“Bruchpilot“- Molecular And Functional Characterization Of A Novel Active Zone Protein At The *Drosophila* Synapse

Dissertation zur Erlangung des
Naturwissenschaftlichen Doktorgrades
der Bayerischen Julias-Maximilians-Universitäte Würzburg

vorgelegt von

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Würzburg, 2005

Eingereicht am: .....

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Tag des Promotionskolloquiums: .....

Doktorurkunde ausgehändigt am: .....….

I N D E X

F I G U R E S

T A B L E S

ZUSAMMENFASSUNG

Die chemische Signalübertragung an Synapsen ist ein komplexer Prozess mit zentraler Bedeutung für die Funktion von Nervensystemen. Man nimmt an, dass er auf einem Zusammenspiel hunderter verschiedener Proteine beruht. Diverse Synopsenproteine haben sich für die Neurotransmission als relevant erwiesen und viele davon sind in der Evolution hoch konserviert, was einen universalen Mechanismus der Neurotransmission wahrscheinlich macht.

Dieser Prozess ist in zahlreiche aufeinander folgende Schritte unterteilt, wie die Neurotransmitteraufnahme in Vesikel, den Transport von Vesikeln in die Nähe von Calciumkanälen, die Ausbildung einer Fusionspore zur Transmitterausschüttung und schließlich die Wiederaufnahme von Vesikeln durch Endozytose. Jeder dieser Teilschritte wird momentan gezielt erforscht und spielt für sich genommen eine zentrale Rolle für das Verständnis des gesamten Prozesses.

Die Calcium-induzierte Transmitterausschüttung findet an spezialisierten Membranstrukturen der Synapsen statt, den aktiven Zonen. Diese sind hoch organisierte, elektronendichte Gitterstrukturen und bestehen aus verschiedenen Proteinen, die den synaptischen Vesikeln bei der Verlagerung in die Nähe von Calciumkanälen behilflich sind. Alle Proteinmodule, die für diese Prozesse nötig sind, scheinen eng aneinandergereiht an den aktiven Zonen vorzuliegen. Nur von wenigen konnte bisher bei Vertebraten die Funktion an der aktiven Zone charakterisiert werden.

Ein Fokus der Arbeitsgruppe, an der diese Doktorarbeit durchgeführt wurde, besteht in der Charakterisierung des molekularen Aufbaus der Synapse von *Drosophila*. Die Taufliege ist aufgrund eines reichen Angebots höchsteffektiver genetischer Methoden und vielfältiger Verhaltensparadigmen ein exzellentes Modellsystem, um die neuronale Signalübertragung zu untersuchen. Monoklonale Antikörper (MAKs) aus einer Hybridomabank gegen das *Drosophila* Gehirn werden standardmäßig verwendet, um neue Gehirnproteine mittels der „reverse genetics“- Methode zu identifizieren. Dazu wird der entsprechende genetische Lokus charakterisiert und eine detaillierte Untersuchung der Proteinfunktion initiiert. Diese Vorgehensweise war besonders hilfreich bei der Identifizierung von Synapsenproteinen, die bei der „forward genetics“-Methode aufgrund des Fehlens eines beobachtbaren Phänotyps übersehen würden. Proteine wie CSP, Synapsin und Sap47 wurden so gefunden und charakterisiert.

MAK nc82 stammt aus dieser Hybridomabank und wird in vielen Labors als allgemeiner Neuropilmarker aufgrund seiner hervorragenden Färbungseigenschaften in Gehirnpräparaten verwendet. Doppelfärbungen der larvalen neuromuskulären Synapse mit dem Antikörper nc82 in Kombination mit anderen prä- und postsynaptischen Markern deuteten stark auf eine Lokalisierung des Antigens an der aktiven Zone hin.

Die Synapsenarchitektur von *Drosophila* ist auf der ultrastrukturellen Ebene gut verstanden. Jedoch sind die molekularen Details vieler Synapsenkomponenten, besonders die der aktiven Zone, nicht bekannt. Die vermutete Lokalisierung des nc82 Antigens an der aktiven Zone war daher der Ansatzpunkt, eine biochemische Charakterisierung zu initiieren und das entsprechende Gen zu identifizieren.

In der vorliegenden Arbeit wird durch 2-D Gelelektrophorese und Massenspektrometrie gezeigt, das das nc82 Antigen ein neues Protein der aktiven Zone ist, welches von einem komplexen Genlokus auf Chromosom 2R kodiert wird. Durch RT-PCR wurde gezeigt, dass die Exons von drei offenen Leserastern, die bisher als getrennte Gene annotiert wurden, ein Transkript von mindestens 5,5 kb Länge kodieren. Northern Blots ergaben ein deutliches Signal bei 11 kb und ein schwächeres bei 2 kb. Das von dem 5,5 kb Transkript resultierende Protein ist hoch konserviert in der Gruppe der Insekten und weist an seiner N-terminalen Domäne eine signifikante Homologie zu den bisher beschriebenen Vertebratenproteinen der aktiven Zone ELKS/ERC/CAST auf. Bioinformatische Analysen sagen „coiled-coil“ Domänen vorher, die über die gesamte Sequenz verteilt sind. Dies deutet stark auf eine Funktion bei der Organisation oder der Aufrechterhaltung der präsynaptischen Struktur hin. Die große C-terminale Region ist zwar bei Insekten hoch konserviert, zeigt aber keine eindeutige Homologie zu Proteinen von Vertebraten.

Für die Funktionsanalyse dieses Proteins wurden transgene Fliegen, die UAS-RNAi Konstrukte in ihrem Genom tragen und durch entsprechende GAL4-Linien getrieben werden können, freundlicherweise von der kollaborierenden Arbeitsgruppe von S. Sigrist (Göttingen) zur Verfügung gestellt.

Der pan-neuronale „knock-down“ des nc82 Antigens durch transgene RNAi-Expression führt zu embryonaler Letalität. Eine schwächere RNAi-Expression führt bei adulten Fliegen zu Verhaltensdefekten, wie instabilem Flug und beeinträchtigtem Laufverhalten. Aufgrund dieser Phänotypen, die in den ersten „knock-down“ Studien beobachtet wurden, wurde das Gen „*bruchpilot*“ (*brp*) und das zugehörige Protein „Bruchpilot“ (BRP) genannt. Die pan-neuronale, sowie die retinaspezifische Reduktion des Proteins führt zu einem Verlust der ON und OFF Transienten des Elektroretinogramms, was auf

nichtfunktionelle Synapsen hindeutet. Die retinaspezifische Reduktion des Proteins hat eine Beeinträchtigung der optomotorischen Reaktion zur Folge. Außerdem scheint auf der ultrastrukturellen Ebene die Bildung der charakteristischen T-förmigen „ribbons“ der aktiven Zonen beeinträchtigt zu sein, jedoch ohne signifikante Veränderungen der Gesamtarchitektur der Synapse (in Kollaboration mit E. Asan).

Von Basson, einem Protein der aktiven Zone bei Vertebraten, ist bekannt, dass es an der Anheftung der synaptischen „ribbons“ an den aktiven Zonen beteiligt ist. Es fungiert als Adapter zwischen RIBEYE und ELKS/ERC/CAST, zwei weiteren Proteinen der aktiven Zone. Die Mutation von Bassoon hat zur Folge, dass die synaptischen „ribbons“ frei im Zytoplasma treiben. Für Bassoon ist kein homologes *Drosophila*-Protein bekannt. Die Reduktion von BRP bedingt ebenfalls ein Fehlen befestigter „ribbons“ an der aktiven Zone. Dies könnte auf eine Art Adapterfunktion von BRP hindeuten. Jedoch hat das Fehlen von BRP zusätzlich zum strukturellen Phänotyp auch deutliche Verhaltensabnormalitäten und starke physiologische Beeinträchtigungen zur Folge. Eine noch stärkere Reduktion bedingt außerdem embryonale Lethalität, wohingegen Mausmutanten ohne Bassoon lebensfähig sind. Daraus ergibt sich, dass BRP eine weitere, wichtige Rolle während der Entwicklung und für die Funktion von Synapsen bei *Drosophila* und möglicherweise auch bei anderen Insekten einnimmt. Es muss aber noch geklärt werden, auf welche Weise BRP die synaptische Signalübertragung reguliert und welche anderen Proteine in diesem BRP-abhängigen Pfad involviert sind. Derartige Studien werden mit Sicherheit in der Zukunft eine bedeutende Rolle spielen.

SUMMARY

Chemical neurotransmission is a complex process of central importance for nervous system function. It is thought to be mediated by the orchestration of hundreds of proteins for its successful execution. Several synaptic proteins have been shown to be relevant for neurotransmission and many of them are highly conserved during evolution- suggesting a universal mechanism for neurotransmission.

This process has checkpoints at various places like, neurotransmitter uptake into the vesicles, relocation of the vesicles to the vicinity of calcium channels in order to facilitate Ca^{2+} induced release thereby modulating the fusion probability, formation of a fusion pore to release the neurotransmitter and finally reuptake of the vesicles by endocytosis. Each of these checkpoints has now become a special area of study and maintains its own importance for the understanding of the overall process.

Ca^{2+} induced release occurs at specialized membrane structures at the synapse known as the active zones. These are highly ordered electron dense grids and are composed of several proteins which assist the synaptic vesicles in relocating in the vicinity of Ca^{2+} channels thereby increasing their fusion probability and then bringing about the vesicular fusion itself. All the protein modules needed for these processes are thought to be held in tight arrays at the active zones, and the functions of a few have been characterized so far at the vertebrate active zones.

Our group is primarily interested in characterizing the molecular architecture of the *Drosophila* synapse. Due to its powerful genetics and well-established behavioural assays *Drosophila* is an excellent system to investigate neuronal functioning. Monoclonal antibodies (MABs) from a hybridoma library against *Drosophila* brain are routinely used to detect novel proteins in the brain in a reverse genetic approach. Upon identification of the protein its encoding genetic locus is characterized and a detailed investigation of its function is initiated. This approach has been particularly useful to detect synaptic proteins, which may go undetected in a forward genetic approach due to lack of an observable phenotype. Proteins like CSP, Synapsin and Sap47 have been identified and characterized using this approach so far.

MAB nc82 has been one of the shortlisted antibodies from the same library and is widely used as a general neuropil marker due to the relative transparency of immunohistochemical whole mount staining obtained with this antibody. A careful observation of double stainings at the larval neuromuscular junctions with MAB nc82 and

other pre and post-synaptic markers strongly suggested an active zone localization of the nc82 antigen.

Synaptic architecture is well characterized in *Drosophila* at the ultrastructural level. However, molecular details for many synaptic components and especially for the active zone are almost entirely unknown. A possible localization at the active zone for the nc82 antigen served as the motivation to initiate its biochemical characterization and the identification of the encoding gene.

In the present thesis it is shown by 2-D gel analysis and mass spectrometry that the nc82 antigen is a novel active zone protein encoded by a complex genetic locus on chromosome 2R. By RT-PCR exons from three open reading frames previously annotated as separate genes are demonstrated to give rise to a transcript of at least 5.5 kb. Northern blots produce a prominent signal of 11 kb and a weak signal of 2 kb. The protein encoded by the 5.5 kb transcript is highly conserved amongst insects and has at its N-terminus significant homology to the previously described vertebrate active zone protein ELKS/ERC/CAST. Bioinformatic analysis predicts coiled-coil domains spread all over the sequence and strongly suggest a function involved in organizing or maintaining the structure of the active zone. The large C-terminal region is highly conserved amongst the insects but has no clear homologues in veretebrates.

For a functional analysis of this protein transgenic flies expressing RNAi constructs under the control of the Gal4 regulated enhancer UAS were kindly provided by the collaborating group of S.Sigrist (Göttingen).

A strong pan-neuronal knockdown of the nc82 antigen by transgenic RNAi expression leads to embryonic lethality. A relatively weaker RNAi expression results in behavioural deficits in adult flies including unstable flight and impaired walking behavior. Due to this peculiar phenotype as observed in the first knockdown studies the gene was named "*bruchpilot*" (*brp*) encoding the protein "Bruchpilot (BRP)" (German for crash pilot). A pan-neuronal as well as retina specific downregulation of this protein results in loss of ON and OFF transients in ERG recordings indicating dysfunctional synapses. Retina specific downregulation also shows severely impaired optomotor behaviour. Finally, at an ultrastructural level BRP downregulation seems to impair the formation of the characteristic T-shaped synaptic ribbons at the active zones without significantly altering the overall synaptic architecture (in collaboration with E.Asan).

Vertebrate active zone protein Bassoon is known to be involved in attaching the synaptic ribbons to the active zones as an adapter between active zone proteins RIBEYE and

ERC/CAST. A mutation in Bassoon results in a floating synaptic ribbon phenotype. No protein homologous to Bassoon has been observed in *Drosophila*. BRP downregulation also results in absence of attached synaptic ribbons at the active zones. This invites the speculation of an adapter like function for BRP in *Drosophila*. However, while Bassoon mutant mice are viable, BRP deficit in addition to the structural phenotype also results in severe behavioural and physiological anomalies and even stronger downregulation causes embryonic lethality. This therefore suggests an additional and even more important role for BRP in development and normal functioning of synapses in *Drosophila* and also in other insects. However, how BRP regulates synaptic transmission and which other proteins are involved in this BRP dependant pathway remains to be investigated. Such studies certainly will attract prominent attention in the future.

C h a p t e r 1 : I N T R O D U C T I O N

1.1 The Concept Of Neurotransmission

Fundamental concepts for learning, memory, cognition and behavior are based on the primary function of neurotransmission in particular at chemical synapses. Upon arrival of an action potential, synaptic vesicles release their content to the extracellular matrix of the synaptic cleft. Binding of the neurotransmitter molecules to receptors on the postsynaptic membrane represents an essential step in this form of communication. Chemical neurotransmission is a complex process and involves hundreds of proteins for its successful execution. With the aid of molecular biology, technical advances in electrophysiology and functional imaging significant progress has been made in our understanding of this process in detail.

Drosophila has attracted scientific enquiry for nearly a century due to valuable genetic tools and robust assays to probe deeper into the formation and functioning of this organism. The completion of genome sequencing and the availability of bioinformatics software have eased the search for genes of interest.

In the following few introductory chapters, an outline of the knowledge accumulated on the process of neurotransmission, on the synaptic vesicle cycle, and on presynaptic active zones will be given.

The functional point of contact between a nerve terminal and its target cell (this could be another nerve cell, an endocrinal organ or a muscle) is defined as a synapse (Sherrington, 1897). Hence, the part of the synaptic membrane where synaptic vesicles dock, fuse and release their content is the presynaptic membrane and the membrane that receives the vesicular content by means of receptors is the post- synaptic membrane. Both membranes are separated by the specialized extracellular matrix (fig.1.1a).

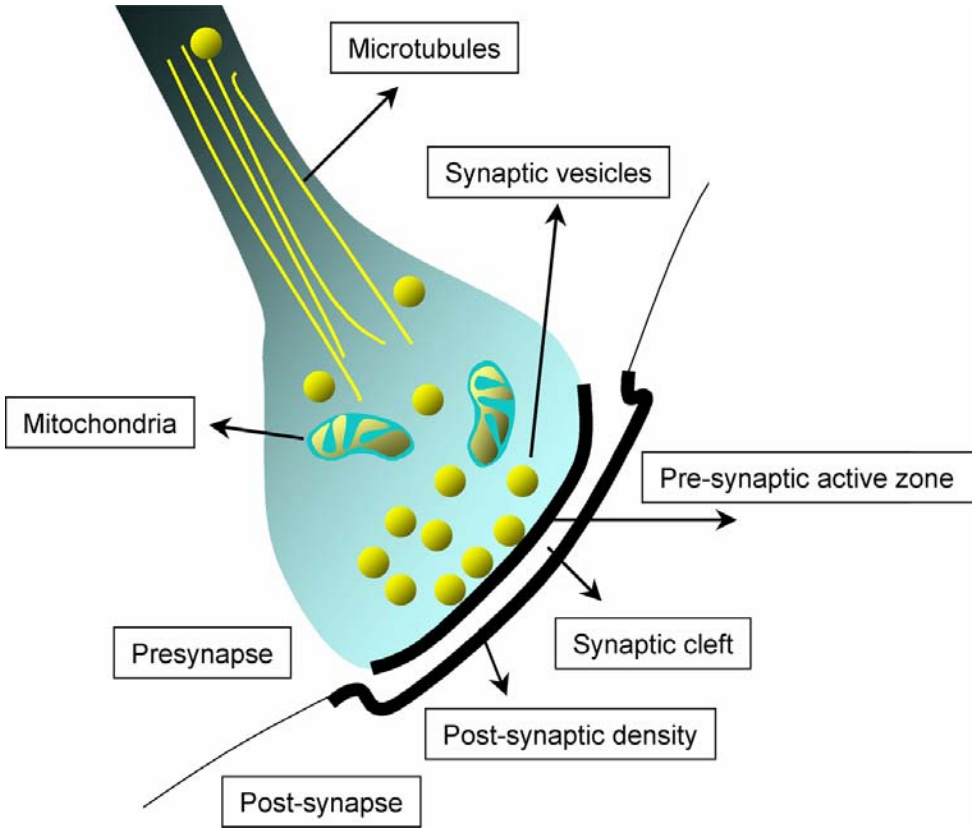

Fig.1.1a- General structure of a synaptic terminal.

Specifically at the "active zone" of a presynaptic terminal synaptic vesicle fuse and release their neurotransmitter (NT) content (Katz, 1969). When the presynaptic membrane is depolarized e.g. by an action potential, Ca^{2+} channels open and allow Ca^{2+} to flow into the terminal which triggers this fusion (fig. 1.1b).

A constant pool of synaptic vesicles (SVs) is required to facilitate sustained release of neurotransmitter. To serve that purpose, after they release their content synaptic vesicles are endocytosed for a new round of exocytosis.

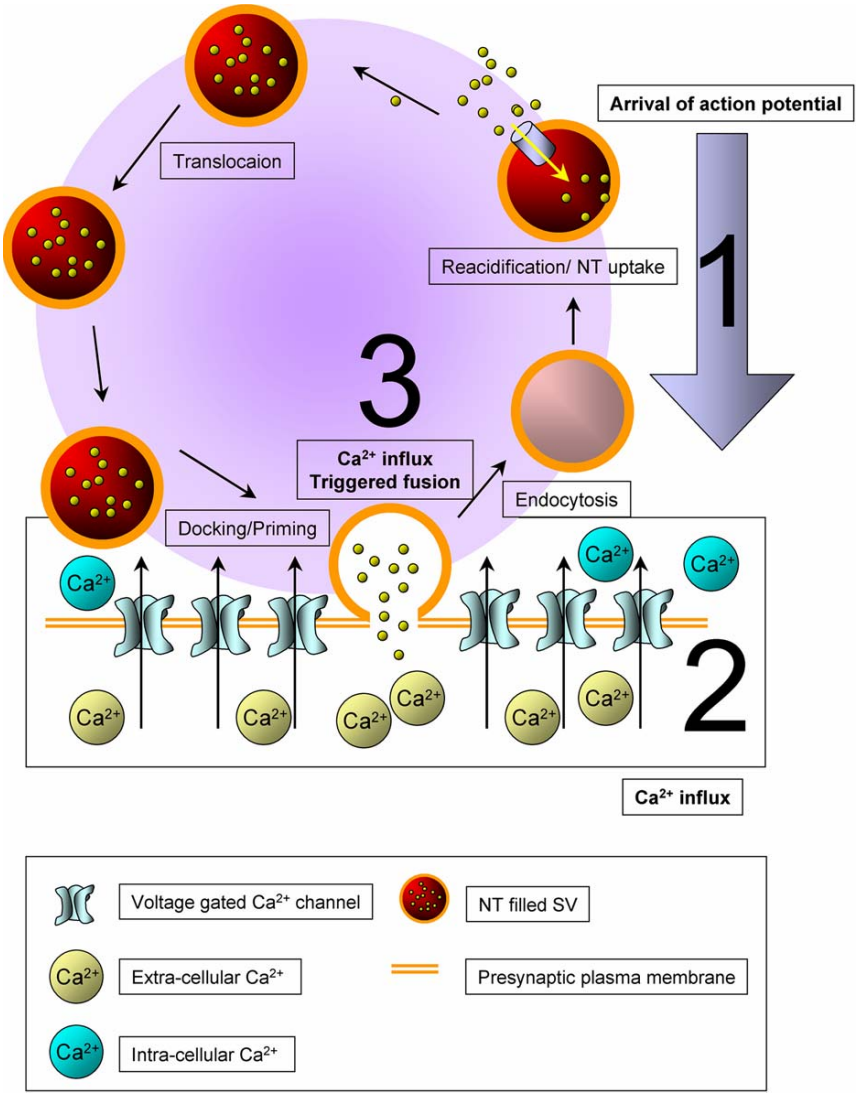

Fig.1.1b- Sequence of events at the presynapse upon arrival of an action potential.

