrometric identification of the antigen candidates

The bands cut out from the gel of the IP experiment (boxed in Fig. 4) were analyzed by mass spectrometry after proteolytic digestion and signals were matched. As is common for 1D-PAGE separations, several different *Drosophila* proteins were identified in each band, of which 12 had a score>300 (Table 2). Three of the identified proteins are known components of the peri-active zone of *Drosophila* synapses and thus in view of the localization data are preferred candidates: Eps15, shibire (dynamin) and α-adaptin. Furthermore, Dap160 was identified as a low scoring but significant component of lane 3 (score 83, 3 peptides matched). For a detailed list of all identified proteins please refer to table S1. Dap160/intersectin is a prominent binding partner of Eps15 [35]. This suggests that one of these proteins could be the desired antigen while the other three may have been co-immunoprecipitated and, due to similar molecular weights, enriched in the cut-out gel pieces.

The gel piece analyzed from the NEPHGE 2D gel (encircled in Fig. 5B) contained proteins which could be matched to three entries of the of the *Drosophila* proteome (Table 3). Since Hsc70Cb and Bicaudal D have not been reported to match to the synaptic neuropil or the synaptic localization of the mAb ab52 antigen these proteins presumably are false positive hits leaving Eps15 as a strong candidate. We next demonstrated that a Western blot of an adult $eps15^{\Delta 29}$ null mutant escaper produced no signal with mAb ab52 but normal SAP47 loading control signals (Fig. 5C) and that $eps15^{\Delta 29}$ null mutant larvae showed no synaptic neuropil staining with mAbs aa2 or ab52 (Fig. 6A,B). Note that with mAb ab52 there is a gradient of staining intensity from the periphery to the center of the neuropil, indicating the (large) IgM penetrates whole mounts less easily than the IgG aa2. Finally, we show that the immunohistochemical signals generated within synaptic boutons of larval nerve-muscle preparations by mAb aa2 exactly match the signals generated by anti-Eps15 antiserum (Fig. 6C).

We thus conclude that mAbs aa2 and ab52 of the Würzburg Hybridoma Library indeed recognize the same protein, Eps15 of *Drosophila*, a protein of the peri-active zone required for normal synaptic bouton development and synaptic vesicle recycling [34,35,45,46]. The two mAbs are of different isotypes produced by two distinct hybridoma lines. mAb aa2 being an IgG1 is more suitable for applications like whole mount stainings and immunoprecipitation (IgMs usually do not bind to protein-A or –G), while for immunostainings on sections or motor neuron terminals and Western blots mAb ab52 (being an IgM) is also suitable.

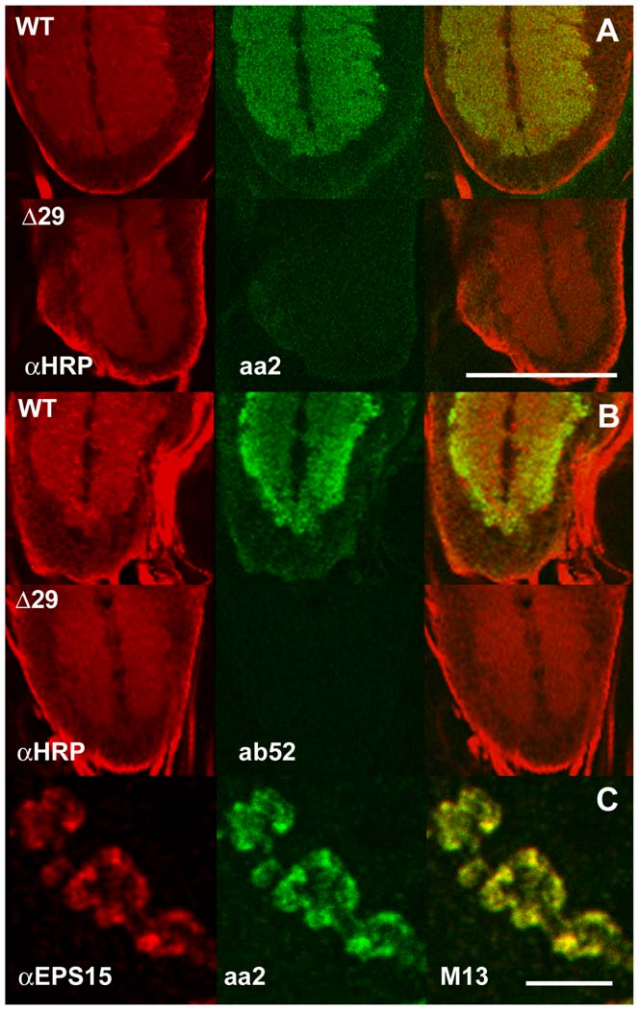

Figure 6. Verification of the prime candidate from MS, EPS15, as the antigen recognized by mAbs aa2 and ab52. Immunohistochemical staining of larval synaptic neuropil with mAbs aa2 (A) and ab52 (B) is present in wild type (WT) but absent in $eps15^{\Delta 29}$ null mutants (Δ29) and perfectly matches the distribution of Eps15 in synaptic boutons, here shown on muscle M13 (C). Scale bar in A for A and B: 100 μm; in C: 5 μm.
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Supporting Information

Table S1 Extended MS results for boxed bands from Figure 4 lane 2 and 3 and for encircled spot of 2DE gel in Figure 5B.
(XLS)

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Author Contributions

Conceived and designed the experiments: EB PH. Performed the experiments: JB EB YC PH UL CW. Analyzed the data: EB PH AH UL AS. Contributed reagents/materials/analysis tools: MCD. Wrote the paper: EB PH.

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Identification and Structural Characterization of Interneurons of the *Drosophila* Brain by Monoclonal Antibodies of the Würzburg Hybridoma Library

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Abstract

Several novel synaptic proteins have been identified by monoclonal antibodies (mAbs) of the Würzburg hybridoma library generated against homogenized *Drosophila* brains, e.g. cysteine string protein, synapse-associated protein of 47 kDa, and Bruchpilot. However, at present no routine technique exists to identify the antigens of mAbs of our library that label only a small number of cells in the brain. Yet these antibodies can be used to reproducibly label and thereby identify these cells by immunohistochemical staining. Here we describe the staining patterns in the *Drosophila* brain for ten mAbs of the Würzburg hybridoma library. Besides revealing the neuroanatomical structure and distribution of ten different sets of cells we compare the staining patterns with those of antibodies against known antigens and GFP expression patterns driven by selected Gal4 lines employing regulatory sequences of neuronal genes. We present examples where our antibodies apparently stain the same cells in different Gal4 lines suggesting that the corresponding regulatory sequences can be exploited by the split-Gal4 technique for transgene expression exclusively in these cells. The detection of Gal4 expression in cells labeled by mAbs may also help in the identification of the antigens recognized by the antibodies which then in addition to their value for neuroanatomy will represent important tools for the characterization of the antigens. Implications and future strategies for the identification of the antigens are discussed.

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† Deceased.

Introduction

The concept of identifiable neurons represents a unique feature of invertebrate neuroscience. Neuron identity in general is a consequence of differential cell-specific and temporal regulation of gene expression or other epigenetic mechanisms throughout development. Thus in principle it should be possible to label each individual neuron. Groups of neurons that express a common gene can be detected by localizing the corresponding mRNA by in-situ hybridization or they can be labelled by enhancer trap or gene trap techniques. Alternatively, antibodies can be used to label groups of neurons that contain a common gene product or a common metabolic product of cell-specific gene expression, like e.g. a certain neurotransmitter or a particular post-translational modification (PTM). Thus it is not surprising that antibodies against numerous known proteins, their metabolic products, or their PTMs are available. However, since a large percentage of human genes and their homologues in genetic model systems like *Drosophila* are still uncharacterized, an attractive alternative approach to the identification of cell-specifically expressed genes – and thereby characterization of those cells – is the generation of libraries of monoclonal antibodies against whole tissue homogenates. We have used this approach that originally was developed for *Drosophila* by the group of Seymour Benzer [1,2], and obtained antibodies against antigens located in neuronal compartments like the cell body, the axon, or the synaptic neuropil, and antibodies that selectively stained individual cells or cell types in the retina or in the brain [3]. In some cases it has been possible to identify the

proteins recognized by the 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 (reviewed in [4]). However, this approach "from antibody to gene" has worked so far for antibodies that recognize relatively abundant proteins like those found in all synapses, or, as in the case of the PDF precursor, by a candidate approach when the cells identified by the antibody were already known to contain a certain protein or peptide. Here we present a collection of mAbs that can be used to reproducibly identify small subsets of brain neurons and thus represent valuable tools for cellular neuroanatomy. These antibodies may attain particular importance when the same cells are independently labelled by antisera against known antigens, tissue in-situ hybridization, or by GFP expression in enhancer trap or gene trap lines. Recently, the number of lines expressing Gal4 in subsets of *Drosophila* brain neurons has been significantly increased by high-throughput approaches employing fragments of regulatory sequences of genes expressed in the nervous system [5,6] (see also ViennaTiles http://stockcenter.vdrc.at). Whenever Gal4 expression and staining by our mAbs overlap it may become possible to identify even rare cell-specific antigens and the genes responsible for their expression. In cases where a mAb identifies the same Gal4 expressing cells in different lines with otherwise non-overlapping expression patterns, the corresponding regulatory sequences can be used for a split-Gal4 approach [7] to transgene expression exclusively in these cells. Here we present the labelling patterns of ten mAbs and examples of co-expression of the unknown mAb antigens and GFP driven by Gal4 lines.

Materials and Methods

Fly strains

Canton S (CS) wild type was used throughout the experiments except when stated otherwise. The line 386Y contains a Gal4 enhancer trap P-element in the amontillado gene [8]. The stocks of the Janelia Gal4 collection used here were obtained from the Bloomington stock center and are listed in table 1 (5 digit identifier).

Generation and characterization of monoclonal antibodies

Hybridoma cell culture, monoclonal antibody production, and isotyping have recently been described [9].