Thus, nerve terminals are secretory machines dedicated to repeated rounds of release.
Most neurons form > 500 presynaptic nerve terminals that are often widely separated from the neuronal cell bodies. Nerve terminals do not convert reliably every action potential into a secretory signal but are "reliably unreliable" (Goda & Südhof, 1997). In most terminals, only 10%–20% of action potentials trigger release. The relationship between action potentials and release in a nerve terminal is regulated by intracellular messengers and extracellular modulators and is dramatically altered by repeated use of a synapse.

Thus, in addition to secretory machines, nerve terminals are computational units where the relation of input (action potential) to output (neurotransmitter release) continuously

changes in response to extra- and intracellular signals. All presynaptic functions, directly or indirectly, involve synaptic vesicles.

1.2 The Synaptic Vesicle Cycle

Briefly, a typical SV cycle comprises following steps (fig.1.1b):

1. Active transportation of neurotransmitters into the synaptic vesicles.
2. Clustering of synaptic vesicles at the active zone.
3. Docking of SVs at the active zone.
4. Priming of SVs, i.e. conversion into a state of competence for Ca^{2+} triggered fusion pore opening.
5. Fusion and transmitter release into the synaptic cleft.
6. Endocytosis and recycling.

The process of endocytosis has become a specialized area of research within neurotransmission and has been studied extensively. This process can occur by various modes (e.g. kiss and run, kiss and stay and Clathrin mediated endocytosis (Südhof, 2004))

Along with understanding the higher neuronal functions like learning, memory and cognition, understanding the molecular machinery of neurotransmission has turned out to be a major goal of modern neurobiology. More than 1000 proteins have been thought to participate in the process of exocytosis (Südhof, 2004). This makes it necessary to understand the process in detail in order to be able to tell actual players from bystanders.

1.2.1 Synaptic Vesicle Pools

For a sustained, reliable release a continuous supply of SVs is essential. Based on their proximity to the release site as well as quantitative analysis of stimulus dependent depletion and the ability to take up fluorescent dye during endocytosis, definitive pools of synaptic vesicles have been characterized.

After repeated stimulation of the nerve terminal at a high rate vesicular release shows a dramatic drop and assumes a lower steady state level. This use dependent synaptic depression seen in the beginning seems to be the result of depletion of SVs in the readily releasable pool (RRP).

The steady-state level of release corresponds to the rate with which vesicles are replenished into the readily releasable pool by recycling or by recruitment from a reserve pool.

The concept of equating release rates with vesicle pools has been useful, and different pools of synaptic vesicles were defined on the basis of the rates of release under various stimulation conditions. The size of the readily releasable pool that can be exocytosed by high-frequency stimulation generally agrees well with the amount of release obtained upon application of hypertonic sucrose as a mechanical stimulus (Rosenmund & Stevens, 1996) or with the number of vesicles that can be measured "docked" by electron microscopy (Schikorski & Stevens, 2001; Sätzler et al., 2002). The total number of vesicles that participate in exo- and endocytosis during mild prolonged stimulation are referred to as the recycling pool. The large reserve pool finally serves to replenish the recycling pool upon its depletion during excessive or unphysiological stimulation (Rizzoli and Betz, 2005).

Quantification of the available number of vesicles in each pool suggests that the pool size varies from synapse to synapse depending on its type. (e.g. the neuromuscular junction (NMJ) has a different pool size from that of cultured hippocampal neurons). Along with these pools sometimes a larger "resting pool" of vesicles is also observed (Südhof, 2000).

Variations observed in the number of vesicles in each pool as well as comparative sizes of different pools indicate that these definitions are operational. Probably, the vicinity of the vesicle to a Ca^{2+}channel and thus the transient ambience of Ca^{2+}concentration would be the true deciding factors over release probability of a vesicle and may in turn allocate the vesicle to an appropriate pool (fig.1.2.1a).

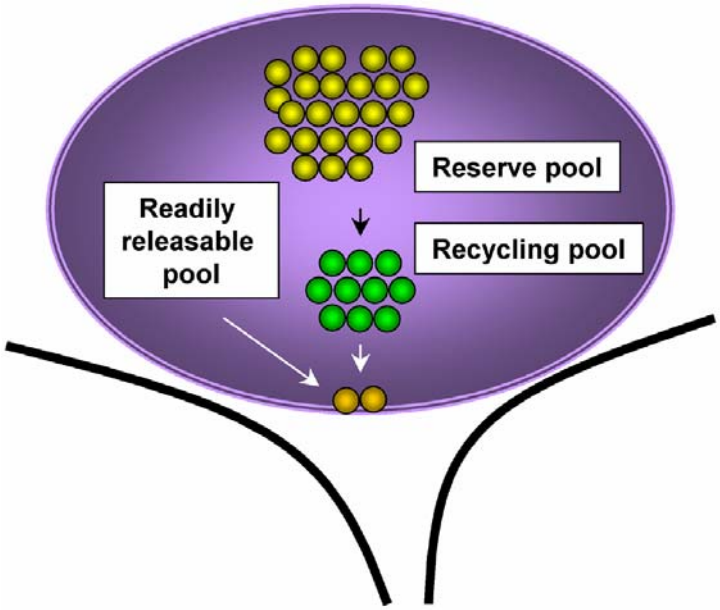

Fig. 1.2.1a- Synaptic vesicle pools (Based on Rizzoli and Betz, 2005).

1.3 The Molecular Architecture Of Synaptic Vesicles

The presynapse is specialized for neurotransmitter release. Hence, all the characteristic cellular functions at the presynapse are directed towards neurotransmission. As a result, all processes in a nerve terminal influence, directly or indirectly, the interaction of synaptic vesicles with the presynaptic active zone. Understanding the composition of synaptic vesicles and of the active zone is a first step towards insight into the molecular mechanisms of release.

1.3.1 Synaptic Vesicles

These are uniformly small organelles (~20-nm radius), and are responsible for the neurotransmitter traffic across the cell membrane. Purified vesicles have a protein: phospholipid ratio of 1:3 with an unremarkable lipid composition. (40% phosphatidylcholine, 32% phosphatidylethanolamine, 12% phosphatidylserine, 5% phosphatidylinositol, 10% cholesterol, wt/wt; (Benfenati et al., 1989).

Two classes of proteins are present on the synaptic vesicles. 1. Transport proteins involved in neurotransmitter uptake and 2. trafficking proteins that participate in vesicular exo-endocytosis and the over all synaptic vesicle cycle (figure 1.3.1a).

Neurotransmitter uptake is initiated by an electrochemical gradient, which is generated by a vacuolar type proton pump. Several neurotransmitter transporter proteins have so far been identified that mediate actual neurotransmitter uptake. The type of neurotransmitter transporter present on the vesicles defines the nature of the synapse (e.g. glutamatergic, GABAergic, cholinergic etc.) and depending on the type of neurotransmitter synapses could be made excitatory or inhibitory (Südhof, 2004).

As compared to the transport proteins the trafficking proteome of synaptic vesicles is complex. It contains proteins, which have transmembrane domains, others are linked to the membrane by post-translational modification, and proteins of a third group are peripherally bound (Figure 1.3.1a). Neither do SV proteins have a comman characteristic that describes them as a class of synaptic vesicle associated proteins nor is their mode of deposition specifically onto the synaptic vesicles known. As summarized in figure 1.3.1a, many but not all of the known synaptic vesicle proteins interact with nonvesicular proteins and are linked to specific functions.

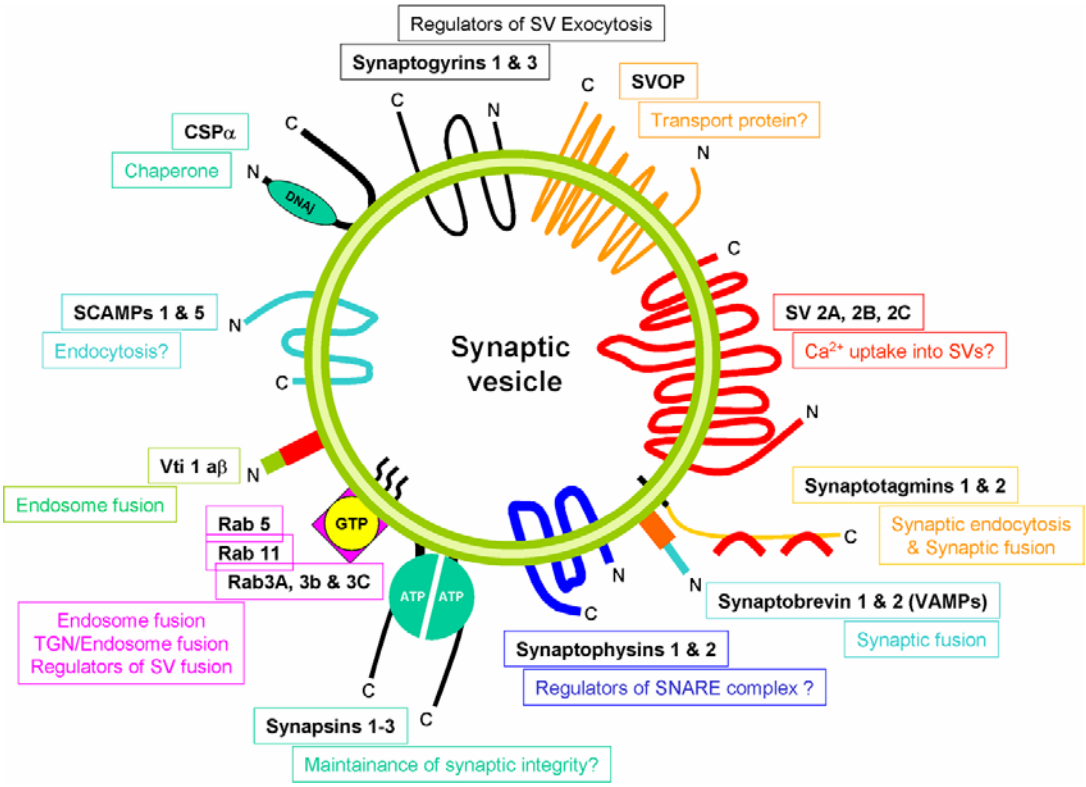

Fig.1.3.1a- Synaptic vesicle and associated trafficking proteins (modified from Südhof, 2004).

1.3.2 Membrane Fusion During Exocytosis

Intracellular membrane fusion is usually mediated by the family of SNARE proteins (Soluble N-ethylmaleimide sensitive factor attachment protein receptor). These proteins are present on both fusing membranes (V-SNAREs and T-SNAREs) and form a tight core complex prior to fusion (Chen & Scheller, 2001; Jahn et al., 2003). SNARE proteins are characterized by a homologous 70-residue sequence called the SNARE motif. The core complex is formed when four SNARE motifs (present in three or four separate SNARE proteins because some SNAREs contain two SNARE motifs) assemble into a parallel four-helical bundle, with the transmembrane regions of the SNAREs emerging on the C-terminus. Core-complex formation may force the membranes on which the SNAREs reside into close proximity, thereby initiating membrane fusion. Synaptic exocytosis is

mediated by three SNARE proteins: Synaptobrevin (also called vesicle-associated membrane protein-VAMP) on synaptic vesicles, and Syntaxin 1 and SNAP-25 on the presynaptic plasma membrane (Söllner et al., 1993).

SNARE complex formation at the synapse and in other intracellular fusion reactions is probably controlled by a class of essential fusion proteins called SM proteins for Sec1/Munc18-like proteins (Jahn et al., 2003). SM proteins often interact with Syntaxin-like SNAREs. Munc18-1, the SM protein that controls synaptic fusion, binds to a conformation of Syntaxin that is closed (Dulubova et al., 1999) and blocks its SNARE motif from participating in SNARE complexes. Thus Munc18-1 must dissociate from Syntaxin for SNARE complexes to form.

Another class of proteins that may regulate SNARE function at the synapse is Synaptophysins, abundant synaptic vesicle proteins that bind directly to Synaptobrevin (Johnston & Südhof, 1990, Calakos & Scheller, 1994, Edelmann et al., 1995, Washbourne et al., 1995).

1.3.3 Ca^{2+} Sensors At The Synapse

Ca^{2+} entry upon arrival of the action potential to the nerve terminal triggers the exocytosis. It has been demonstrated that Ca^{2+} binding sites of vesicular proteins Synaptotagmin 1 and Synaptotagmin 2 have 5 Ca^{2+} binding sites with affinity to Ca^{2+} at micromolar concentration and can mediate this trigger for fast exocytosis (Meinrenken et al., 2003).

1.3.4 The Rab3 Cycle

Rab proteins are a family of GTPases, which are associated with the vesicles in a GTP-bound form but dissociated, in a GDP-bound form. These proteins have a key role to play in different stages of SV release and reuptake.

Rab3 undergoes a cycle of synaptic vesicle association and dissociation in parallel with synaptic vesicle exo- and endocytosis (Fischer von Mollard et al., 1991). Rab3 is attached

to synaptic vesicles in the GTP-bound state via covalently linked geranylgeranyl moieties (Johnston et al., 1991). During or after synaptic vesicle fusion, GTP on Rab3 is hydrolyzed to GDP, and the resulting GDP-bound Rab3 is dissociated from synaptic vesicles by GDI (named GDP dissociation inhibitor, although its general function is to dissociate Rab proteins from membranes; Araki et al., 1990). The soluble GDI/GDP-Rab3 complex is then reattached to synaptic vesicles by a poorly understood process that involves GDP to GTP exchange. Rab3 dissociation from vesicles depends on Ca^{2+}-triggered exocytosis of synaptic vesicles (Fischer von Mollard et al., 1991), which suggests that the Rab3 cycle ensures directional interactions of Rab3 with effector proteins during exocytosis.

1.3.5 Rab3 Effectors

Two classes of Rab3 effectors that bind only to GTP-Rab3 but not to GDP-Rab3 have been identified, Rabphilin (Shirataki et al. 1993; Li et al., 1994) and RIM1α/2α (Wang et al. 1997a, 2000;Wang & Südhof, 2003). Both effectors have a similar N-terminal zinc-finger domain that interacts with all Rab3 isoforms, include central phosphorylation sites for PKA, and contain two C-terminal C2 domains. Otherwise, however, Rabphilin and RIM1α/2α are very different (Wang et al., 1997a). Rabphilin is a soluble protein that requires Rab3 for binding to synaptic vesicles (Geppert et al., 1994b, Li et al., 1994) and binds Ca^{2+} via its C2 domains (Ubach et al., 1998). RIM1α/2α, in contrast, are larger, biochemically insoluble active-zone proteins whose C-terminal C2 domains lack predicted Ca^{2+}-binding sites. Rabphilin exhibits biologically interesting properties (Ca^{2+} binding, cycling on and off-synaptic vesicles in a manner dependent on Rab3, stimulation-dependent phosphorylation by multiple kinases; see Shirataki et al. 1993; Li et al. 1994). The binding of Rab3A on synaptic vesicles to RIM1α in the active zone suggests a docking function (figure 1.3.1a), but RIM1α knockout (KO) mice did not exhibit a change in the number of docked vesicles (Schoch et al., 2002), consistent with a lack of change in docking in the Rab3A KO mice (Geppert et al., 1997). Viewed together, these data suggest that RIM1α (and probably RIM2α) regulates neurotransmitter release via interactions of its N-terminal domain with Rab3 and Munc13-1, and possibly via interactions of its PDZ domains with ERCs and its C-terminal C2 domain with α-Liprins and Synaptotagmin-1 (Betz et al., 2001; Ohtsuka et al. 2002; Schoch et al. 2002; Wang et al. 2002).

1.4 The Presynaptic Active Zone: A Comparative Analysis Of The Structural-Molecular And Functional Architecture

The conversion of the electric impulse to a chemical signal described above occurs at the special vesicle fusion sites of the presynapse called active zones (AZ). The term "active zone" was coined in 1970 by Couteaux and Pecot-Dechavassine. Ultrastructural studies of synapses in different organisms have revealed a few conserved morphological features among active zones, regardless of their size, location, or types of neurons and their targets:

1. An electron-dense plasma membrane, suggesting a proteinaceous nature of the AZs.
2. Observation that SVs cluster, tether and fuse at the active zones (Couteaux et al., 1970; Heuser et al., 1973).
3. The active zone is in close and precise alignment with the post-synaptic density (PSD) area, spanning the same width as the PSD. AZ and PSD are separated by a synaptic cleft, which could be as narrow as 30 nm (Lagnado et al., 2003).

1.4.1 The Structure Of Active Zones

Active zones could be dissected in three functionally distinct components. A. The plasma membrane juxtaposed to the PSD where synaptic vesicle fusion occurs. B. The cytomatrix immediately internal to the plasma membrane where synaptic vesicles dock. C. The electron-dense projections extending from the cytomatrix into the cytoplasm. Synaptic vesicles are tethered to these projections (figure 1.4.1b).

1.4.2 The Plasma Membrane Of The Active Zones

The plasma membrane of AZs harbors two ports. One for the entry of Ca^{2+} upon arrival of an action potential and the other one for vesicular fusion which is triggered by Ca^{2+} entry and in turn results in neurotransmitter release. As the time delay between Ca^{2+} entry and vesicular fusion is very short (0.2 ms, Parsegian, 1977; Stanley, 1997) and probabilistic analysis estimates that for a decent release probability the distance of a fusion competent vesicle to the presynaptic membrane should be <50 nm (Atwood et al., 2002; Bennett et al., 2000; Stanley, 1997), these two ports are thought be present in very close proximity to

each other. Localization of Ca^{2+} channels in proximity to active zones has been demonstrated by various immunohistochemical studies (Kawasaki et al., 2004; Robitaille et al. 1990; Zhang et al., 2000). Studies on the frog, lizard and mammalian NMJs by freeze fracture techniques elegantly demonstrate the arrangement of Ca^{2+} channels at the AZs (fig1.4.1a. Ellisman et al., 1976; Heuser et al., 1974; Walrond et al., 1985).

Other important components of the plasma membrane are the adhesion molecules by which the precise alignment of the active zone with the PSD is most likely mediated. Several classes of adhesion molecules have been shown to be present at the active zone: Cadherins (Shapiro et al., 1995; Yagi et. al, 2000), Protocadherins (Frank et al., 2002), Nectins (Mizoguchi et al., 2002; Takai et al., 2003), Neural cell adhesion molecule (Rougon et al., 2003)/Fasciclin II (Davis et al., 1997)/aplysia cell adhesion molecule (Mayford et al., 1992), Down syndrome adhesion molecule (Schmucker et al., 2000), Syndecans (Hsueh et al., 1998), L1/Neuroglian (Walsh et al., 1997), Integrins (Chavis et al., 2001), Neurexins (Missler et al., 1998), and Sidekicks (Yamagata et al., 2003; Yamagata et al., 2002). All adhesion molecules share common protein motifs: an extracellular domain that mediates binding with the postsynaptic counterparts or extracellular matrix, a single-pass transmembrane domain or membrane anchor, and often an intracellular domain that binds to the cytoskeleton or the intracellular scaffolding proteins (Gottardi et al., 2001, Sheng and Sala, 2001). All of these adhesion molecules except Neurexin, which is expressed presynaptically and binds its postsynaptic receptor Neuroligin (Yamagata et al., 2003), are expressed in both pre- and postsynaptic terminals, and adhesion is formed through homophilic interactions. In short, the plasma membrane at the active zone mediates fusion of SVs upon Ca^{2+} entry during neurotransmission. An array like organization of the Ca^{2+} channels and the adjacent localization of the fusion machinery facilitates the process.