Wholemount confocal fluorescence microscopy

Different authors contributing to the stainings in Figures 1–6 used slightly varying procedures for antibody staining of adult or larval whole-mount brains of *Drosophila*. BM (Figure 1A, B, D E, H) followed the detailed description given in [10]. The procedure used by MB and FE (Figure 1F, I, J) has been described in [11,12] and was followed with minor modifications: Brains were removed from the head capsule in PBS containing 0.5% Triton X-100 (PBS-0.5T) at room temperature (RT) and fixed for 1 h in 4% paraformaldehyde (PFA) in PBS-0.1T. They were washed five times in PBS for 10 minutes each, blocked in PBS-0.5T and 5% normal goat serum (NGS) for 3 h at RT or overnight at 4°C. The brains were incubated for 36 h at 4°C with the primary antibodies diluted as indicated in Table 1 in PBS-0.5T with 5% NGS, washed five times 10 min in PBS-0.5T at RT and incubated overnight at 4°C with two of the following secondary antibodies diluted in PBS with 5% Normal Goat Serum and 0.5% Triton X-100: Alexa Flour 488 goat anti-rabbit IgG (1:200), Alexa Flour 488 goat anti-mouse IgG (1:200), Alexa Flour 555 goat anti- rabbit IgG (1:200), Alexa Flour 555 goat anti-mouse IgG (1:200) (all from Life Technologies). Finally, the brains were washed 5×10 min in PBS-0.5T at RT and once in PBS-0.1T and mounted in Vectashield™ (Vector Laboratories) for fluorescence microscopy. The brains were examined by confocal microscopy (Leica TSC SPE All pictures were taken at 20×. BBR and MKS (Figures 1C, G, 2, 3, 4H-M, 5, 6F-H) followed the procedure described in [13] with some modifications: Brains of adults or wandering third instar larvae were dissected in PBS, fixed for 30 min – 1 h in 4% PFA in 0.04 M phosphate buffer, washed 3×5 min in PBS-0.3T, blocked at RT for 2 h in 5% NGS in PBS-0.3T, incubated in primary antibody (including chicken anti-GFP 1:1000) for 48–60 h on a horizontal shaker at 4°C, followed by 6×20 min washes in PBS-0.3T, incubation in secondary antibody as above at dilutions 1:400 (or Alexa Fluor 488 goat anti-chicken 1:400, Alexa Flour 568 goat anti-mouse IgG 1:400) for 12–24 h on a horizontal shaker at 4°C, 6×20 min washes in PBS-0.3T at RT and mounting in Vectashield™. The brains were imaged using confocal microscope Olympus FV1000-IX81 (Tokyo, Japan). Staining patterns clearly similar to the ones shown in Figure 1 were obtained with: ab47 five times in seven stained brains (5/7), ab135 (3/4), ab158 (4/5), ca8 (12/14), fb20 (4/4), nb139 (10/11), nb168 (3/3), nb169 (7/7), nc24 (9/11), nc53 (12/14).

Cryosections

The procedure for immunostaining of cryosections has recently been described [9]. Dilutions of antibodies used: nb169 1:2; anti-serotonin (Millipore AB938) 1:500; anti-GFP (Life Technologies A11122) 1:3000; DAB staining with Vectastain kit (mouse) according to manufacturer's protocol. Secondary antibodies for immuno-fluorescence: Alexa-488 anti-rabbit (goat) 1:400 (Life Technologies A11008); Dylight 549 anti-mouse (donkey) 1:200 (Jackson Laboratories 715-505-151).

Results and Discussion

Figure 1 depicts 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) clearly 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 arborisations in tangential layers of the medulla. Each staining pattern is characteristic of the particular antibody and was obtained at least three times (Table 1). Three of the antibodies (ca8, nb168, nb169) also produced reliable stainings in the larval nervous system (Figures 2, 4K-M).

The similarity of mAb nb168 staining (Figure 1G) to the known distribution of pigment dispersing factor (PDF) in *Drosophila* [14,15] is obvious. Indeed, double stainings of wholemount brains with mAb nb168 and an antiserum against PDH [16] (which cross-reacts with PDF) reveal that the same neurons are labelled by the two antibodies (Figure 3A1-3). Mutation of the *Pdf* gene eliminates mAb nb168 staining and thus demonstrates that mAb nb168 very likely recognizes PDF or its precursor protein (data not shown). Since the hybridoma clones secreting mAb nb168 and mAb nb33, which recognizes PDF precursor protein but not the mature PDF [4], 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 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

Table 1. 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)).

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)	''	R49C04 [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)	''	R49C04 [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]	(+)
''	(11:20)	''	R49C04 [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)	''	R49C04 [50415]	(+)
nc53	(1:5)	IgM	anti-PDH	–
''	(1:5)	''	anti-NPF	–
''	(1:5)	''	anti-PER	–

doi:10.1371/journal.pone.0075420.t001

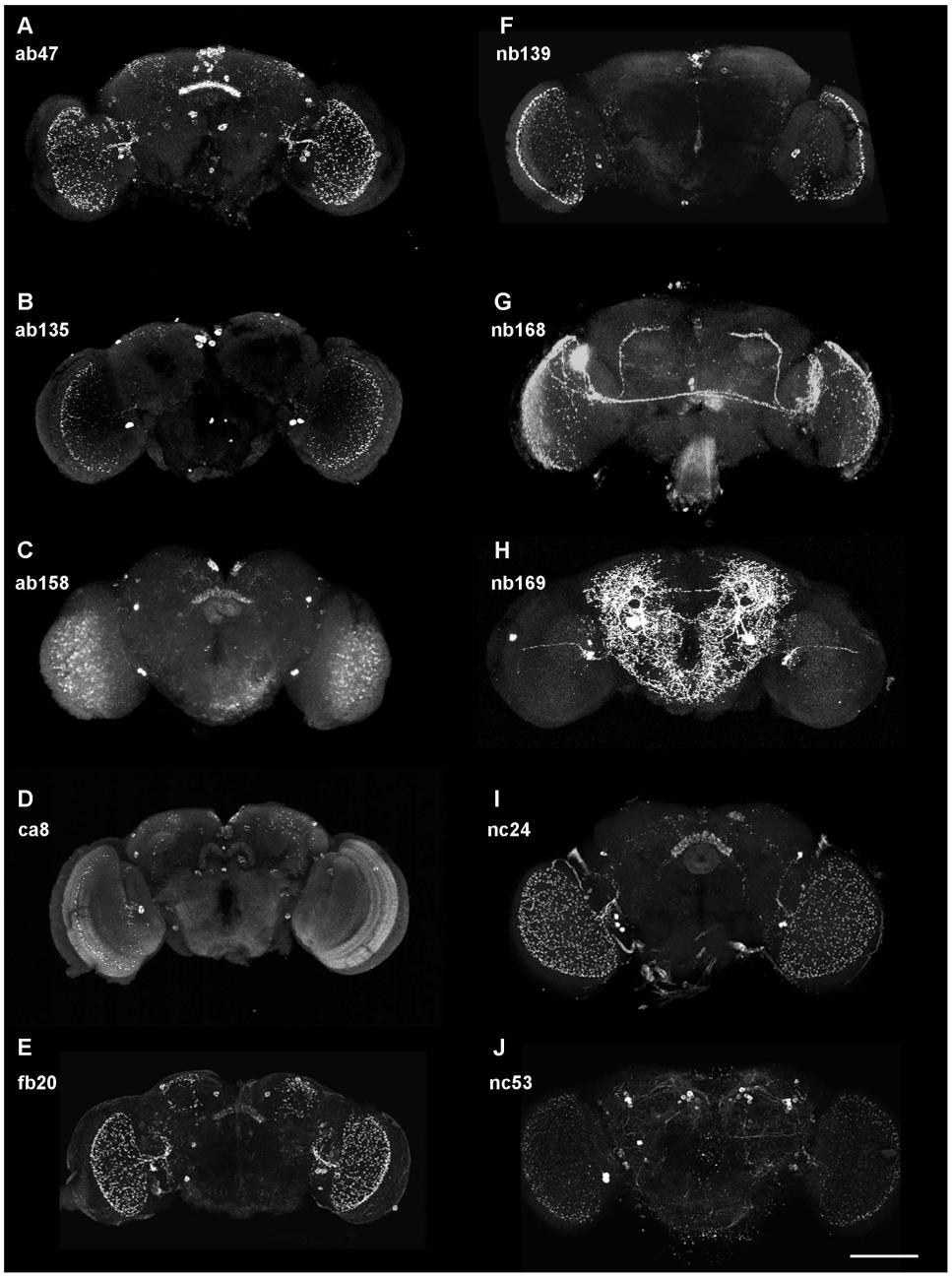

Figure 1. Z-projections of confocal stacks of whole-mount adult *Drosophila* brains (frontal view) stained with ten different monoclonal antibodies of the Würzburg hybridoma library. Note in (H) that the bright spot at the left rim of the projection is due to a staining artefact from outside the brain. Scale bar 100 μm.
doi:10.1371/journal.pone.0075420.g001

addition to a small number of cell bodies in the dorso-lateral protocerebrum and the visual system (Figures 1H, 3B2). The distribution of the corresponding antigen was investigated in more detail. Horizontal diamino benzidine (DAB)-stained frozen sections reveal two immuno-positive cell bodies of the lateral protocerebrum with some of their arborisations at the dorso-ventral level of the fan-shaped body (Figure 4A), 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 4B), as well as the labelling of cell bodies in the visual system with fine arborisations and varicosities in the accessory medulla, in a tangential layer of the medulla and, conspicuously, in the cell body layer of the lamina (Figure 4C, D). These features were partly reminiscent of stainings using antisera against serotonin [17]. Thus we wanted to determine if the cells identified by mAb nb169 contain serotonin (5HT). Fluorescent double staining with anti-5HT serum and nb169 (Figure 4E, 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 arborisations in the cellular cortex of the lamina (compare Figure 4C with F, G). This group of cells has been denoted as lamina tangential or wide-field neurons (Lat [18] or Lawf [19]).