1.4.3 The Cytomatrix Underlying The Plasma Membrane Of The Active Zone

The cytomatrix at the active zone (CAZ) is an electron dense structure and displays a web like pattern (Bloom et al., 1968; Pfenninger et al., 1972). By electron microscope tomography of the CAZ at frog NMJs an array-like structure has been observed (Harlow

et al., 2001). It consists of "beams" and "ribs" that connect docked synaptic vesicles with putative Ca^{2+} channels at the plasma membrane (figure 1.4.1a).

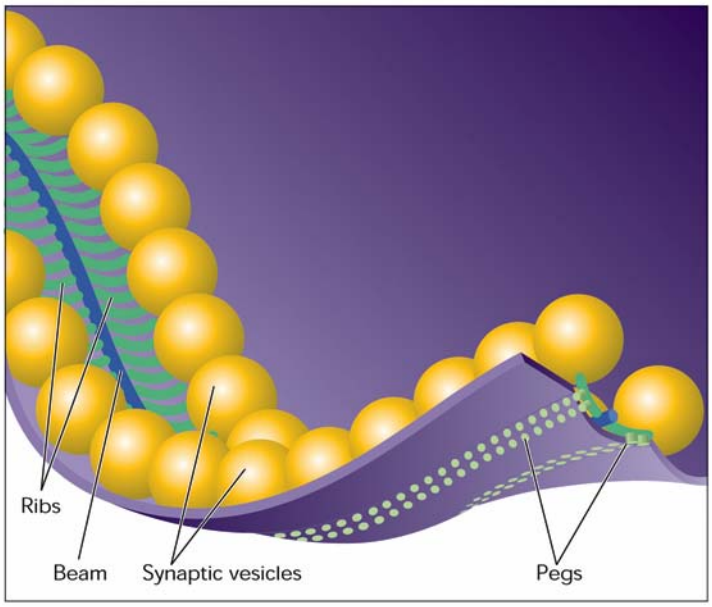

Fig.1.4.1 a- Schematic of CAZ arrangement at the frog NMJ. The "pegs" are assumed to be the Ca^{2+} channels (Zhai and Bellen, 2005).

The beams run along the midline of the presynaptic ridge parallel to the ridge's long axis, and the ribs extend from the beams and connect the synaptic vesicles near the vesicle-plasma membrane interface. In addition, the ribs are connected to the intramembrane macromolecules resembling the putative Ca^{2+} channels seen in freeze-fracture studies. This organization allows alignment of each docked vesicle with at least one Ca^{2+} channel which could provide release with high probability.

On the basis of their function or putative function, the proteins identified in the active zone cytomatrix can be classified into three categories (also summarized in fig. 1.4.1c).

1. The classical cytoskeletal proteins corresponding to Actin, Tubulin, Myosin, Spectrin α-chain and β-chain, and β–Catenin (Burns et al., 1995; Hirokawa et al., 1989; Phillips et al., 2001) are the fundamental elements of the framework of active zone cytomatrix.
2. The known scaffolding proteins include SAP90/PSD95/Dlg, SAP97, and CASK/LIN-2 (Hata et al., 1996; Kistner et al., 1993; Koulen et al., 1998; Muller et al., 1995). These proteins are not restricted to active zones because they also participate in clustering of postsynaptic receptors and are involved in the organization of a variety of cell junctions (Fanning et al., 1999; Garner et al., 2000; O'Brien et al., 1998). If the cytoskeleton

proteins form a grid-like structure at the active zone, these scaffolding proteins probably link the ion channels and the fusion machinery onto the grid to ensure proper active zone function. For example, CASK interacts with α-Neurexin, Syndecan 2, Ca^{2+} channels, the cytosolic protein Veli/LIN- 7, and the Munc18/n-Sec1-interacting protein Mint1 (Butz et al., 1998; Hata et al., 1996; Hsueh et al., 1998; Maximov et al., 1999).

3. The active zone-specific proteins including RIM1, Munc13/unc13, Bassoon, Piccolo/Aczonin, and ELKS/CAST/ERCs (Brose et al., 1995; Dieck et al, 1998; Fenster et al., 2000, Ohtsuka et al., 2002; Wang et al., 1999; Wang et al., 2002; Wang et al., 1997). Their active zone-specific localization and their multidomain structure allow them to participate in modulating synaptic vesicle docking, priming, and fusion, as well as the initiation of the assembly of the active zone structure. In short, CAZ is a protein dense complex made up of cytoskeletal and scaffolding proteins that are responsible for the formation of a web like structure which has slots for synaptic vesicle docking, and CAZ also consists of proteins that mediate vesicular priming and fusion.

1.4.4 The Electron-Dense Projections Extending From The Cytomatrix Of The Active Zone: Synaptic Ribbons

At certain active zones thin, electron dense projections can be seen extending 0.5 to 1 μm from CAZ into the cytoplasm (Lagnado et al., 2003; Lenzi et al., 2002; Von Gersdorff, 2001). Their shape, appearance and even visibility varies greatly across species and sometimes the type of synapse. These are known as the synaptic ribbons. Apart from their characteristic appearance, ribbons have always synaptic vesicles tethered to their surface (figure 1.4.1b).

Morphologically, dense projections have been observed in various types of synapses in different species. At *Caenorhabditis elegans* NMJs, dense projections in the shape of a plaque have been described (figure 1.4.1b). In *Drosophila*, T-shaped dense projections can be seen in NMJs, the tetrad synapses of the visual system in CNS synapses (Meinertzhagen, 1996; Yasuyama et al., 2002). In crustacean NMJs, dense projections appear to be cylindrical (figure 1.4.1b) (Govind et al., 1979). In vertebrate NMJs, dense projections have been described in frog, lizard, and mammals (figure 1.4.1b) (Ellisman et al., 1976; Heuser et al., 1974; Walrond et al., 1985). In mammalian CNS synapses, dense projections were also noticed in electron microscopic studies as early as the 1960s and

recently have been visualized in great detail (figure 1.4.1b)(Bloom et al., 1968; Phillips et al., 2001). Although synaptic ribbons vary greatly and although it is conventionally believed that they are specific to only certain synapses that require a constant, tonic release, a modern view is emerging which suggests the universality of these structures (Lenzi et al., 1999; Von Gersdorff, 2001). Synaptic ribbons could be an evolutionarily conserved structure whose primary function might be tethering of synaptic vesicles at the active zones.

Analysis of vertebrate synaptic ribbons has shown that they are composed of several components which may have functional distinction. RIBEYE, a protein with homology to the C-terminal Binding Protein 2 (CtBP2) at its B domain and which also has an ability to assemble into large structures mediated by its A domain has been shown to be a major componant of the photoreceptor synaptic ribbons in vertebrates (Schmitz et al., 2000; tom Dieck et al., 2005). Motor protein KIF3A also has been shown to be a component of the ribbons (tom Dieck et al., 2005) and is likely to mediate the tethering of vesicles. A detailed model sketch will be described in the discussion (figure 4.3.5a) including the localization of its components. Most of the information about the composition of active zones is obtained from vertebrates. The molecular composition of the invertebrate active zone is largely unknown. In *Drosophila* and crayfish, synaptic vesicles cluster around T-bars, although the mechanism of tethering is not known (figure 1.4.1b).

Recently the dense projections of mammalian CNS active zones have been biochemically purified and molecularly characterized (Phillips et al., 2001). These dense projections are ~50 nm in size, are pyramid like and contain synaptic vesicle binding proteins such as synapsin and RIM (Hilfiker et al., 1999; Phillips et al., 2001; Wang et al., 1997).

It seems possible that because of their peculiar structure(s) and ability to tether vesicles synaptic ribbons could bind a large number of vesicles, thereby increasing the readily releasable pool, without increasing the area occupied by CAZ and PSD at the synapse (figure 1.4.1b). This feature is particularly important in sensory synapses, because sustained release upon continuous stimulation requires a large readily releasable pool and a capacity for efficient synaptic vesicle replenishment, while the defined portion of an individual sensory neuron e.g. in the vertebrate retina or along the cartridge of an insect lamina restricts the size of each terminal. In contrast, at many NMJs, where stimulations

are not continuous, the size of nerve terminals is not restricted and the active zone must expand as the muscle grows, so dense projections are relatively small. Interestingly, in *Drosophila* and crustacean NMJs, active zones with prominent T-bars can be seen adjacent to those without T-bars within the same presynaptic nerve terminal. It has been proposed that the active zones with prominent T-bars have a stronger output, possibly because more synaptic vesicles are released upon stimulation. Supporting evidence comes from crustacean studies showing that high-output NMJ terminals have a threefold higher density of dense projections than the low-output terminals arising from the same excitatory motor axon, although no difference was observed in total synaptic area (Govind et al., 1979; 2001). In summary, although the morphology of dense projections varies greatly among different types of synapses, the primary function of dense projections is to tether synaptic vesicles at the active zone. Larger dense projections tether more synaptic vesicles and therefore increase the size of the readily releasable pool.

1.4.5 Active Zone Assembly And The Regulation Of Active Zone Density And Spacing

Active zone assembly begins after initial axon target recognition and contact takes place and commences as the neurotransmitter release sites are established. In cultured hippocampal neurons it has been shown to require ~30 min (Ahmari et al., 2000, Friedman et al., 2000). According to the unitary assembly model active zone precursors are packaged into the transport vesicles and delivered to the nascent synaptic contact site. Fusion of these vesicles with the plasma membrane establishes deposition and localization of active zone proteins. 2-3 transport vesicles have been shown to be sufficient for the formation of one active zone (Shapira et al., 2003, Zhai et al., 2001). According to this model the average active zone has 10-15 vesicle release sites or “grid units” thus each active zone transport vesicle (PTV (Shapira et al. 2003)) should carry building material for 4-5 release sites.

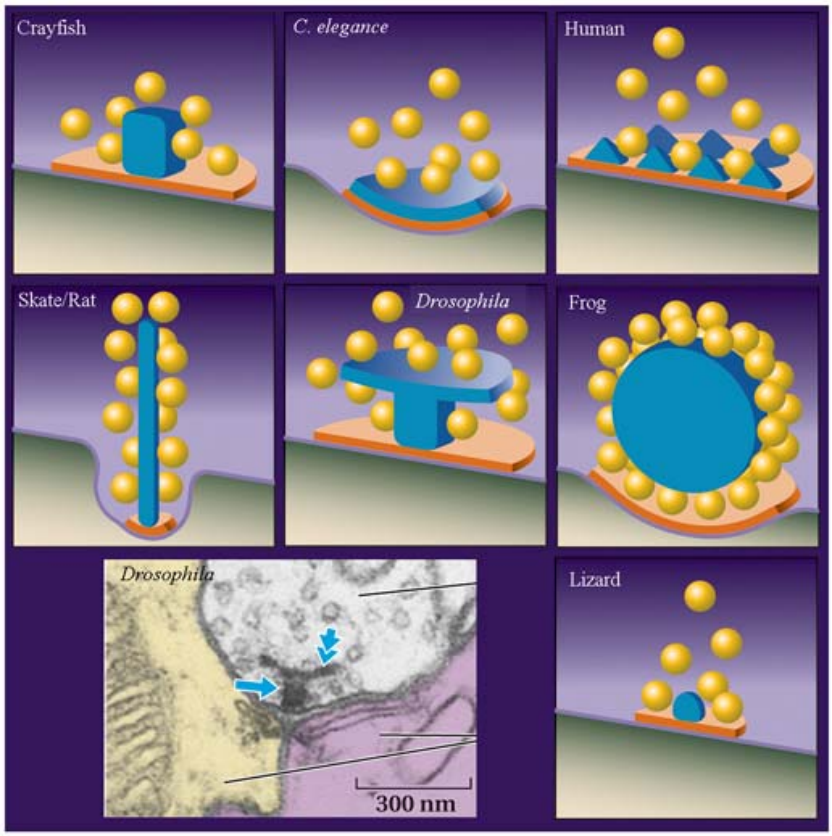

Fig.1.4.1b- A comparative analysis of active zone structures in different synapses (based on Zhai and Bellen, 2005).

1.4.6 Genetic Analysis Of Invertebrate Active Zones

Although much information about the molecular architecture of the invertebrate active zones is still fragmentary, recent genetic analysis has helped identifying a few molecular players at the invertebrate CAZ.

Loss of function mutants for *syd-2* gene in *C. elegans* have lengthened NMJ terminals which are less electron dense as well (Zhen et al., 1999). The Syd-2 protein is localized to active zones and is a member of the Liprin protein family, which contains coiled coil and sterile α-motif domains (Serra-Pages et al., 1998). Liprins interact with the Lar family of receptor protein tyrosine phosphatases (RPTPs) and cluster RPTPs to focal adhesions (Serra-Pages et al., 1998). *Drosophila* Liprin-α is also localized to active zones at NMJs, and in flies mutant for Liprin-*Dlar*, the size of active zones are ~2.5-fold bigger than

normal and the morphology is more irregular (Kaufmann et al., 2002). In *Drosophila*, loss of *wishful thinking* (*wit*) causes a reduced number of boutons, an increased number of active zones per bouton, and freely floating T-bar structures in the cytoplasm (Aberle et al., 2002). Wit is a BMP type II receptor that is expressed in a subset of neurons, including motor neurons. However, the mechanism as to how Wit regulates active zone assembly is not understood (Aberle et al., 2002; Marques et al., 2002).

Active zones are plastic structures and their number varies during development as well as in adult nervous system. In tetrad synapses of the *Drosophila* visual system, the number of presynaptic ribbons/ T-bars changes with alterations in light stimulation (Brandstatter et al., 1999; Rybak et al., 1997).

In crustacean NMJs, high-frequency stimulation-induced long-term facilitation also correlates with an increase in the number of active zones and dense projections (Wojtowicz et al., 1994). In mammalian hippocampal neurons, long-term potentiation also correlates with the expansion or “division” of active zones (Harris et al., 2003; Weeks et al., 2000).

Synaptic molecules that are known in vertebrates and invertebrates are summarized in table 1.4 along with their possible functions wherever applicable.

Vertebrates	**Function/Proposed function**
Exocytosis	
Synaptobrevin/VAMP/SNAP25, Syntaxin	Components of SNARE complex involved in synaptic vesicle docking and fusion
NSF, α and β SNAPs	Dissociation of SNAREs
Synaptotagmins	Calcium sensors; interact with syntaxin and RIM
N and P/Q type Ca^{2+} channels	Calcium influx
Munc 18	Binds and negatively regulates syntaxin and synaptic vesicle fusion
Munc 13	Involved in synaptic vesicle priming; interacts with RIM; displaces Munc18
RIM1 α	Involved in synaptic vesicle priming; interacts with Munc 13, RIM BPs and Synaptotagmin
Rab3A	Regulates synaptic vesicle cycle; interacts with Rabphilin, DOC2, PRA 1 and RIM
Complexin	Binds and regulates SNARE complex
Endocytosis	
Clathrin	Involved in synaptic vesicle endocytosis; interacts with dynamin, AP2, amphiphysin and other molecules
Dynamin	GTPase involved in pinching off synaptic vesicles during endocytosis
Amphiphysin	Binds dynamin and is involved in synaptic vesicle endocytosis
Active zone proteins	
CASK	CAMKII domain containing MAGUK; forms a complex with MINTs, Veli and calcium channels, neurexin and SynCAM
MINTs	Munc18-interacting molecules; found in complex with CASK and Veli
Veli	Found in complex with CASK and MINTS
Bassoon and Piccolo	Large structural proteins of the CAZ that interact with PRA 1, Profilin, Abp1 and ERC/CAST
Synapsins	Anchoring of synaptic vesicles to actin; regulation of reserve pool of synaptic vesicles

α-Liprin	Scaffold proteins that bind RIM, ERC/CAST and LAR
Spectrin	Cortical cytoskeletal protein; interacts with actin; cell-adhesion molecules and receptors
ERC/CAST	CAZ proteins that interact with Piccolo, Bassoon, RIM and Liprin
RIMBP	RIM binding protein; component of CAZ
RIBEYE/CtBP2	C-terminal binding protein, component of synaptic ribbons
KIF3A	Motor protein; component of the ribbon; aids tethering of vesicles

Invertebrates	**Fly**	**Worm**	**Proposed function**
Exocytosis			
CAPS	*dcaps*	*unc-31*	Role in late stage of DCV fusion
Unc-13	*dUnc- 13*	*unc-13*	Required for SV priming via control of syntaxin conformational state
RIM	CG7301, CG7305, CG7321	*unc -10*	Involved in SV priming; coordination of Rab-3 and Unc-13 dependant functions
SNAP-25/t-SNARE, membrane associated protein	*Snap25ts*	*ric4*	Component of core SV fusion machinery and calcium channel inhibitor ATPase required for SNARE complex disassembly following vesicle fusion
Syntaxin	*syx*	*Unc-64*	Essential component of core SV fusion machinery and calcium-channel inhibitor
NSF	*comatose*	?	ATPase required for SNARE complex disassembly following vesicle fusion
Synaptotagmin	*syt-1*	*snt-1*	Ca^{2+} sensor for SV fusion and facillitatory role in endocytosis
SLO-1	*slow poke*	*slo-1*	Ca^{2+} activated K^{+} channel regulating SV release duration
Synapsin	*Syn*		Required for normal learning and memory

SAP47	*Sap* ^{47}		?
CSP	*Csp*		SV associated co-chaperone.
Endocytosis			
Clathrin heavy chain	*chc*	?	Clathrin mediated endocytosis
Endophilin	*Dendo-A*	?	Required for Clathrin mediated SV endocytosis but not for the "kiss and run" mode.
Eps-15	CG16932	*ehs-1*	Facillitatory SV endocytosis role, possibly through a dynamin interaction
Stoned A/B	*stoned*	*Unc-41*	Endocytosis; sorting and recycling of synaptotagmin
Dynamin	*Shibire* ^{ts}	?	Endocytosis
α- ADAPTIN	*dada*	?	Endocytosis
Lap (Like AP180)	*lap*	*Unc-11*	May regulate clathrin coat assembly and the SV size
Abnormal wing disc	*awd*	?	Endocytosis
Amphiphysin	*amphiphysin*	?	Role in muscle organization, excitation-contraction coupling, but not SV endocytosis
Dynamin associated protein 160	*Dap160*	?	Stabilizing scaffold required for synaptic development and endocytosis (Koh et. al., 2004).
Liquid facets	*lqf*	?	Clatherin mediated endocytosis
Stoned	*stoned*	?	SV recycling
Synaptojanin	CG6562-PB	*Unc-26*	Clathrin mediated endocytosis

Active zone proteins			
Ca^{2+} channel	*Cacophony*	?	Calcium influx
Liprin	*Dlar*	*Syd-2*	Regulation of the bouton size, formation of active zones?
Bruchpilot	*brp*	*Cecast*	Normal synaptic functioning, adapter for synaptic ribbons?

Table 1.4. List of known vertebrate and invertebrate (*Drosophila* and *C. elegance*) proteins involved in synaptic transmission as well as proteins present at the active zone. (Adapted from Ziv and Garner, 2005; Richmond and Broadie, 2002; Koh et al., 2004 and Zhang et al., 2003).