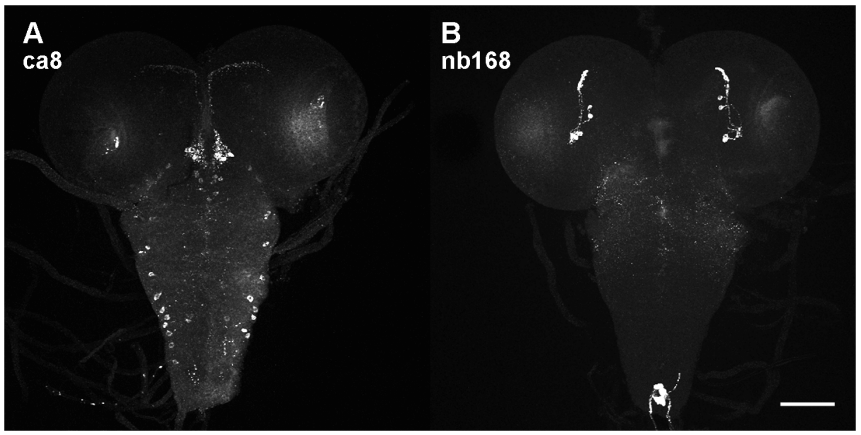

Figure 2. Z-projections of confocal stacks of larval nervous systems stained with two of the monoclonal antibodies shown in Figure 1. Larval staining of nb169 is shown in Figure 4K-M, the other seven antibodies did not produce reliable staining in the larval nervous system. Scale bar 100 μm.
doi:10.1371/journal.pone.0075420.g002

(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 [20,21,22] and are assumed to serve a paracrine function [23]. In the larval ventral ganglion mAb nb169 selectively labels five neurons on each side (Figure 4K-M). Screening patterns of GFP expression driven by subsets of the Janelia Gal4 collection [5,6] (http://flweb.janelia.org/cgi-bin/flew.cgi) 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

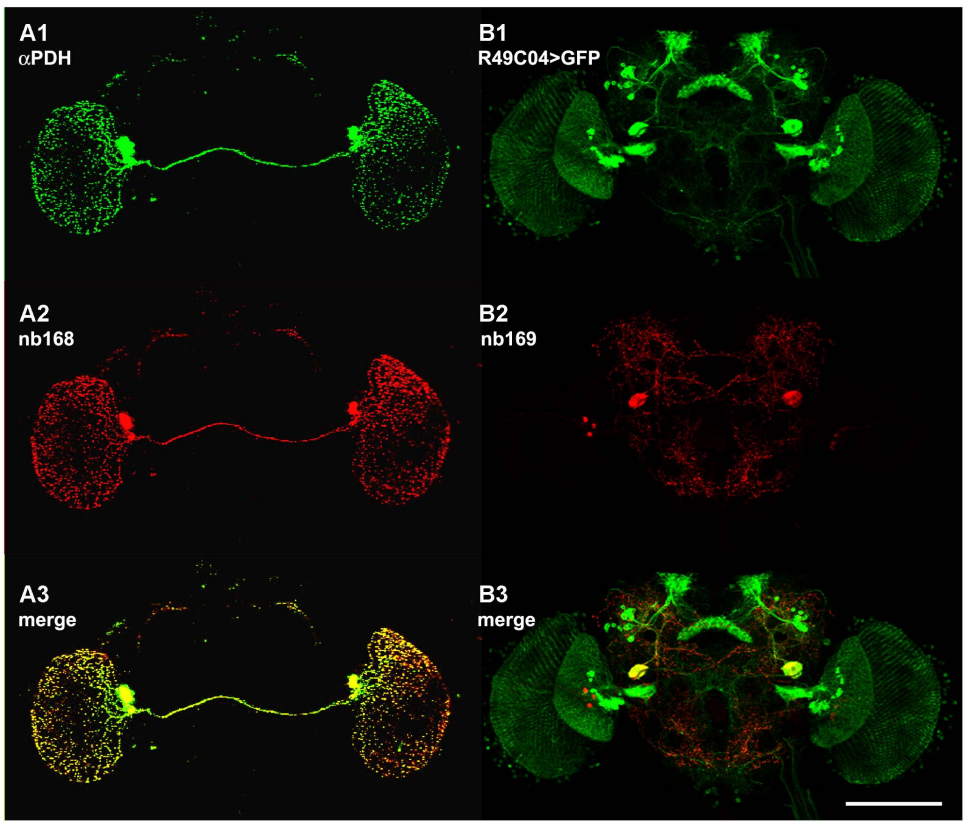

Figure 3. Z-projections of confocal stacks of whole-mount brains double-stained with mAb nb168 and anti-PDH antiserum (A1-3) or mAb nb169 and anti-GFP in a fly expressing GFP driven by Janelia Gal4 line R49C04 (B1-3). The perfect co-localization in A3 indicates that nb168 could recognize *Drosophila* PDF or its precursor protein, whereas co-localization in B3 is restricted to the two very large cells stained by nb169 in the posterior protocerebrum. Scale bar 100 μm.
doi:10.1371/journal.pone.0075420.g003

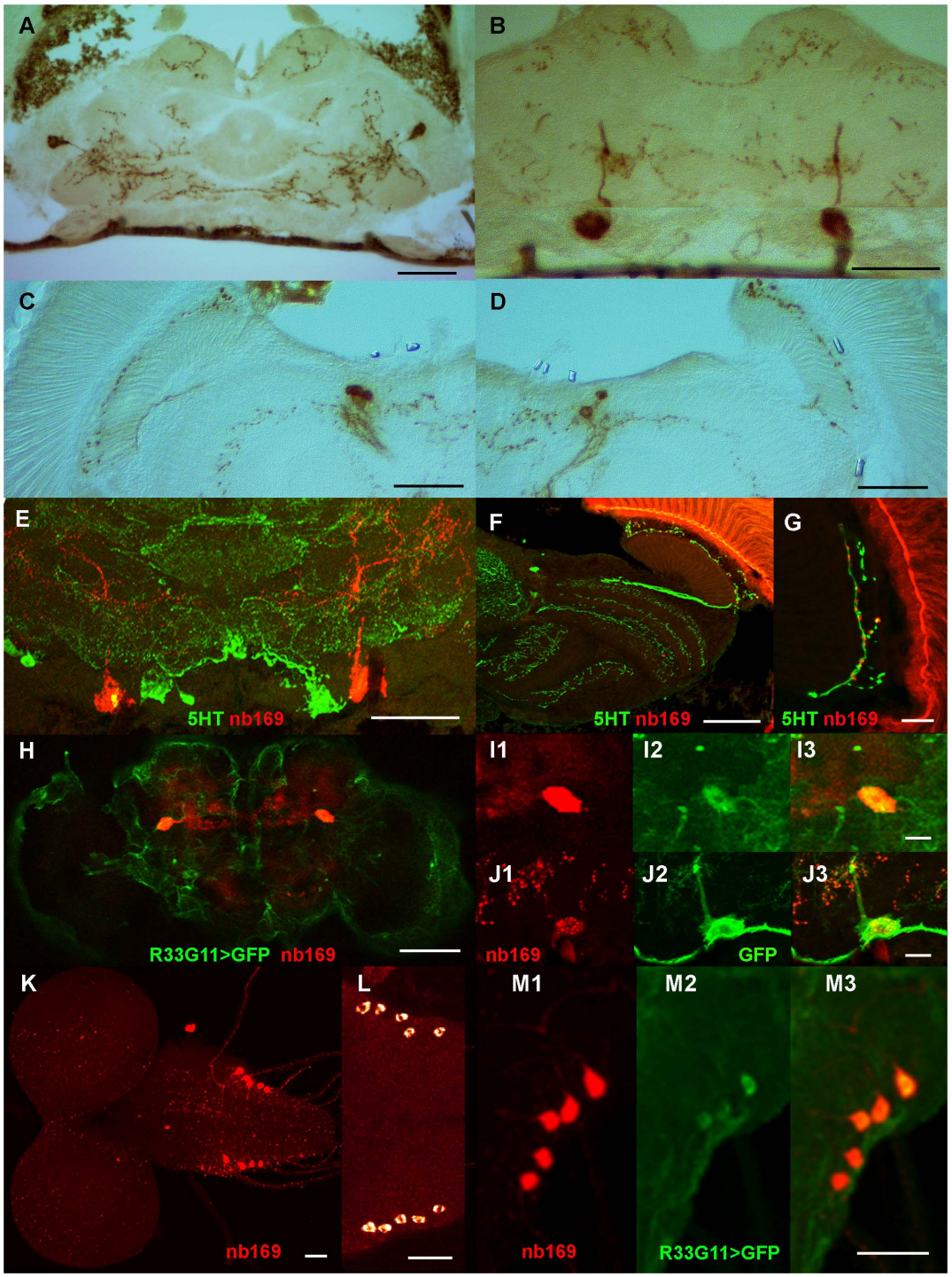

Figure 4. Staining details for mAb nb169. (A-D) The horizontal cryo-sections stained with mAb nb169 and DAB development show the cell bodies in the lateral protocerebrum at the dorso-ventral level of the fan-shaped and ellipsoid bodies (A), the giant neurons in the caudal cellular cortex with their main neurite (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 R33G11 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 R33G11-Gal4 (M, enlargement of K). Scale bars in A-F, H, K-M: 50 µm; in G, I, J: 10 µm.
doi:10.1371/journal.pone.0075420.g004