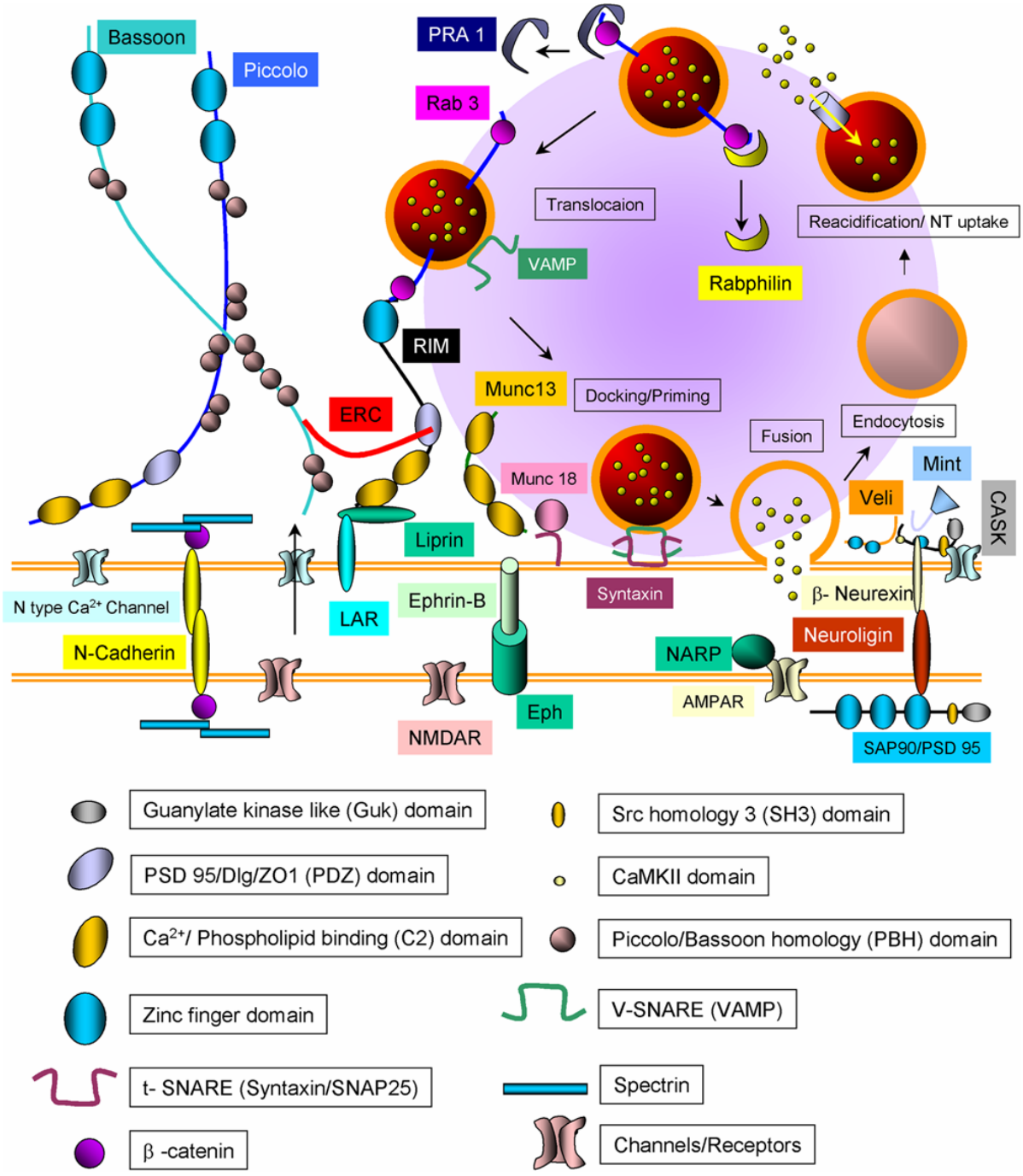

Fig. 1.4.1c. Synaptic vesicle cycle and cytomatrix at the active zone (Based on Ziv and Garner, 2004).

1.5 Motivation For Investigating The nc82 Antigen And Identifying Novel Candidates At The Invertebrate CAZ

In the past decades *Drosophila* has emerged as an excellent model system to study the nervous system with respect to its genesis, assembly and function. However, molecular analysis of its components is not complete. Synaptic structures are difficult to analyze biochemically. Yet, over the years a large amount of valuable data about the synaptic vesicular cycle, processes of neurotransmission and some aspects of exo- and endocytosis have been accumulated.

Several molecules which are components of the synaptic machinery have been characterized and mutants for their respective genetic loci have been obtained to aid in functional studies (summarized in table 1.4).

However, numerous unanswered questions remain and many important components as well as their functions need to be characterized. Presynaptic active zones of invertebrates are one such structure that is poorly understood at the molecular level.

According to recent reviews (Zhai and Bellen, 2005; Ziv and Garner, 2005) conflicts still persist with respect to the problem if there exists a common minimal synapse and if active zone assembly takes place by a generalized mechanism. Such questions require a comparative study of different organisms at an ultrastructural as well as molecular level. So far valuable ultra-structural data have been obtained from several organisms which highlight the diversity and complexity present at the active zone structures.

In vertebrates, although sufficient material could be obtained to study *in vitro* protein–protein interactions, *in vivo* validation of these interactions could take very long due to cumbersome genetics. In invertebrate model systems, although their powerful genetics has been extremely beneficial for such studies, molecular analysis is far from trivial. The availability of the whole genome sequence for *Drosophila* has greatly helped overcoming this problem. Homologues for already known proteins could be searched and simultaneous forward and reverse genetic approaches can be taken to address relevant questions.

Primary focus of the research group where the present study was carried out has been to understand the molecular architecture of the synapse. To address this problem an efficient reverse genetic approach has been developed in the pre-genomic era. A hybridoma library was obtained after *Drosophila* brain homogenates were injected into mice (Hofbauer, 1991). Monoclonal antibodies were selected by an immunohistochemical screen on adult brain sections for interesting/unusual staining patterns. Out of these, the antibodies that could reliably recognize their respective antigens on immunoblots were further shortlisted. An identification of the antigen by various genetic and/or biochemical methods, followed by characterization of its genetic locus was launched. Perturbation of the genetic locus by classical mutagenesis, identification of interaction partners and a collective functional analysis of the molecule under investigation was carried out. This, so far has proved to be a valuable and economical approach supplementary to forward genetic screens which would identify only genes that lead to a strong phenotype when mutated. The group has so far described CSP (Zinsmaier et al., 1991, 1994), Synapsins (Godenschwege et al., 2003) and SAP47 (Funk et al., 2004) using these approaches.

The Monoclonal antibody MAB nc82 is one such antibody shortlisted in the screen described above. The nc82 antigen seems to be pan-neuronally expressed and staining quality is crisp.

At the NMJs unlike other synaptic markers nc82 has sharp puncta of staining. This property presumably makes the staining on adult brains also crisp. Optical sections of such stainings are transparent and hence nc82 has been a very popular synaptic marker amongst *Drosophila* neuroanatomists, as it serves as an excellent scaffolding marker.

Because of the primary indications about its subcellular localization at the active zones, lack of knowledge of the AZ proteins in invertebrates and our interest in studying synaptic architecture at the molecular level, we decided to investigate the molecular nature and function of this candidate active zone protein in detail.

Our investigation identified the nc82 antigen as one of the first invertebrate active zone protein. It has conserved domains similar to the vertebrate active zone protein ERC/CAST. Functional analysis by RNAi knockdown lead us to name the protein Bruchpilot (German for “crash pilot”). The present study suggests that Bruchpilot

expression at the presynapse is essential for normal structure and function of chemical synapses.

Chapter 2: MATERIALS AND METHODS

2.1. Nucleic Acid Isolation

2.1.1 DNA Isolation

DNA isolation was primarily carried out for the following purposes.

A. Isolation and purification of plasmid DNA as an empty vector or with insert by doing plasmid miniprep for smaller amounts (100ng/ul) and midipreps for larger amounts of DNA (1000ng/ul).

B. Purification of DNA digestion, PCR and RT PCR products which might serve as cloning fragments, templates for Southern and northern blot probes or templates for sequencing.

C. Isolation and purification of genomic DNA which may serve as a template for genomic PCRs or for blotting in order to make Southern blots.

A. Plasmid Purification

Minipreps: Up to 1 ug of DNA was prepared using the miniprep method. Bacterial colonies were grown in LB medium supplemented with appropriate antibiotic at 37 or 30^0C at recommended time periods. Routine alkaline lysis method (Sambrook et al., 1989) was used for plasmid purification.

QIAprep Spin Miniprep Kit (QIAGEN) was used alternatively. Manufacturer's instructions were follwed without significant alteration (refer to QIAGEN® Plasmid Purification Handbook(s) available with the commercial plasmid purification kits from Qiagen limited).

Larger amounts of plasmid DNA were made using the midiprep method. QIAGEN Plasmid Midi Kits (QIAGEN) were used for the same (refer to QIAGEN® Plasmid Purification Handbook(s)). DNA was eluted in the given amount of TE buffer or dH_2O and stored at

-20^0C for future use. DNA content was estimated photometrically with appropriate dilutions or visualized on a gel along with standard molecular weight markers (2 log ladder, NEB).

The elution procedure for miprpeps was modified slightly in order to obtain higher yields of the DNA. Elution columns were incubated with smaller amounts of dH_2O or elution buffer (10 mM Tris, pH 8.5) and columns were incubated at 68^0C for 5 minutes. Columns were centrifuged for 2 minutes. The elution procedure was repeated in a similar fashion if desired using fresh dH_2O or elution buffer and combining the eluates at the end.

B. PCR Product Purification And Gel Extraction

PCR and RT-PCR products were purified using commercially available silica gel based QIAquick PCR Purification Kit (QIAGEN). Purification procedure as recommended by the manufacturer was followed with slight modifications at the elution step. DNA was eluted in small amounts of dH_2O or elution buffer (10 mM Tris pH 8.5), columns were incubated at 68^0C for 5 minutes and centrifuged for 2 minutes in a tabletop centrifuge at maximum speed. For gel extraction the DNA was fractionated on an agarose gel (using lowest possible concentration of agarose in the gel for the given species of DNA), visualized under UV light, excised with a clean blade and subjected to gel extraction using the QIAquick Gel Extraction Kit from QIAGEN. Manufacturers' instructions were generally followed except for the elution step, which was modified as described for the miniprep and PCR purification methods.

C. Isolation And Purification Of Genomic DNA

Genomic DNA was primarily isolated to serve as a template for the genomic PCRs, as a template for preparing radiolabelled probes and mostly for making southern blots in order to screen the jump out locus. Flies were collected and frozen at -80^0C. 50 flies/preparation were homogenized in 1 ml of homogenization buffer (100 mM NaCl, 100 mM Tris 50 mM, EDTA (pH 8.0), 0.5% SDS). Homogenate was incubated at 68^0C for 30 minutes. Homogenate was incubated on ice for 30 minutes after the addition of 125 ul of 8M calcium acetate. A centrifugation for 10 minutes at 14,000 rpm followed this step and was repeated once after transferring the supernatant to a fresh tube. DNA was precipitated with 2.5 volumes of 100% ethanol for 10 minutes at RT. The pellet was washed with 70%

ethanol, dried and dissolved in the appropriate amount of 10mM Tris pH- 8.0 or dH_2O. This procedure typically yields 15 ug DNA/100 ul. DNA was usually stored at $–20^0$C in elution buffer or under 100% ethanol for longer duration.

2.1.2 RNA Isolation

RNA was isolated for following purposes:

A. To serve as a template for reverese transcription.
B. To serve as a raw material for poly A^+ selection.
C. For Northern blot analysis of transcripts.

RNA was isolated from flash frozen WT *Drosophila* heads using RNeasy Midi Kit (QIAGEN). 250 mg of flyheads per midi column were obtained by passing the frozen heads over sieves placed in liquid nitrogen. Heads were powdered in a prechilled mortor to make a fine powder and then subjected to homogenization with 10 strokes in buffer RLT supplemented with 10ul/ml of 2-mercaptoethanol. Homogenate was centrifuged at 35000 rpm for 1 hour in order to remove all insoluble particles which would clog the silica membrane during purification. The rest of the protocol was carried out as recommended by the manifacturer. RNA was extracted in water and stored at $–80^0$C for long term storage. RNA content was photometrically estimated. Usually 800ng/ul of total RNA was obtained. For poly A^+ selection of the RNA Oligotex mRNA Mini Kit available from QIAGEN was used. Manifacturer's instructions were followed without any modification. RNA was eluted in elution buffer (10mM Tris pH -7.5). RNA content was photometrically and visually estimated (1% agarose gel, run for 15 minutes, stained with EtBr). RNA was stored at -20^0C under 100 % EtOH after precipitating with 4 M LiCl.

2.2. Nucleic Acid Amplification

2.2.1 PCR

PCR was primerily used for following purposes

A. To amplify a certain region from genomic DNA which would subsequently be used for cloning or as a template for labelling reaction.
B. To amplify a single stranded DNA reversely transcribed from mRNA in order to make cDNA
C. To amplify products that would be used for sequencing.

A few variations on the following aspects of polymerase chain reaction were routinely applied in order to increase the specificity and good yield of the PCR product.

Primers

Primers were designed using commericially available primer design programs (e.g. primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)).

%CG concentration was usually kept around 50. Melting tempratures of the primer pairs were adjusted to be similar within an interval of 1-2 ^{0}C and primer length was usually restricted to 20 –22 bases. Melting tempratures were kept between 55-62 ^{0}C.

For linker PCR restriction sites were added to the primers. Restriction sites were flanked by the recommended number of bases in order to facilitate direct digestion of the PCR product without subcloning (NEB catalogue, MBI catalogue).

Primer stocks were appropriately diluted and kept frozen. Frequent subjection to freeze-thaw cycles of the master stocks was avoided as much as possible.

Template

Depending on the length of the expected PCR product the amount of template DNA was adjusted. For 0.1 to 10 kb genomic DNA targets 50 –100 ng of template gDNA was used. For 0.2 to 10 kb plasmid DNA targets 0.01 to 10 ng of template DNA was used (adopted from a support protocol from Eppendorf).

dNTPs

A 10 mM stock of dNTPs was prepared and always stored as 20 ul aliquotes at –20^{0}C as dNTPs are prone to degradation upon frequent freeze-thawing.

PCR Conditions

Denaturation: Usually template DNA was denatured for 4 minutes at 95^{0}C initially followed by 20 –30 sec denaturation at each cycle.

Annealing : Annealing temprature was kept approx. 5^{0}C lower than the melting temperature of primers and this step was usually allowed to take place for 20 –30 sec. A range of temperatures were almost always tested using the gradient option for annealing temperatures.

Extension: This step was usually carried out at 72^{0}C. However, for long range PCRs the temperature was reduced to 68^{0}C in order to decrease the damage to the template. Elongation time was set for each reaction assuming that each 1000 base amplification requires 1 minute.

Number of cycles : Typically of 25 to 35 cycles were found to yield a good amount of product for most of the reactions.

Reamplification : In case a very weak product was obtained, the PCR was repeated using 1/20th to 1/50th of the weak product as a template.

Extension at the end of the last cycle: In order to ensure the completion of amplified product an 8 minutes elongation step at the end of the last cycle was added to the program. For TA cloning its necessary for the amplicon to have an A overhang which is ensured by this step (see TA cloning in method 2.4.2).

Salt concentration: For most of the reaction 1.5 mM $MgCl_2$ turned out to be suffient. However, for certain reaction increasing the salt concentration to up to 3-4.5 mM produced better results.

Visualization, estimation, purification and storage : 1/20th of the PCR product was visualized on an agarose gel after EtBr staining, purified by QIAquick PCR Purification Kit (QIAGEN) and stored at –20^{0}C until further use.

2.2.2 RT-PCR

Reverse transcription was carried out using either oligo dT or a specially designed gene specific internal primer. Superscript II (Invitrogen) or MMLV H^- RT (Fermentas) reverse transcriptases were used with comparable results. 0.5 to 2.5 ug of Total RNA or 0.1 to 1 ug of poly A^+ RNA was used. DTT was incorporated whenever instructed by the manufacturer. Reaction was carried out at 42^0C for 1 hr and the enzyme was inactivated by heating the reaction for 15 minutes at 70^0C. $1/4^{th}$ of the RT pcr product was directly used as a template for the PCR reaction. Primer nested to the primer used for RT was sometimes used as a reverse primer for second starnd synthesis and subsequent amplification of the cDNA (see method 2.2.1 for PCR).

Products were used for sequencing, for labelling templates or cloning fragments after PCR purification. A list of primers used for PCR, RT-PCR and linker PCR for various purposes is given in Appendix 1.

2.3. Nucleic Acid Detection

2.3.1 Agarose Gel Electrophoresis

DNA was fractionated on 0.8%, 1% or 2% agarose gels. Ethidium bromide (EtBr) was incorporated (0.1 ul of 10 mg/ml stock) in the gel prior to casting or the gel was stained with EtBr in 1X TBE for 1 hr before visualization.

2.3.2 Southern Blots

The P element mobilization screen was carried out using the southern transfer and detection method (fig. 2.3.2a & b).

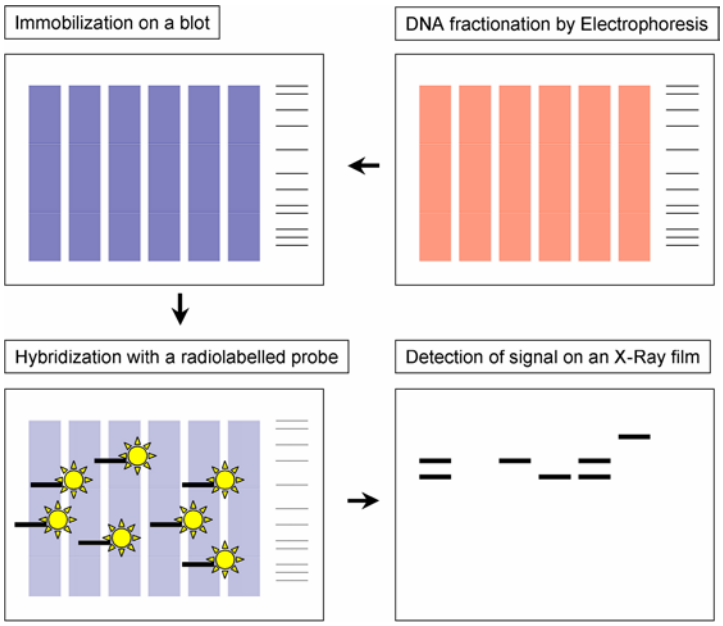

Fig. 2.3.2a –Basic principle of a southern blot.

(Based on : http://www.bio.davidson.edu/courses/genomics/method/Southernblot.html)

0.8 % agarose gel was used to fractionate ca. 4 ug appropriately digested and phenol purified genomic DNA from entire flies. Amersham Hybond NX was used as a membrane for the southern transfer. DNA was electrophoratically fractionated overnight along with the DNA marker (2 log ladder, NEB), lightely stained with EtBr, documented along with a scale, depurinated, denatured, neutralized and subjected to overnight blotting by cappilary action. DNA was crosslinked (auto cross-link mode, Stratalinker, Stratagene) and hybridized with an appropriate radiolabelled probe (see method 2.3.4 for radiolabelling) in Church buffer at 65^0C for 24 hours. Excess of probe was washed off with 2X, 30' low stringency (2X SSC, 0.2%SDS, RT) and 2 X, 30' high stringency (0.2X SSC, 0.2% SDS, 68^0C) washes and exposed for 2-4 days at -80^0C. Blots were kept moist until reprobing or stripping and stored under SARAN wrap at -20^0C. (see method 2.3.5 for stripping the Southern/Northern probes).

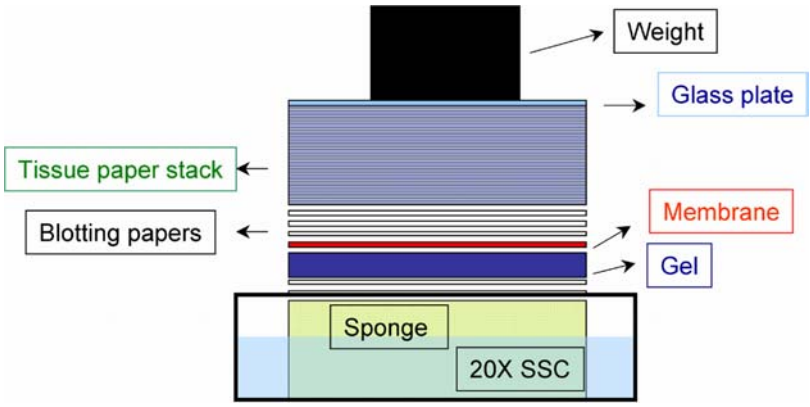

Fig- 2.3.2b- Southern transfer by capillary action.