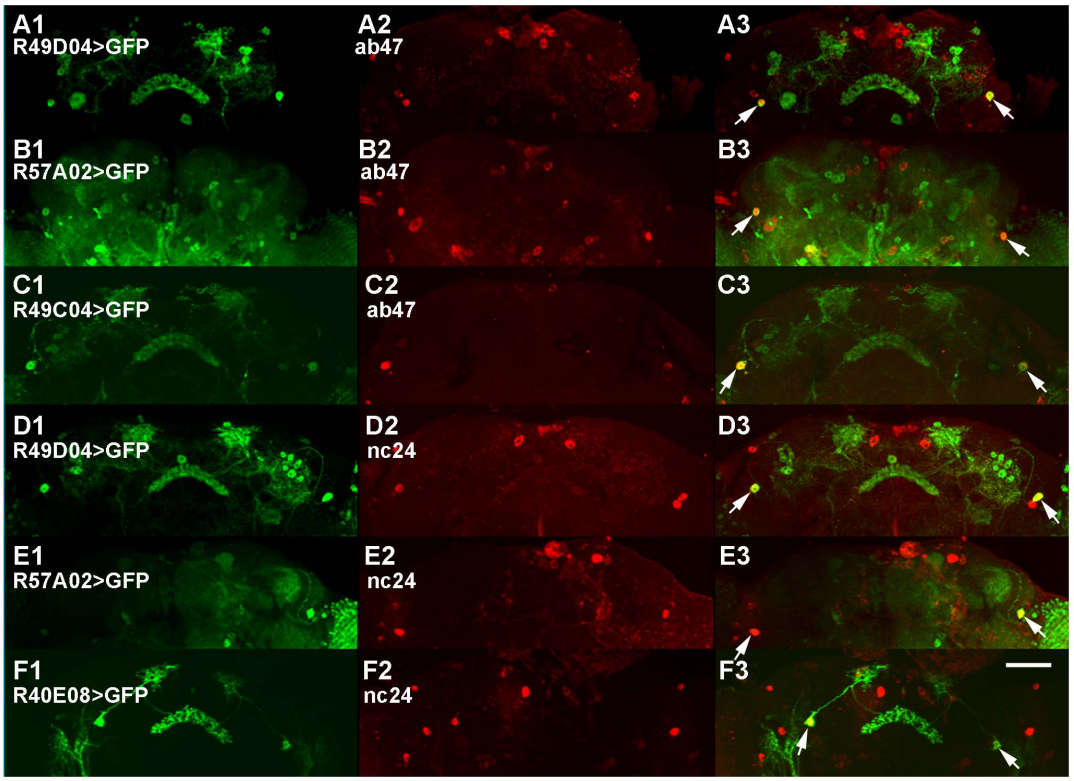

Figure 5. Examples for the co-localization of staining by mAbs ab47 (A-C) and nc24 (D-F) and GFP driven by selected Janelia Gal4 lines. Due to the variability of cell body positions in different individuals it is only suggestive that the Gal4 expression patterns overlap. Scale bar 100 μm.
doi:10.1371/journal.pone.0075420.g005

the same two perikarya (Figure 3B1-3 for line R49C04, Figure 4H, 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 3B1, 4H, J2). The co-localization of the nb169 antigen and GFP driven by line R33G11 is also seen in the larval ventral nerve cord (Figure 4M1-3). GFP driven by line R49C04 in larvae, on the other hand, does not co-localize with nb169 antigen. 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. Obviously, it will be fascinating to learn more about the neurons and the antigen recognized by mAb nb169. The discovery of two Gal4 lines with very different expression patterns overlapping apparently only in the pair of giant neurons labelled by mAb nb169 suggests that it might be possible to generate a line expressing a transgene of choice only in these two giant neurons by split-Gal4 technique [7]. The co-localization results of all tested mAb/Gal4 pairs are summarized in Table 1. Figure 5 shows examples for co-localization of GFP expression driven by Janelia Gal4 lines with stainings by mAb ab47 (Figure 5A-C) and nc24 (Figure 5D-F).

Strategies towards identifying the antigens for mAbs

Earlier attempts to identify the antigen of mAb nb33 [4] by extensive screening of a cDNA expression library had failed (K. Zinsmaier, unpublished), whereas screening of the same library with mAbs recognizing synaptic proteins was successful [24,25]. Also, for none of the antibodies described here a reliable Western blot signal from brain homogenate could be obtained, defeating any attempts to identify the antigens by protein purification and mass spectrometry [26,9]. Thus the classical approaches to the identification of antigens for mAbs cannot be applied. We therefore here present the distribution of the antigens in the *Drosophila* brain and make them available for use in neuroanatomy and for comparison with cell identification by other techniques mentioned above. We first selected a small number of Gal4 lines from the Janelia collection [5,6] that showed similarity between their expression patterns revealed by GFP and the staining patterns of our mAbs in the adult fly brain. We then investigated by double stainings whether there was any overlap detectable. Table 1 summarizes the results. For mAb nb169 and Gal4-lines R33G11 and R49C04 we find clear co-localization of GFP and antibody binding as described above. Frequently, however, in spite of apparent similarity of staining patterns in the neuropil, e.g. in the fan-shaped body or in horizontal layers of the medulla, 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 the Gal4-line indicated ((+) in Table 1). The stainings shown in Figure 5 suggest that Gal4 expression from different Gal4 lines may overlap as their GFP reporter is observed in mAb-stained cells at similar positions. However, the positions of cell bodies are somewhat variable from fly to fly such that additional evidence for the overlap will be required.

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. In general, neuropeptides are cleaved from precursor proteins by proteolytic enzymes. Thus many neuropeptide-containing cells can be labelled by GFP driven by