(Based on : http://fugu.hgmp.mrc.ac.uk/Protocols/Biology/southern_blot.gif)

2.3.3 Northern Blot

A protocol as described in the Unit 4.9 of current protocols in molecular biology was followed for northern blots. All the solutions except for Tris were DEPC treated and the glassware was baked at 180^0C overnight in order to remove any RNAse contamination.
Tris was made into DEPC treated water. Gel chamber and gel cast were treated with 3% hydrogen peroxide for 10 minutes and then washed thoroughly with DEPC treated Mili-Q water. RNA was isolated from wild type fly heads as decribed in method 2.1.2. Bulk quantities were stored at -80^0C for longer storage. Alternatively small aliquotes were pelleted down and stored under 100% ethanol at -20^0C. 3 – 10 ug of poly A^+ RNA was fractionated using formaldehyde gel elecrtophoresis in 1X MOPS buffer. Only the fractionated RNA marker was cut out from the gel stained with EtBr and photographed along with a scale for future comparison with the blot. Amersham Hybond NX was used as a blotting membrane. Northern transfer was carried out overnight (refer to figure 2.3.2b), RNA was crosslinked to the membrane (autocroslink mode, Stratalinker, Stratagene). Church buffer was used as a prehybridization and hybridization buffer. 2000 – 5700 bp DNA probes were used. Probe DNA was excised from the plasmid, gel purified, roughly visually quantified on an agarsose gel and then subjected to radiolabelling (see method 2.3.4 for labelling). Hybridization was carried out at 65^0C for 24 hours following 1 hour prehybridization at the same temperature in hyridization tubes.
Excess of label was washed off 2X, 30’ each with 2X SSC, 0.2% SDS and 2X, 30’each with 0.2 X SSC, 0.2% SDS at 65^0C in hybridization tubes. Moist membrane was covered with a transperency cut to the the size of the support film and the blot was exposed to an X-Ray film for a duration of 24 hours to up to 21 days at -80^0C. For their reuse either the blots were left at -80^0C for longer duration in order to allow the label to disintegrate or alternatively the blots were stripped prior to reprobing (refer to method 2.3.5).

2.3.4 Radiolabelling

Hexalabel nucleotide labelling kit (Fermentas) was used for radioactive labelling of the probes for southern and northern blots. Manufacturer's protocol was followed without any significant modification. 100 ng of DNA (visually estimated on an agarose gel) was used as a template. The reaction mixture was denatured by boiling for 10 minutes prior to setting-up the hybridization. γ ^{32}P dCTP (Amersham and Hartmann analytic) was used as the radioactive dNTP.

To test the extent of incorporation of the label 1ul of the final reaction volume of the labelling reaction was added to 950 ul of 3.5 % perchloric acid, 100 mM NaPPi and 50 ul of carrier DNA. A GF/C paper (Schleicher & Schuell Bioscience GmBH) was wet with 1N HCl, 10mM NaPPi and 1ml of the above mentioned mixture + 50 ul of carrier DNA (Sheared Salmon Sperm DNA, Eppendorf) was applied to it. The paper was washed throughly with 1N HCl, 10mM NaPPi and the radioactivity determined with a Geiger counter.

2.3.5 Reprobing

Southern blots

Blots were boiled in 0.1 X SSC, 1% SDS in a microwave oven for 10 minutes. The extent of stripping was estimated by exposing the blots overnight.

Northern blots

Blots were incubated at 65^{0}C for one hour. Often the blots were just stored at –80^{0}C in a moist condition until the label had disintigrated.

2.4 Cloning

Cloning procedures were used in order to achieve the follwing purposes.

A. To use the cDNA for subsequent bacterial expression.

B. To incorporate fragments of cDNA in oppposite orientations with a spacer and fragments of genomic DNA and cDNA in opposite orientations in a pUAST vector for the RNAi studies.

C. To incorporate southern blot probes in bluescript vector to facilitate easy storage and amplification.

Cloning Procedures

2.4.1 Blunt/Cohesive End Ligation

Both insert and vector were digested with appropriate enzymes for not more than 2-3 hours at recommended tempratures. DNA samples were fractionated by agarose gel electrophoresis, visualized briefly under UV light (preferably the low power UV torch), cut out with a clean blade and gel purified. DNA was usually estimated by visualization in the gel (preferred) and/or by photometric measurement.

For cohesive/blunt end ligations 1:5 or 1:3 was used as a vector:insert ratio. In extreme cases the ratio 1:10 was also used. For blunt end ligations 4% PEG was incorporated in the ligation mixture as recommended by the manufactureres. The amount of vector and insert needed was calculated using the formula: P moles of DNA = (ug of vector x 1500) / bp of the DNA. Typically 20-50 ng of vector was used for the ligation reactions. Ligations were performed for 1 hr at RT or overnight at 18 ^{0}C followed by transformation. DNA was isolated from the colonies (blue-white selection whenever possible) and after minipreps digested with appropriate enzymes.

2.4.2 TA Cloning Using TOPO Cloning Kit

Concept of TA cloning is illustrated in figure 2.4.2a.

Manufacturer's instructions were followed while using the TA cloning.

The insert and vector mixture was incubated together for up to half an hour whenever bigger inserts (> 2 KB) were used. Similar yield of positive clones was obtained when the amount of vector used was reduced to half the value than recommended in the standard protocol. Transformants were always subjected to blue/white selection prior to screening.

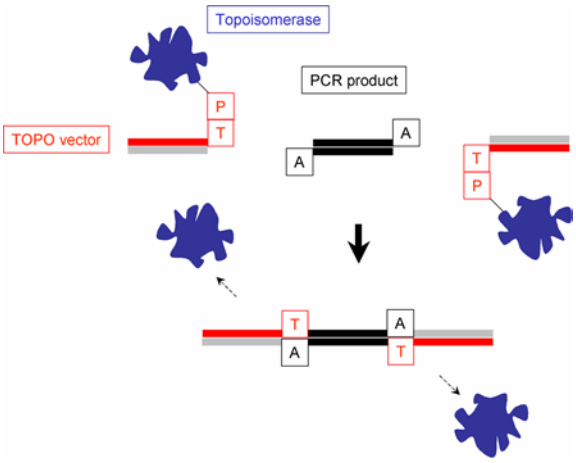

Fig.2.4.2a- TA cloning.

In order to ensure that the insert contains A overhangs, the PCR program was modified allowing an extra 8 minute elongation step at 72^0C. In cases where Taq polymerase was not used for the PCR, the product was incubated with taq and dNTPs (or dATPs) for 10-15 minutes at 72^0C in a water bath or heating block prior to cloning.

Protocol for TOPO – TA cloning: (Modified from invitrogen).

PCR product	0.25-2 ul
Salt solution	0.5 ul
TOPO vector	0.5 ul
dH_2O	add to 3 ul
Final volume	3 ul

2.5. Protein Analysis

2.5.1 SDS PAGE

The Laemmli buffer system was used for SDS-PAGE. Depending on the size of the protein to be fractionated 5%, 7.5% and 12.5% running gels were used. 2X or 5X sample buffer was used for loading protein samples onto the gel.

2.5.2 Protein Staining In The Gel

Coomassie Staining

Gels were stained with Coomassie stain (1%BPB, 40% methanol, 20% acetic acid) for two hours under shaking. 40%methanol + 20% acetic acid was used to destain the gel. Usually a ball of tissue paper was included with the gel in the destaining jar to accelerate the process.

Silver Staining

For visualizing weaker bands silver staining was used. A standard procedure was applied and care was taken not to touch the gel with bare hands. The gel was soaked in distaining solution (40% methanol, 20% acetic acid) for 30 minutes, washed in Mili-Q water, followed by soaking the gel in solution 1 (0.3% sodium thiosulphate, 0.15% potassium ferricynide and 0.05% sodium carbonate) for 90 seconds under shaking. A wash with MiliQ water was included to remove the colour. The gel was incubated in solution 2 (0.1% $AgNO_3$) for 30 minutes in the dark. The gel was washed 2X, 5 minutes each with solution 3 (2.5% sodium carbonate) with constant shaking. The gel was left in solution 4 (2.5% sodium carbonate, 100ul/100ml formaldehyde) until bands appeared. The reaction was terminated with 1% acetic acid.

Chloroform Staining

As a quick staining method gels were immersed in chloroform and the fractionated proteins were visualized under UV as faint white bands.

Gel Drying

Gels were left in the gel drying solution (3% glycerol, 20% methanol) overnight, followed by covering them in a cellophane paper (with sufficient water to exclude air bubbles), sandwiched between two sheets of blotting paper and vacuum dried overnight.

2.5.3 2D-PAGE For The Isolation Of The nc82 Antigen From Fly Head Homogenate

Drosophila head homogenate was fractionated it two dimensions based on charge and mass properties of the proteins. Non Equilibrium PH Gradient Electrophoresis (NEPHGE) was used as the 1st dimension and 12% SDS PAGE was used as the 2nd dimension. Basic working principle of the method is shown in figure 2.5.3a.

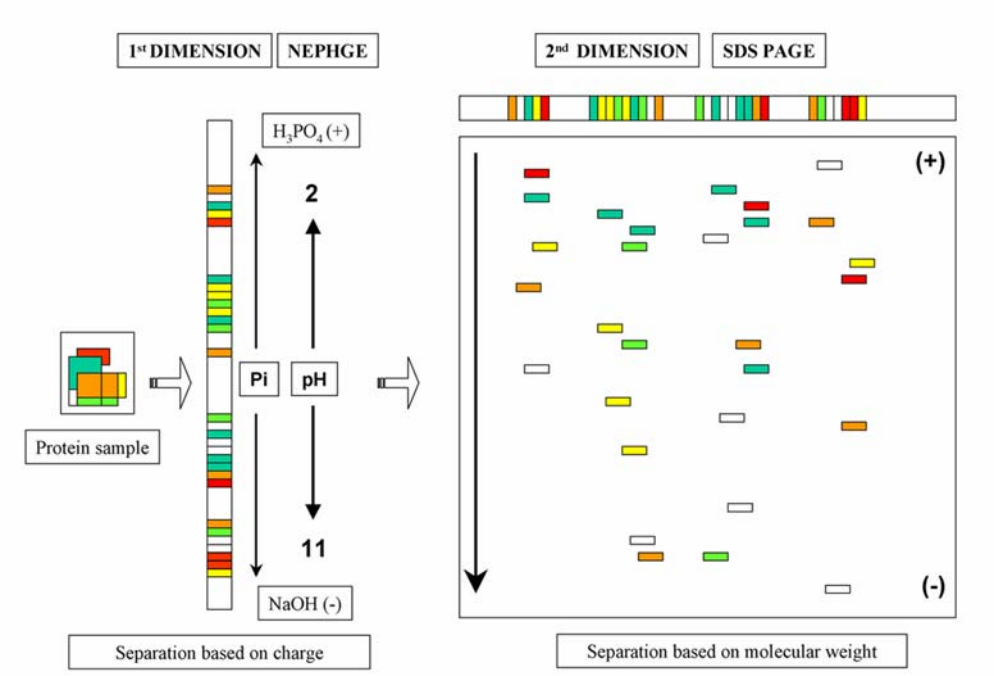

Fig. 2.5.3a- 2-D PAGE set up and its working principle.

500 fly heads were homogenized in 200 ul 2X modified Laemmli sample buffer (without bromophenol blue and glycerine) with 10 strokes in a glass homogenizer, centrifuged at 10,000 rpm for 10 minutes at 4^0C follwed by ultracentrifugation at 35,000 for 1 hour at 4^0C. Protein in the supernatant was precipitated overnight by adding 9 volumes of chilled acetone at

–20^0C. The pellet was washed 2X with 90% chilled acetone and dried under vaccum for 5-10 minutes. The pellet was redissolved in minimum amount (200 ul for 500 heads) of Lysis buffer (I1, see Appendix 3) an equal amount of Lysis buffer 2 (I2, see Appendix 3) was added to the redissolved pellet. The homogenate was loaded onto already prepared rod gels (see Appendix 3). Prior to rod gel preparation, glass tibes were rinsed with

isopropanol, rinsed with Na-Silicate and baked in an oven at 110^0C for 1 hr. The tubes were marked from the bottom to 12 cm and the bottom was sealed with parafilm. After rod gel casting 100-200 ul of sample was loaded on top of the gel rods alongwith Cytochrome C as a marker in one of the rods. Samples were overlayed with 20-40 ul of overlay solution/buffer (J, see Appendix 3). 10mM H_3PO_4 (running buffer, M, see Appendix 3) and 20mM NaOH (N, see Appendix 3) were the electrolytes at the two poles as anode and cathode respectively. Electrophoresis was carried out at 200V for 15 minutes follwed by 300V for 30 minutes and finally at 400V for 90-120 minutes. Gels were removed from the glass rods and processed for the second dimension prior to sealing them on top of 12.5% SDS-PAGE (view SDS-PAGE for details). SDS–PAGE was immunoblotted and the blots were probed with anti-nc82 to detect the spots (refer to method 2.6.1 for western blots).

2.6 Protein Detection, Purification And Localization

2.6.1 Western Blots

For high molecular weight proteins routine western blotting protocol was modified as follows.

The western blot buffer was stored at 4^0C. The nitrocellulose membrane (0.45 um, Schleicher & Schuell Bioscience GmBH) sandwitched between the filter papers was soaked into the western blot buffer for at least 15 minutes prior to blotting. The stacking gel was separated and discarded from the running gel. The running gel was briefly washed in the western blot buffer to remove the foam created by SDS in the running buffer. Blotting was typically carried out for 2 hours at room temprature. Efficiency of the western transfer was qualitatively determined by immersing the membrane for 1 minute in Ponsue – S solution (Sigma) and washing it thoroughly under distilled water afterwards.

The blot was marked for lanes with a ball point pen or pencil. Blocking was carried out by immersing the the membrane in warm (Ca. 50^0C) blocking solution (5% milk powder in 1X TBST) and incubation at 4^0C overnight or for 1 hour at RT.

Excess blocking solution was washed off by 3X washes of 10 minutes each with 1X TBST. Primary antibody was appropriety diluted in 1X TBST. Staining was carried out in glass/plastic dishes or sealed plastic bags for 1 hour at room temperature. Blots were washed 3X for 10 minutes each with 1X TBST follwed by 2^{nd} antibody incubation (diluted in 1X TBST) for 1 hr at RT or overnight at 4^0C. Blots were washed 3X, 10 minutes each with 1X TBST and subjected to ECL detection. ECL solutions (Amersham Biosciences) were mixed following the manufacturer's instruction, blots were covered with the mixture for 1 minute, excess of the solution was drained and the blots were exposed to X ray films for 5 – 10 minutes. Films were submerged in developing solution (2min.) – flowing water – fixing solution (2 min) – flowing water and dried at 37^0C.

Marker proteins from the blot were traced on the blots along with the borders of the nitrocellulose membrane after orienting the developed film with the original blot.

2.6.2 ECL Detection

When the blot membrane is incubated with peroxide and luminol local generation of light takes place wherever the peroxidase coupled 2nd antibody is bound. This luminescence is trapped on an x-ray film for visualization and permanent record.

Membranes were kept moist between reprobings. ECL detection kit available from Amersham biosciences was used for western blots.

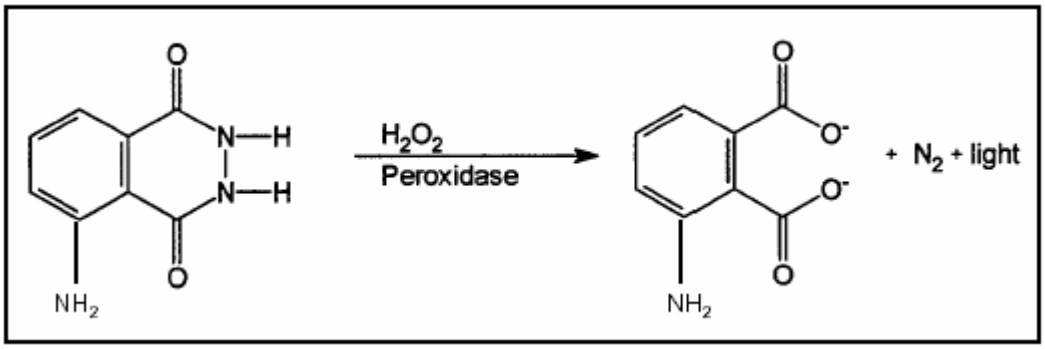

Fig. 2.6.2a – Enhanced chemiluminescence.

2.6.3 Protein Staining On Nitrocellulose Membrane

Ponsue-S Staining

To ensure the extent and quality of protein transfer blots were immersed in commercially available Ponsue-S stain (0.1% Ponsue –S w/v in 0.5% acetic acid, Sigma) for a few minutes and then washed under distilled water prior to blocking in order to remove excess stain. Bands could be marked with a pencil at this stage.

Amido Black/India Ink Staining

0.15% India Ink (Pelican GMBH) in 1X TBST (with 0.5% Tween 20) was applied to the blots overnight prior to washing the excess stain away with TBST. Blots could be stored as permanent records after drying.

Coomassie Staining

Coomassie stain (see staining gels) was alternatively used to stain the blots by applying the staining solution to the blot for a few seconds prior to washing with distaining solution/water. Blots could be stores as permanent records after drying.

2.6.4 Immunoprecipitation

An immunoprecipitation protocol using high salt buffers (Burridge and Bray, 1975) was standerdized and used to immunoprecipitate BRP protein from *Drosophila* brains (developed in colaboration with Roland Jeblick, F2 laboratory course, Oct-Dec 2003).
1000 heads were homogenized in 2 ml of buffer A or buffer B (see Appendix 3) freshly supplimented with 0.5mM final concentration of PMSF and Complete^{TM} protease inhibitor tablet (Roche)(1 per 50 ml of buffer) in a glass homogenizer with 10 strokes after powdering in a prechilled mortar. Homogenate was centrifuged for 10 minutes at 14,000 rpm at 4^0C. 100-500 ul of the MAB nc82 (monoclonal hybridoma supernatant) was added to the supernatant and incubated for 30 minutes to 1 hour at 37^0C. 20 ul of protein-A agarose beads (Invitrogen) were added to the mixture and incubated at RT for 30 min to 1 hour or 4^0C overnight. Beads were washed with buffer A or B (with 0.2% Triton-X 100) and eluted in 2-5X lammli sample buffer. Samples were fractionated by 7.5% PAGE and immunoblotted. Blots were probed with 1:100 MAB nc82 and subjected to ECL detection.

2.6.5 Immunohistochemistry

Flies were cooled down on ice and were glued to a plastic stick with their wings and abdomen prior to dipping them in prechilled 4% freshly prepared paraformaldehyde (USB) in phosphate buffer (pH: 7.4, Ashburner, Protocol 115). Proboscis and air sacs were removed with the help of a tweezer and sharp blade. Flies were fixed at 4^0C for 3-4 hours and the fixing solution was replaced with 25% sucrose in *Drosophila* ringer, overnight. Fly heads were immersed in carboxymethylcellulose gel on a peg and frozen in melting LiN_2 after orienting as desired and marking the position with a marker pen. Frozen carboxymethylcellulose was cut into a rectangular manner to adjust the spacing between serial sections. Cryosections were collected on pre-chilled SuperFrost™ Plus (Menzel-Glaser GmBH) slides and left at -20^0C for a minimum of 20 minutes prior to drying them at RT for another 20 minutes. Slides were marked with a grease pen around the sections. Sections were blocked with normal serum (Vectastatin™ ABC kit, Vector laboratories) at RT. 1st antibody was applied for overnight incubation at 4^0C. After washing in PBST 2X, 10 minutes each, appropriately diluted 2nd antibody (Vectastatin™ ABC kit, Vector laboratories) was added to the sections for 1 hour at 37^0C.