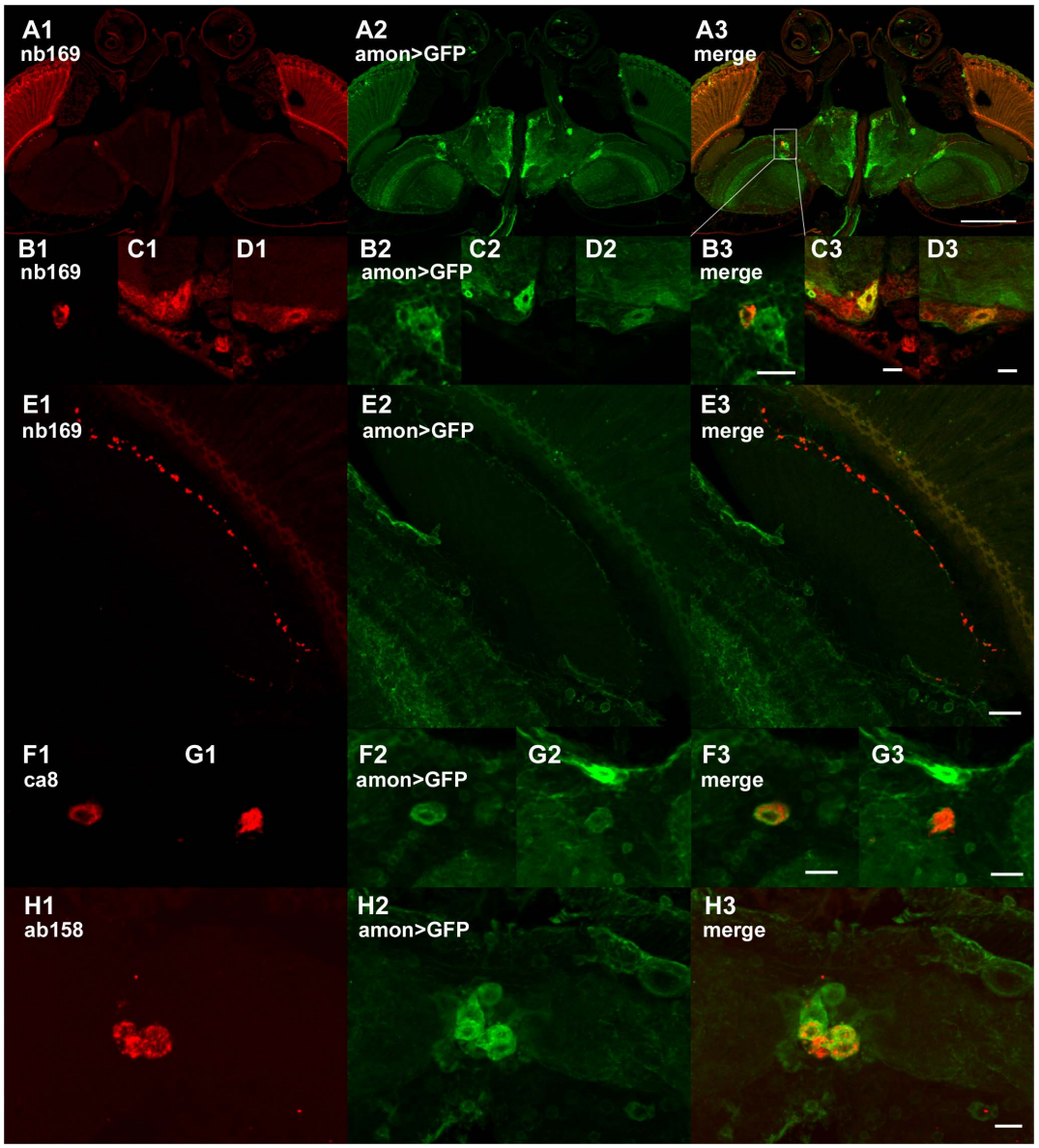

Figure 6. The antigens detected by mAbs nb169, ca8, and ab158 may be related to neuropeptides. (A-E) The cell stained by nb169 (red) (A, enlarged in B), and the giant cells in the caudal protocerebrum (C and D from different sections) all are positive for GFP (green) expressed under the control of 386Y 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.
doi:10.1371/journal.pone.0075420.g006

386Y-Gal4, a Gal4 enhancer trap construct under regulatory control of the prohormone convertase 2 gene (*amon*) [8,27,28]. Figure 6 shows the co-localization of nb169, ca8, and ab158 antibody staining with amon>GFP expression, supporting a speculation that these three mAbs may perhaps bind to neuropeptide-related antigens.

The interpretation of co-localization (or its lack) of any Gal4-driven GFP expression with a staining pattern obtained with an antibody against an unknown antigen remains difficult. Several mechanisms may be responsible for discrepancies between the Gal4-driven GFP expression patterns and the antigen distributions even if the antigen is the product of the corresponding gene. First, enhancer, silencer, and promoter elements may be widely separated in a regulatory region of a gene and usually combine in a complex, non-additive fashion to produce the final expression pattern. Second, the GFP of the mCD8-GFP construct expressed by Gal4 is targeted to all membranes such that the entire cell is labelled, whereas the antigen recognized by the mAb may be specifically and efficiently targeted to a specific cell compartment and might be detected by the antibody only there. Third, the antigen recognized by the mAb may perhaps be a processed or post-translationally modified version of a gene product and therefore be present only in a subset of the cells expressing the gene. Thus, for each of the mAbs shown here considerable efforts

will be necessary in any attempt to identify the antigen. However, in cases where neuronal cell bodies identified by a mAb can be labelled in vivo by GFP in principle it can become feasible to separate these cell bodies and isolate their mRNAs for gene expression profiling [29] or generation of a cell-specific cDNA expression library which can be screened with the mAb. This approach to the identification of unknown antigens labelled by monoclonal antibodies only in a few cells in the brain can now be attempted e.g. with mAb nb169.

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Author Contributions

Conceived and designed the experiments: VR KVR MR DR CF AH EB. Performed the experiments: BBR MB PH FE MKS BM FE EB . Analyzed the data: KVR MR DR CF AH EB. Contributed reagents/materials/analysis tools: AH CW. Wrote the paper: EB.

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