ABC complex (Vectastatin™ ABC kit, Vector laboratories) was added to the sections for 1 hour at 37^0C after washing the 2nd antibody away with PBST, 2X, 10 minutes. A 1X, 5 minutes wash with PBST followed prior to the DAB reaction (DAB substrate kit, LINARIS ltd). After monitoring the colour development, and obtaining the desired staining, sections were washed with 1X PBST and dH_2O to stop the reaction and were mounted in the vectashield mounting medium.

2.6.6 Fixes For Electron Microscopy

Mouthparts were removed as mentioned in method immunohistochemistry. Fly heads were bisected from midline and transferred to modified Karnovsky's fixative (see Appendix 3) (Fröhlich and Meinertzhagen, 1982) for 2 hours at 4^0C. Fixative was later replaced with 4.5% Sucrose in 0.1M Na-cacodylate, 7mM $CaCl_2$ at 4^0C, overnight.

2.7 Electro Retinogram (ERG)

An experimental set up as recommended (Heisenberg, 1971) was used without much alteration. Flies were glued to the stand with nail-polish. Electrodes were filled with *Drosophila* ringer. The ground electrode was inserted into the thorax. The recording electrode was placed as described in figure 2.7a. Oscilloscope traces were photographed with a digital camera and processed in photoshop.

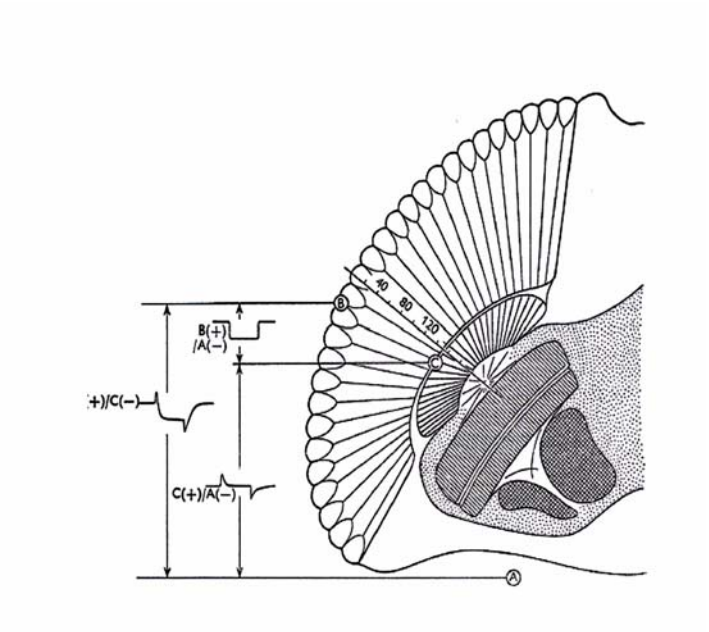

Fig.2.7a- ERG – origin of the receptor potential and ON/OFF transients (Heisenberg, 1971).

2.8 Microinjections And Transgenics

Flies were allowed to lay eggs on apple agar plates at 25^0C for 25 minutes. Embryos were dechorionated with 7% sodium hypochlorite solution for 45 seconds, washed and arranged on a grid in a row of ca.70-100 embryos. Embryos were transferred onto a glas slide precoated with heptane and allowed to stay in a dessicator for 25 minutes. Embroys were covered with injection oil. Plasmid carrying the DNA of interest and the helper plasmid (3:1) were dissolved in the injection buffer, centrifuged in order to remove the suspended material which might clog the injection needle and filled into the injection needle with an ultrathin pasteur pipette. The needle was broken close to the tip thereby adjusting the volume of DNA released per injection. Embroys were allowed to stay at 18^0C for 48 hours. Larvae were collected in a drop of injection oil after 2 days and left at the bottom of a fresh cornmeal medium vial. Larvae were allowed to develop at 25^0C. Emerged flies were back-crossed to the source strain w^{1118} and the F1 was screened for the presence of red eye colour. Transformants were crossed to balancer stocks in order to determine the chromosomal location of the insertion(s). Transformants were inbred to obtain homozygous transgenic stocks.

2.9 Behavioral Assays

Animals were investigated for their locomotor and flying abilities by using different behavioral assays.

2.9.1 Negative geotaxis

Prior to the assay animals were cooled down on ice and their wings were clipped. An empty food vial was caliberated from the bottom. A single fly was kept in the vial and allowed to walk up the wall within the time period of 30 seconds. Assay was repeated thrice for each animal.

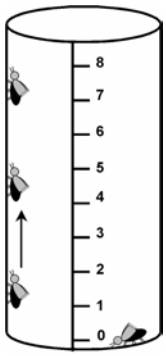

Fig. 2.9.1a – Experimental set up for negative geotaxis.

2.9.2 Walking Behavior

Prior to the assay animals were cooled down on ice and their wings were clipped. A grid of 2 cm unit square was marked on the outer surface of a large petri plate (145 mm). A single fly was released roughly in the center of the plate and allowed to walk for a period of 30 seconds. Sometimes walking was induced by gentle tapping. Everytime the animal crossed a line on the grid a mark was made. Each fly was tested thrice. The number of crossings by each fly was averaged afterwards. Experiment was carried out in red light.

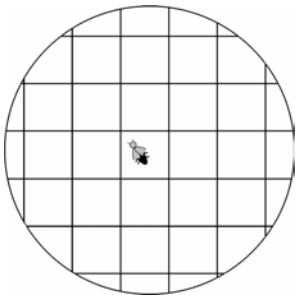

Fig. 2.9.2a – Experimental set-up for the walking behavior.

2.9.3 Flight Tester

A 500 ml calibrated glass cylinder was coated with praffin oil on the inner walls (Benzer, 1973, figure 2.9.3a). Flies were tapped into the cylinder using a funnel. The number of flies sticking to wall at each 50 ml interval as well as those fallen to the bottom were counted. The data was normalized by calculating percentage of animals for each group.

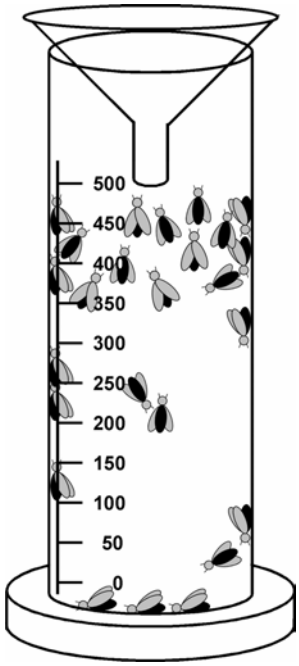

Fig. 2.9.3a- Experimental set-up for the flight tester (not drawn to scale).

2.10 Fly Genetics

Throughout the project wild type as well as mutants of the fruit fly *Drosophila melanogaster* were used for genetic and biochemical analysis. Flies were grown on standard corn meal medium (Appendix 3) and were maintained at 25^0C, 18^0C and 29^0C wherever mentioned.

For bulk isolation of proteins and RNA, stocks of wild type flies were expanded at 25^0C, flies were collected in LiN_2 filled falcon tubes (50 ml) and stored at -80^0C till further use (flash freezing).

Table 2.10- The following stocks were used for different experiments.

Nr.	Stock
1	WT-B
2	Cs
3	w^{1118}
4	w^-; Sp/CyO; Δ2-3 TM2-UBX/TM3 Tb-Sb
5	w^-; Sp/CyO; TM2-UBX/TM3 Tb-Sb
6	y[1]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}KG04653a P{SUPor-P}KG04653b/CyO; ry[506]
7	Df (BSC29)
8	*brp*^{69}/Cyo-gfp (S. Sigrist)
9	UAS –*brp -gfp* 2nd chromosome (S. Sigrist)
10	UAS- *brp -gfp* on 3rd chromosome (S. Sigrist)
11	UAS – *brp* on X chromosome (S. Sigrist)
12	UAS – *brp* on 3rd chromosome (S. Sigrist)
13	UAS – *brp* RNAi (B12) on X chromosome (S. Sigrist)
14	UAS – *brp* RNAi (C8) on 3rd chromosome (S. Sigrist)
15	UAS – *brp* RNAi (DW1)
16	UAS – *brp* RNAi (DW2)
17	*elav* Gal4 on X chromosome
18	*Nina E-gmr* Gal4/CyO

Most of the comman lab chemicals were obtained from USB chemicals, Sigma, MERCK and Roth. Enzymes for molecular biology were obtained from MBI-Fermentas, New England Biolabs and Invitrogen. Commericial sequencing was performed at MWG biotech. Mass spectroscopic analysis of the BRP protein was carried out at Toplabs (Munich). Primers were obtained from MWG biotech and Metabion internatonal ltd.. Some special reagents were obtained from companies wherever mentioned. 2-D gel electrophoresis was carried out at the department of cell biology and zoology at the biocenter in Prof. Dabauvalle's lab. Radioactivity was obtained from Amersham Biosciences and Hartmann analytic ltd. Flystocks were obtained from the Bloomington stock center.

C h a p t e r 3: R E S U L T S

3.1 Identification Of The Protein Recognized By MAB nc82

3.1.1 MAB nc82 Specifically Labels Pre-Synaptic Active Zones

MAB nc82 is widely used as a neuropil marker in confocal images of *Drosophila* brain, providing a structural framework for the "standard brain" and in conjunction with cell-specific stainings (Laissue et al., 1999; Rein et al., 1999; Jefferis et al., 2004; Wilson et al., 2004). For adult brain the staining seems to be uniform, labeling all neuropil. Due to its property to allow a high transparency in immunofluorescent wholemount stainings, this antibody is an ideal tool for 3-D reconstructions and optical sections deep below the brain's surface using confocal microscopy. Fig.3.1.1a shows a confocal section of the adult brain of a transgenic fly expressing the GFP- based sensor cameleon in dopaminergic neurons. The preparation was double stained with nc82 (red) and GFP antiserum (green).

In larval neuromuscular junctions the antigen seems to be primarily present at the presynaptic boutons. In stainings of larval body wall muscles nc82 selectively labels discrete small spots (fig.3.1.1b) surrounded by Dynamin, a GTPase of the peri-active zone known to be involved in synaptic vesicle endocytosis. Distribution of MAB nc82 appears complementary to Dynamin. This suggests that MAB nc82 localizes at the presynaptic active zones.

In order to investigate this finding further, postsynaptic molecules viz. glutamate receptor subunit DGluRIIC (Marrus et al., 2004) and DPAK (Harden et al., 1996) were stained to label the postsynaptic density region (PSDs) at individual synaptic sites (fig. 3.1.1b B and C). The MAB nc82 label always directly faces the center of the postsynaptic densities from the inside and is aligned with the center of PSDs.

These observations strongly suggested that MAB nc82 labelled spots represent the area of presynaptic active zones, the regions of presynaptic plasma membrane, where synaptic vesicles dock, fuse and release their contents into the synaptic cleft upon arrival of an action potential.

Based on its pan-neuronal expression as well as its subcellular localization it was concluded that nc82 antigen could be a potential candidate for an active zone protein. A detailed investigation into the biochemical characterization of the antigen was therefore initiated.

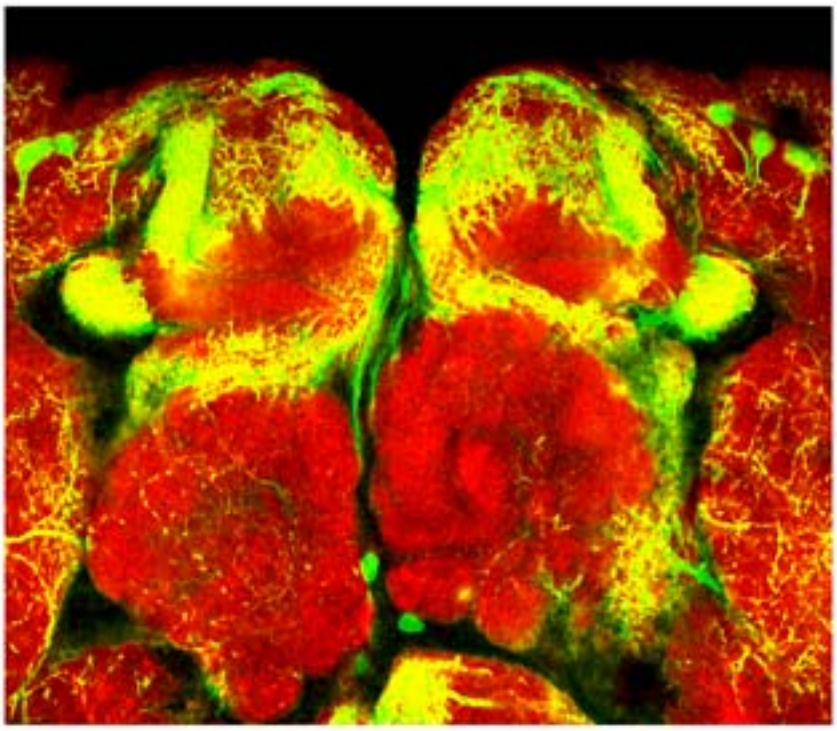

Fig. 3.1.1a- MAB nc82 label as a scaffolding marker (Green- *TH* Gal 4 X UAS-*Cameleon GFP*, kindly provided by A. Fiala).

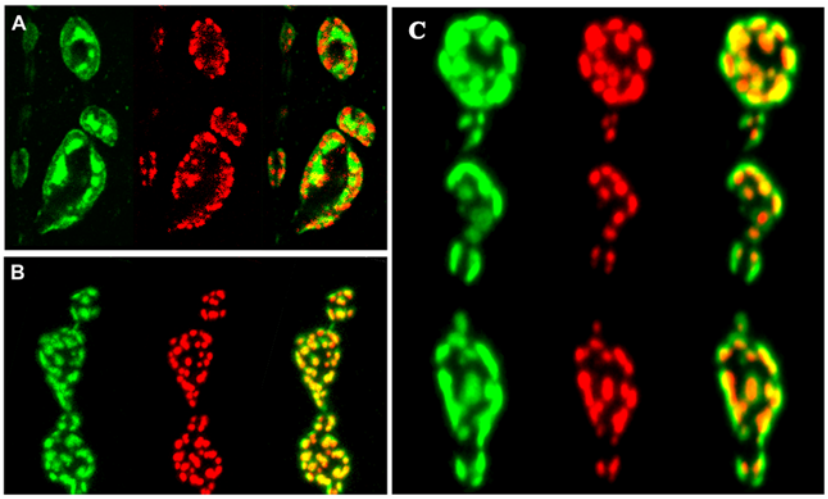

Fig. 3.1.1b- Active zone localization of the nc82 antigen. A: nc82 (red) and anti-dynamin (green) double labelling at the NMJ bouton. (Kindly provided by S. Buchner, E. Buchner and V. Rodrigues) B: nc82 (red) and anti-DGluRII (green) labelling. C: nc82 (red) and anti-DPAK (green) labelling. Third figure in each panel is a merge of both markers used. (C- kindly provided by S. Sigrist).

3.1.2 MAB nc82 Identifies A Protein Of About 200 Kda On Western Blots

The nc82 antibody labeled two prominent bands at 190 and 180 kDa, respectively, a weak irregular band at about 60 kDa, and a few very low molecular weight bands.

Thorough washing of the blot often resulted in the disappearance of the weak bands without affecting the two high molecular weight bands (see Discussion).

Protein extracted from 4-8 wild type heads (males and females) was sufficient to obtain the characteristic nc82 signals. Chilled western blot transfer buffer and prolonged transfer time at RT (2 hours) resulted in consistent results. Heating samples prior to loading did not seem to enhance the western blot signals significantly (fig. 3.2.1a).

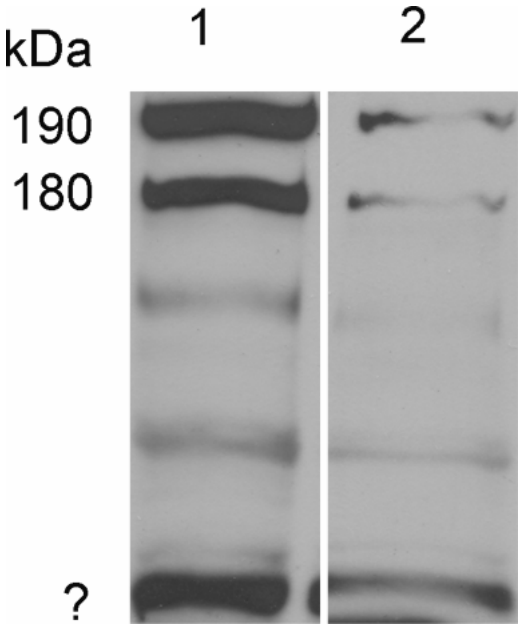

Fig. 3.1.2a – The nc82 Immunoblot from wild-type animals. Fly head homogenate from 8 heads (lane 1) and 4 heads (lane 2) was fractionated on a 5% PAGE and stained with 1:100 MAB nc82 (5 minutes exposure).

3.1.3 MAB nc82 Identifies 2 Spots At About 200 kDa On A 2D (NEPHGE-PAGE) Western Blot

In order to investigate the nc82 antigen further, it was necessary to isolate the antigen from the mixture of brain proteins after head homogenization. Due to the lack of knowledge about its biochemical properties, initial attempts to purify the protein by immunoprecipitation failed. The relatively unconventional approach of 2D-gel electrophoresis was taken in order to achieve this goal. Mass scale isolation of fly heads, homogenization and sample preparation was carried out as described in Materials and Methods (2.5.3). Proteins fractionated on the basis of charge were refractionated based on their molecular weights by SDS-PAGE prior to western blotting. Blots were stained with 1:100 nc82 antibody and the gel was silver/Coomassie stained in parallel. Due to shrinkage during drying scanned pictures of gels had to be resized to match the blots in order to locate the spots on the gel. Two spots at 190 and 180 kDa were observed by Coomassie and Silver staining (fig. 3.1.3a). The experiment was repeated twice. Spots were excised from a Coomassie stained gel and were analyzed by mass spectrometry (Top Labs GMBH, Munich).

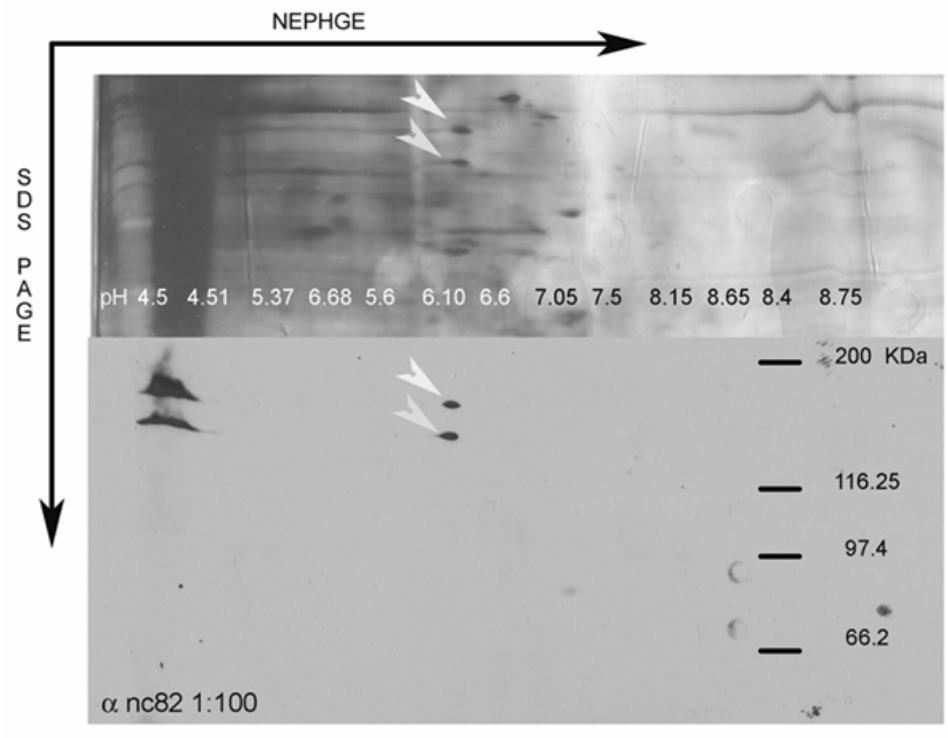

Fig. 3.1.3a – A silver stained 2-D (NEPHGE –SDS PAGE (10%)) of fly head homogenate and subsequent western blot probed with 1:100 MAB nc82.

3.1.4 The nc82 Antigen Solubilizes In High Salt Buffers And Can Be Immunoprecipitated From *Drosophila* Brain Using MAB nc82

Comparison of the peptide masses of the trypsin digested nc82 antigen with expected trypsin fragments of open reading frames (ORFs) of the *Drosophila* genome revealed that the computed gene CG30337 encodes a protein contained in both isolated spots. However, since CG30337 codes for a protein of only 127.4 kDa molecular weight (see Result-3.1.5), it was assumed that the locus has annotation errors and the expected start codon would be upstream to the annotated one. In order to identify the full length mRNA sequence different approaches were taken (RT-PCR, please refer to Results-3.2.1 & 3.2.2). Alternatively, if available in sufficient amounts, pure protein could also be subjected to Edman degradation in order to sequence the N-terminus of the protein. Information about the first 5-10 amino acids of the antigen could have helped to identify the start codon of the gene. To obtain 20-30 pmoles (clearly visible bands on a Coomassie stained gel) standardization of a large scale protein purification method was required. Bioinformatic analysis of the amino acid sequence revealed the presence of several coiled-coil motifs throughout the sequence (see 3.2.3 for more details) and similarity to the myosin heavy chain domain. Solubilization of myosin like proteins is well characterized and documented (Burridge and Bray, 1975). The solubilization protocol for myosin like proteins using high salt buffers as described by (Burridge and Bray, 1975) was followed in order to solubilize the antigen in the absence of ionic detergents like SDS. 600 mM KCl or NaCl was incorporated in the homogenization buffer (see 2.6.4). To confirm the true solubilization of the antigen in the high salt buffers homogenates were ultra centrifuged (35,000 rpm) for 1 hr in order to remove cell debris and membranes as shown in the flowchart below (figure 3.1.4a). After confirming the solubilization of the antigen immunoprecipitation was attempted. Protein-A coated agarose beads were incubated with the antibody at RT for 1hr or extended time periods and head homogenate supernatant was incubated with the antibody bead complex at RT for 1 hour or more or at 4 ^{0}C overnight. Beads were washed with homogenization buffer + 0.1% Triton-X100. Protein was eluted by incubating the beads with Laemmli sample buffer (see 2.6.4). Samples were subjected to western blotting and stained with MAB nc82 after fractionating on a SDS-PAGE (fig. 3.1.4b).

Two bands characteristic of the nc82 antigen were observed at 190 and 180 kDa respectively. Most of the antigen was retained by the antibody adsorbed beads as very little signal was observed in the washes. Coomassie/silver staining of the gel (not shown) also demonstrated the presence of two prominent bands at the expected molecular weight. However, staining of the gel also revealed the presence of several other weak bands near the nc82 bands (see Discussion).

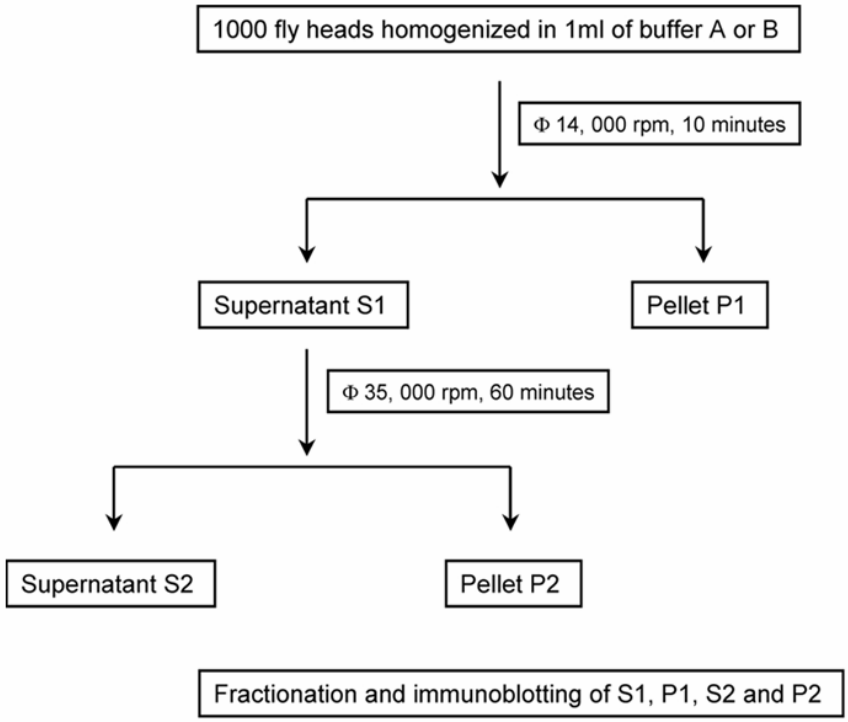

Fig. 3.1.4a – A flowchart showing the homogenization and subsequent centrifugation of the fly head extracts in buffer A or B to confirm the true solubilization in either of the buffers.

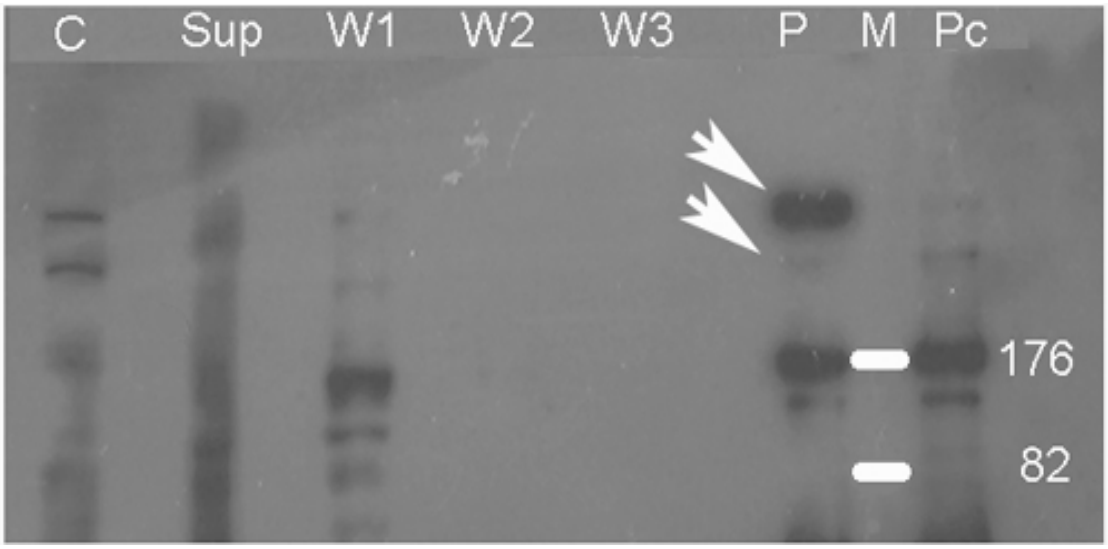

Fig. 3.1.4b - Immunoprecipitation of the nc82 antigen (in buffer A (see Material and Methods 2.6.4)). C – fly heads directly homogenized in the Laemmli buffer, Sup – Post incubation supernatant, W1, W2 and W3 – consecutive washes with buffer A + 0.3% Triton X 100 prior to elution, P – Elution fraction stained with MAB nc82 (1:100), M – Molecular weight marker (in kDa), Pc – Elution fraction stained with only second antibody (goat anti-mouse HRP). (In collaboration with R. Jeblick, F2-student).

3.1.5 The nc82 Antigen Is A Protein Encoded By A Large Genetic Locus On Chromosome 2R

Initial homology searches in the protein database revealed that the ORF encoded by CG30337 has a high homology with the *Anopheles gambiae* annotated protein ENSANGP0000004221 and with the *Apis mellifera* annotated protein XP_392541. CG30337 was also found to be highly conserved in *D. pseudoobscura* (relevant sequences in Appendix 2).

The CG30337 ORF did not show any significant homology to any known vertebrate active zone proteins. In order to verify that the epitope recognized by MAB nc82 was indeed contained in the CG30337 ORF it was decided to express a cDNA of CG30337 in *E. coli*. A cDNA clone (AT09405) corresponding to the exons of CG30337 was available from the Berkley Drosophila Genome Project (BDGP) and was obtained from Resgen. Experiments by I. Schwenkert demonstrated that induction of the expression of this cDNA in *E. coli* produced a protein of about 120 kDa that is recognized by MAB nc82 (fig. 3.1.5a). The translated protein displays similarity to certain conserved domains (Smc ATPase, Myosin heavy chain and ERM (refer to fig. 3.2.2a for gene structure and fig.3.2.3a for domain structure comparison). Biochemically the protein has a pI between 5.9 to 6.4. No transmembrane domains or known nuclear localization signals were detected.

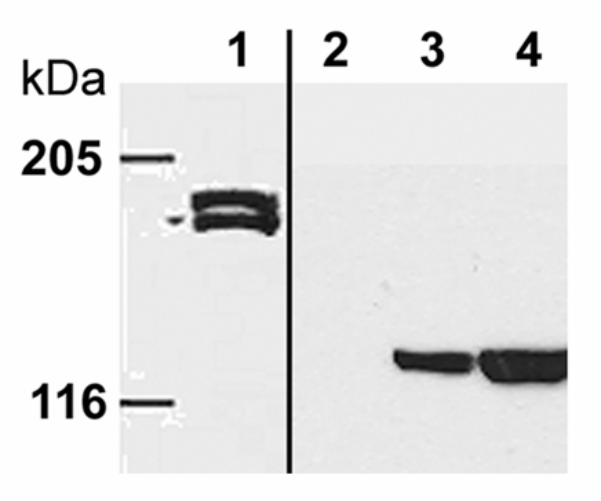

Fig. 3.1.5a- Bacterial expression of cDNA AT09405. Lane 1- Wild type nc82 from fly heads. (Kindly provided by H. Dürrbeck). Lanes- 2, 3 and 4 – 0 minutes, 30 minutes and 60 minutes induction respectively of cDNA AT09405 in *E. coli* (kindly provided by I. Schwenkert).

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Upon comparing the AT09405 translated protein (1133 aa) to its mosquito homologue ENSANGP0000004221 it was observed that though both proteins are highly similar, about 300 amino acids at the N-terminal of the *Anopheles* protein were missing in the *Drosophila* homologue. Mosquito protein was larger in size (1227 aa).

When ENSANGP0000004221 was compared (BLAST) to *Drosophila* proteins it was observed that this protein shows homology with 2 proteins coded by 2 adjacent computed genes on Drosophila chromosome 2R, viz. CG30336 and CG30337 which are apart from each other by about 3 kb. It was also observed that the mosquito protein contains a non-homologous stretch of 34 amino acids that falls between its CG30336 and CG30337 homology regions.

This led to a detailed *in silico* investigation of the intronic region between the CG30336 and CG30337. A 6 frame translation followed by a BLAST search with the "non-homologous" 34 aa stretch from mosquito located a stretch within this intronic region that coded for an almost identical sequence. This stretch was also found to be present in *D. pseudoobscura* (relevant sequences in Appendix 2).

These observations further supported the speculation that the annotation is erratic and would require a detailed experimental analysis of the genetic locus to determine the complete coding region and to identify possible splice variants. An RT-PCR analysis with CG30337 and its neighbouring annotated genes to complete the coding region (see 3.2.1a) and a northern blot analysis (see 3.2.4) to determine the spilce variants were carried out subsequently.

3.2 Characterization Of The Gene Coding For The nc82 Antigen

3.2.1 RT-PCR Analysis Of The nc82 Antigen Coding Locus

Based on conclusions drawn from the discrepancies between observed (127.4 kDa) and expected (180-190 kDa) molecular weights of the protein and from the homology searches with mosquito protein it was thought that CG30336 and CG30337 could indeed be a single gene with a small additional exon present within the large intronic region between the two annotated genes. This hypothesis was tested experimentally by RT –PCR.

Several primer pairs including oligo-dT and as well as gene specific primers were designed in order to investigate the connectivity of the transcripts by RT-PCR (see Appendix 1 for RT- PCR primer sequences).

Total head RNA or poly A^+ selected head RNA served as the template for reverse transcription. Gene specific primers or oligo-dT primers were used for the reverse transcription reaction. RT-PCR products were sequenced and sequences were combined afterwards (figure 3.2.1a summarizes the RT-PCR analysis).

It was concluded that the speculations about CG30336 and CG30337 being part of the same genetic locus and also the presence of the small exon in the annotated intronic region were supported by experimental evidence (fig. 3.2.2a).

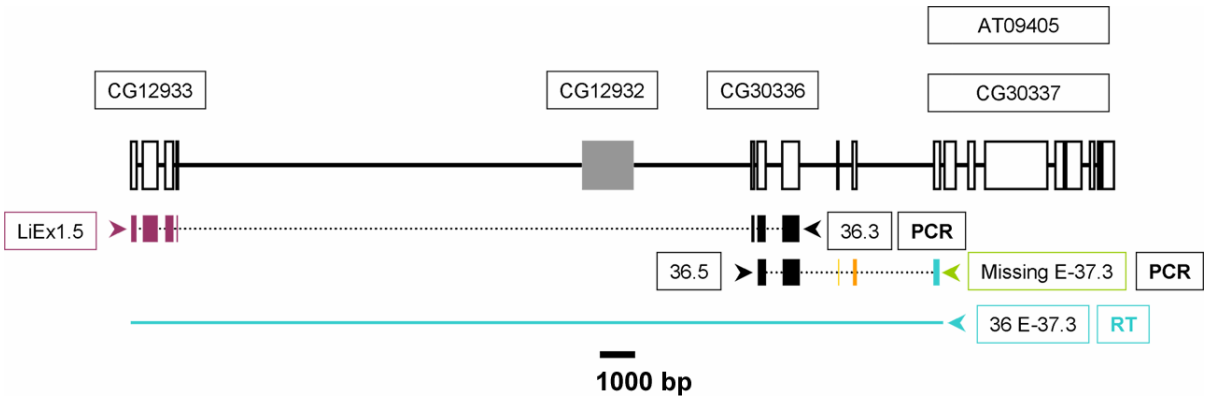

Fig. 3.2.1a- RT-PCR analysis of the genomic region encoding the nc82 antigen. Primer names are indicated next to the arrows.

3.2.2 CG12933, CG30336 And CG30337 (But Not CG12932) Belong To The Same Genetic Locus That Codes For The *Drosophila* Homologue Of Vertebrate Active Zone Protein CAST/ERC

BLAST comparison of CG30337 ORF with the protein database did not reveal any significant homology with known synaptic/active zone proteins of vertebrates. In a reverse approach known vertebrate active zone proteins were compared with the *Drosophila* proteome. A previously described vertebrate active zone protein CAST/ERC (Cytomatrix at the Active zone STructural protein, Ohtsuka et al., 2002; Wang et al., 2002) showed small but significant homology to the ORF of another annotated gene (CG12933) and surprisingly, to the CG30336 ORF.

CG12933 is approximately 22 kb upstream to CG30337 on chromosome 2R. Another annotated gene CG12932 is situated 12 kb downstream to CG12933 between CG12933 and CG30336.

CG30336 and CG30337 are homologous to the predicted *Anopheles* protein ENSANGP00000014221 while CG12933 corresponds to the neighbouring protein ENSANGP00000002918. CG12933 locus is highly conserved in *D. pseudoobscura* also.

In order to determine if CG12933 and CG12932 belong to the same genetic locus that codes for the nc82 antigen, further RT-PCR experiments were carried out.

A gene specific primer (36 E 37.3) designed for the 2nd annotated exon of CG30337 was used to make the 1st strand cDNA, compatible primer pairs designed for the 1st annotated exon of CG12933 (forward primer LiEx1.5) and for the last annotated exon of CG30336 (reverse primer 36.3) were used for the subsequent PCR. The PCR product was sequenced (Appendix 2). Experimental data showed a connection between CG12933, CG30336 and CG30337 in that sequence (fig. 3.2.2a). Attempts to connect CG12932 to CG12933 and CG30336 did not succeed (refer to the northern blot data, 3.2.4).

Also, no splice variants were observed when above mentioned primer combinations were used.

It was concluded that the nc82 antigen is significantly homologous to the vertebrate active zone protein ERC/CAST. It is encoded by a complex genetic locus which comprises the ORFs of CG12933, CG30336 and CG30337. After sequencing the RT-PCR products a composite sketch of the revised coding region was obtained. Fragments were pasted into the AT09405 BDGP cDNA and a new complete-composite cDNA was constructed from cDNA fragments (in collaboration with S. Sigrist). Based on the peculiar instable flight behavior of the RNAi knockdown transgenic animals the nc82 antigen was renamed as "Bruchpilot (BRP)" and its coding region was named as the "*bruchpilot* (*brp*)" locus.

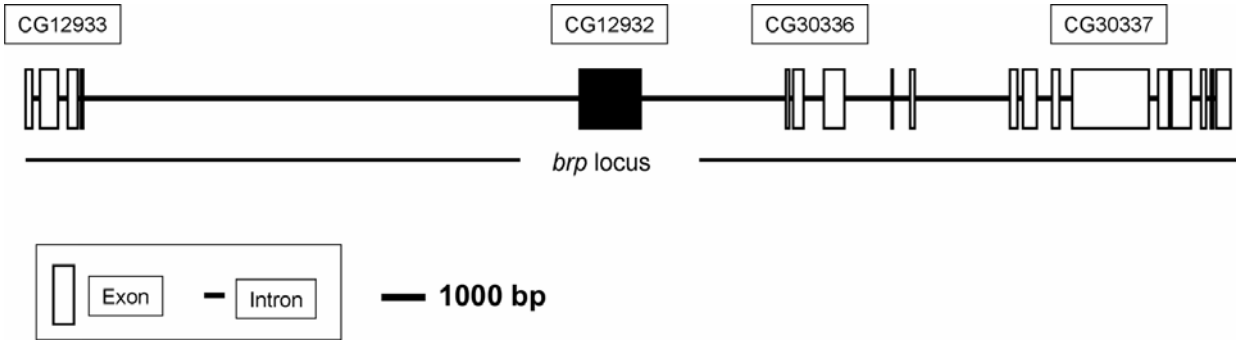

Fig. 3.2.2a – The *brp* locus as determined by RT-PCR analysis, northern blots and homology searches. CG12932 (black box) is situated within the same locus but does not appear to be a part of it.

3.2.3. *Drosophila* BRP Contains A Large C-Terminal Part Not Present In Mammalian CAST/ERC Proteins But Highly Conserved Within Dipteran Insects

The N-terminal part of the BRP protein reveals high homology with all mammalian ELKS/CAST/ERC proteins and the *Anopheles* homolog. The conservation is highest in regions corresponding to the first 5 coiled-coil domains of CAST/ERC (Fig. 3.2.3a, for the domain structure of CAST see Ohtsuka et al., 2002). In *Drosophila* as well as *Anopheles* and *Apis mellifera* the *brp* gene and its homologues encode a large C-terminal region (fig. 3.2.3) which is not present in mammalian CAST/ERCs and for which no homologous proteins apart from insect BRPs are found. The high level of conservation between *Drosophila* and *Anopheles* within this domain, however, indicates that this part is likely to be important for insect BRP function.
MAB nc82 identifies proteins at 190 and 180 kDa from mosquito head homogenates similar to that of the fly (fig. 3.2.3b).

Bioinformatic analysis of the amino acid sequence of BRP predicts a possible nuclear localization (not supported by immunohistochemistry), numerous possible phosphorylation sites, no transmembrane domains, two leucine zipper domains, and a glutamine-rich C-terminus. However, no PDZ interaction motif for RIM interaction as found in several mammalian ELKS/CAST/ERC forms seems to be present in the insect BRP protein family. In addition, significant sequence similarities to Myosin heavy chain, Plectin, and Restin are found, mainly due to coiled-coil regions and leucine zipper domains of the proteins. In the *Drosophila* proteome similarities to LVA (larval lamp), an actin-, spectrin- and microtubule binding protein, to CLIP-190, MTOR (Megator), ZIP (zipper), and MHC (myosin heavy chain) are detected.

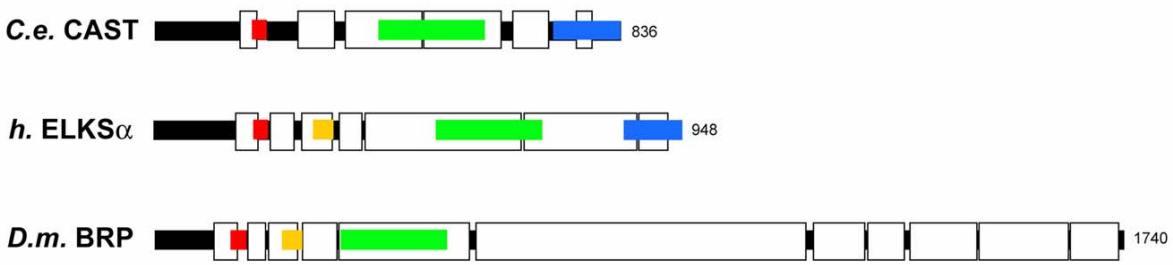

Fig. 3.2.3a – Coiled-coil motifs (white boxes)- as predicted by "Coilscan" (Husar) for *C. elegance* CAST, Human ELKS a and BRP. Red, yellow, green and blue stripes indicate highly similar amino acid stretches respectively (Note: Drawn to scale).

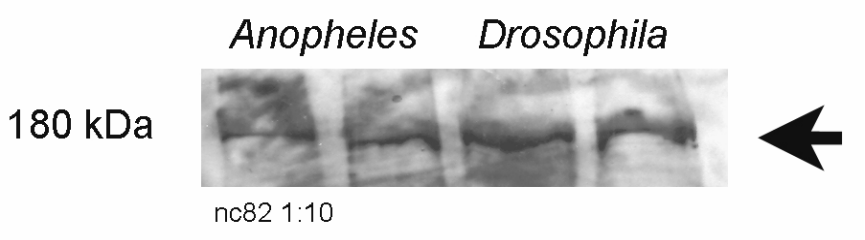

Fig. 3.2.3b- MAB nc82 recognizes an *Anopheles* protein at an identical molecular weight position.

3.2.4 The *Drosophila brp* Locus Codes For A Single 11 kb Transcript And A Smaller 2 kb Transcript

On western blots nc82 antibody recognizes two bands. Mass spectrometric data suggests them to be isoforms of the same protein. It was interesting to have some information about these isoforms. In order to know if they are originating due to splice variants of the *brp* transcripts or whether they are generated by differential post-translational modifications affecting their size and thereby mobility on an SDS-PAGE, northern blot analysis of the gene transcript(s) was carried out.

10 ug of poly A^+ selected wild-type head RNA was blotted on a nitrocellulose after fractionation on a MOPS-formaldehyde gel. Blots were probed with complete cDNA (excised complete *brp* cDNA composed up of CG12933, CG30336 and CG30337 from pUAST vector). A 7 days exposure followed by developing the X-Ray film revealed that the most prominent signal is located at about 11 kb. An additional weak signal was also obtained at 2 kb (see fig.3.2.4a).

Failure to amplify cDNAs of different sizes in any of the RT PCR experiments as well as the detection of only one prominent band on the northern blot indicates the presence of a single major transcript for the *brp* gene and that the two bands obtained on western blots could be due to differential post translational modifications.

In order to further investigate whether CG12932 is also a part of the *brp* locus, a 500 bp genomic PCR product obtained from CG12932 was used for reprobing the blot. A very weak signal at about 4.9 kb was obtained after 21 days of exposure which did not match with the *brp* signal (refer to Appendix 1 for the primer sequences used to make the CG12932 probe).

The *Anopheles* CG12932 homologue lies at a very similar relative genomic position (3R: 52 788 996 : 52 789 985, (Holt et al., 2002)) when compared to the genomic organization of *Drosophila melanogaster*. However, since attempts to connect CG12932 to the *brp* gene by RT-PCR (see 3.2.2) as well as northern blots hybridization failed consistently, it seems unlikely that the CG12932 open reading frame might represent or contain alternatively spliced *brp* exons (fig. 3.2.4a).

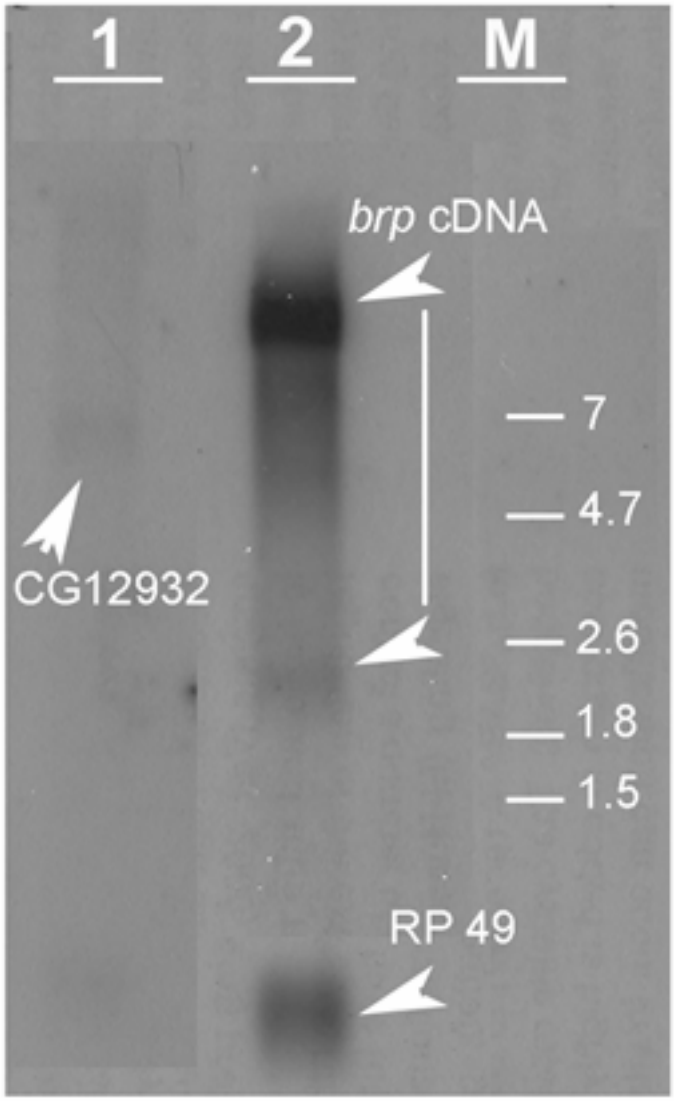

Fig. 3.2.4a- Northern blot analysis of *brp* transcripts from fly heads. 10 ug poly A^+ RNA blotted and probed with *brp* cDNA (7 days exposure, lane 2 top), *rp49* (1 hour exposure, lane 2 bottom) and CG12932 (genomic probe, 21 days exposure lane 1). Molecular weights (lane M) are in kb.

3.3 Functional Investigation Of The Bruchpilot Protein

First hints on the function of a novel protein can be obtained by reducing the expression levels or by the complete elimination of the protein and by studying the phenotypes under these conditions. Genetic manipulations in *Drosophila* facilitate the elimination of a protein by means of destruction of the gene. Usually a transposable element present in the vicinity of the genetic locus can be mobilized in order to create desired deletions in the genetic locus. This may result in a functional null mutation for the protein encoded by that gene. However, finding a transposable element in the vicinity of the gene under study is not always possible. Use of RNA interference (RNAi) to reduce the protein levels has emerged as a powerful technique in modern days (Kalidas and Smith, 2002, Piccin et al, 2001).

A combination of RNAi with the well established Gal4-UAS system (Brand and Perrimon, 1993) has provided a faster alternative when null mutants are not available for a particular gene. This facilitates tissue specific suppression of the protein. After its initial gene structure and protein homology had been clarified, the functional significance of the BRP protein was investigated in detail. Since no P element insertions in proximity to exons of the *brp* locus were available, the above mentioned UAS-RNAi approach was initially used. As the P element insertions in the more distant vicinity of *brp* exons were available, generation of classical null mutants was subsequently initiated. Generation of the transgenic flies expressing UAS-*brp* RNAi, generation of *brp* null mutants, and preliminary functional data obtained from the brain specific RNAi expression will be discussed in next few sections.

3.3.1 Generation Of RNAi Constructs And Transgenic Animals For The UAS-RNAi Knockdown Studies

In order to suppress the expression levels of BRP protein RNAi constructs were generated as described by Kalidas and Smith, 2002 and Piccin et al, 2001. Some reports suggested that the usage of a genomic DNA-cDNA hybrid construct has a stronger effect than cDNA-cDNA hybrids (Kalidas and Smith, 2002) while others show comparable results with cDNA-cDNA hybrids. It was decided to use both methodologies in parallel.

An 862 bp region (DWa) (highlightened in Appendix 2, see fig.3.3.1a) from the AT09405 cDNA (obtained from BDGP) was chosen for the cDNA-cDNA constructs. The selected region was amplified by linker PCR (see Appendix 1 for primer sequences). A 199 bp fragment from an unrelated DNA (coding for GFP) was amplified by linker PCR to serve as a spacer between the inverted cDNA fragments (fig. 3.3.1b).

For the gDNA-cDNA constrcuts the exon-intron region of CG30337 as depicted in fig. 3.3.1a (DWc) was selected and amplified by linker PCR (Primer sequences in the Appendix 1). The corresponding region of the AT09405 cDNA (DWb) was also amplified as above. Regions were selected in such a way that in reverse orientation there are no GTNNNGT splice donor sites.

Fragments were sub-cloned in pBluescript and constructed step by step. Once cDNA-spacer-inverted cDNA and gDNA-intron-inverted cDNA fragments were ready they were excised from the pBluescript and cloned directionally into pUAST. Inserts were confirmed by restriction digestion analysis and sequencing.

Transgenic animals were generated as described in Method 2.8. The cDNA-gfp-cDNA construct incorporated successfully into the germline. No transformants were obtained with gDNA-cDNA construct. 2 flies with orange-red eye colour were obtained. These flies were used to establish individual lines DWT1 and DWT2. When crossed to *elav*-Gal4 both DWT1 and DWT2 did not show any obvious phenotype. Since a collaborating group (S. Sigrist) had obtained RNAi lines (C8 and B12, figure 3.3.1a) with drastic phenotypes when crossed to *elav*-Gal4, our lines were not analyzed further.

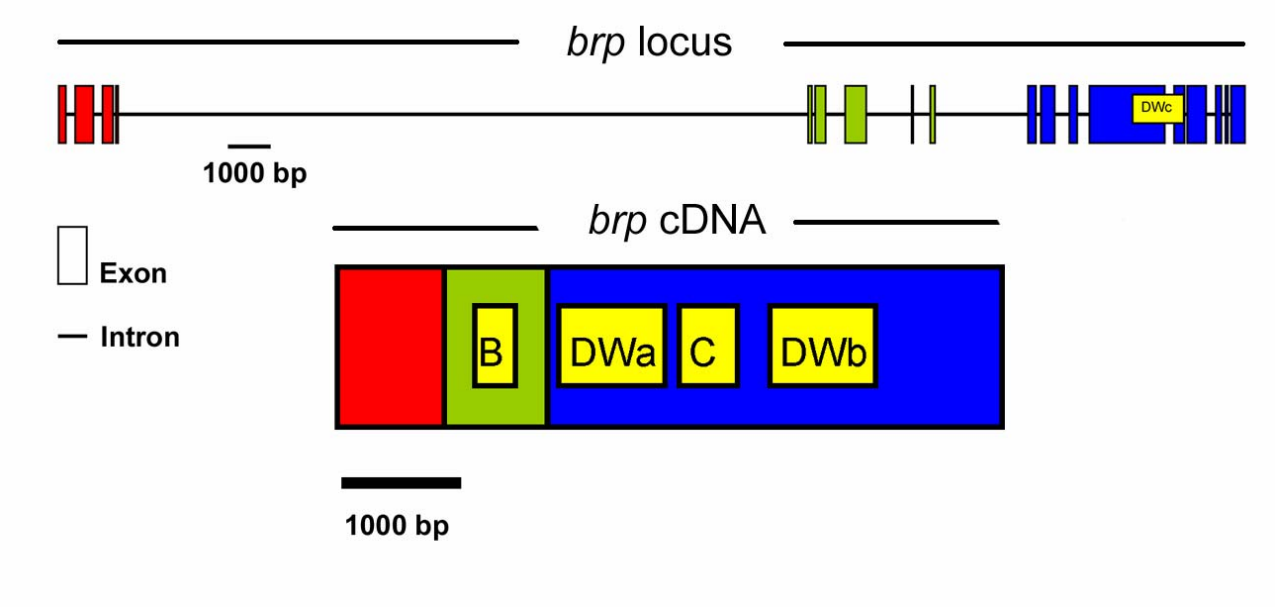

Fig. 3.3.1a- RNAi target regions mapped on *brp* genomic region and cDNA. Regions B and C were targeted to create RNAi lines B12 and C8 respectively (S. Sigrist). Region DWa was used to create cDNA-cDNA RNAi constructs and region DWc and DWb were used in combination to create genomic DNA-cDNA RNAi constructs.

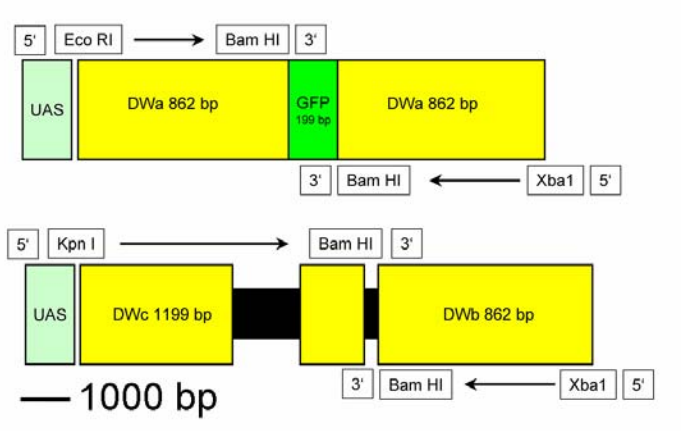

Fig. 3.3.1b- RNAi constructs using DWa, DWc and DWb target regions. The upper sketch depicts the cDNA-cDNA construct while the lower sketch depicts genomic DNA-cDNA construct. Black blocks represent the intronic region of the genomic fragment DWc.

3.3.2 Generation Of Classical Null Mutants By P Element Mobilization Mutagenesis

Since suppression of a protein by RNAi is never complete and useful P insertions were available it was decided to attempt the generation of deletions in the *brp* gene that would eliminate the protein. P element stock 14101 available from Bloomington stock center was used for this P element mobilization experiment. This line was described having 2 P insertions about 100 kb apart from each other on the 2nd chromosome. The genotype of the line is as follows (henceforth referred to as P1P2/CyO).
y[1]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}KG04653a P{SUPor-P}KG04653b/CyO; ry[506] (refer to Materials and Methods 2.10 for a detailed listing of all the fly stocks used for this experiment).

Insertion P1 is described as being in chromosomal segment 45D4-5, proximal to an unrelated gene *wun2* inserted in one of the exons. Insertion P2 is in 45E4, 5kb downstream to the last annotated exon of CG12933 (fig. 3.3.2a).

Other lines that could have been considered for this mobilization experiments (stock numbers and description of all available stocks in table 3.3.2a) had the insertions at least 3-9 kb downstream to the last annotated exon of CG30337.

Bloomington stock No.	Genotype (1; 2; 3)	Distance from the *brp* locus	Break point or insertion site
15570	y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2} CG1888[EY02539]; +	9 kb downstream to annotated last exon of CG 30337	045F01
14960	y[1] w[67c23]; P{y[+mDint2] w[BR.E.BR]=SUPor-P} KG08588; +	9 kb downstream to annotated last exon of CG30337	045F01
13948	y[1] w[67c23]; P{y[+mDint2] w[BR.E.BR]=SUPor-P} KG03268; +	3 kb downstream to annotated last exon of CG30337	045F01
14101 2nd insertion	**y[1]; P{y[+mDint2] w[BR.E.BR]=SUPor-P} KG04653a P{SUPor-P} KG04653b/CyO; ry[506]**	**5 kb downstream to last annotated exon of CG12933**	**045E04**
14101 1st insertion	y[1]; P{y[+mDint2] w[BR.E.BR]=SUPor-P} KG04653a P{SUPor-P} KG04653b/CyO; ry[506]	Near the last annotated exon of *wun2* on 2R, 100 kb from 1st exon of CG12933	045D04

Table 3.3.2a- Description of the P element insertions around the *brp* locus.

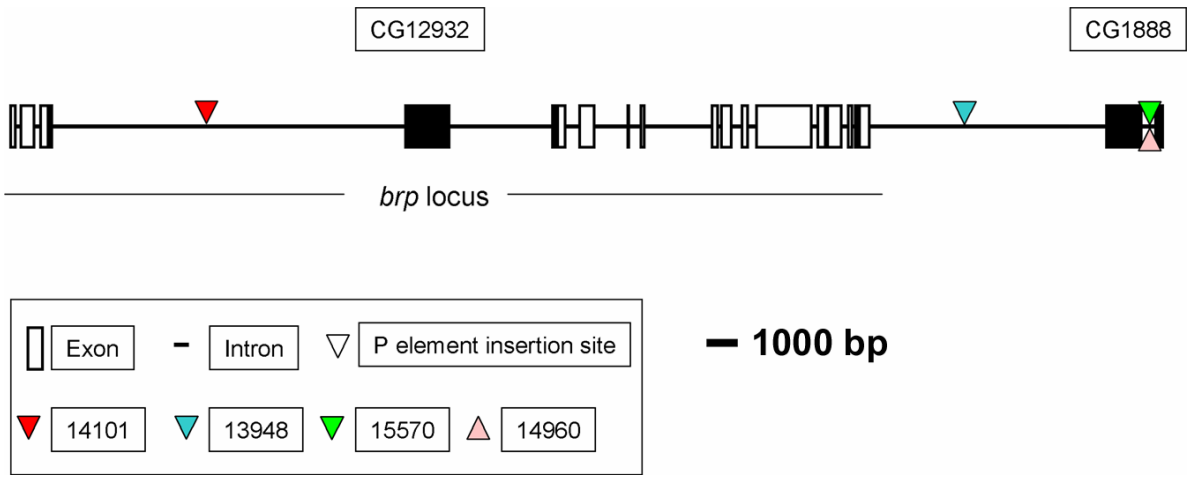

Fig. 3.3.2a – P element insertions flanking the *brp* locus. The first insertion for "14101"-P1 exists about 100 kb upstream on the same chromosome (table 3.3.2a). The insertion "14101" P2 is shown here in red.

Small deletions at the 3' end of the gene usually result in a truncated product which may or may not produce a null mutant. On the other hand, deletions at the 5' end tend to result in functional null mutants more frequently. Therefore, in order to obtain a 5' deletion in the CG12933 locus stock 14101 was used for P element mobilization.

In the F2 generation, vials were screened for flies with white eyes and single lines were set with each scored jump out event.

The genetic crossing strategy was designed as depicted in the following flow chart (fig. 3.3.2b). Jump out lines were set up on the basis of loss of eye colour to white. Flies were balanced on CyO. About 420 individual jump out lines were set up. Due to the usage of a weak double balancer stock a large number of weak lines did not survive and the final number of surviving lines was reduced to about 300 (table 3.3.2b). Lines which were homozygous lethal were considered candidates for null mutation and were selected for further analysis. About 20% of the lines were homozygous viable. The rest were subjected to screening by southern blots.

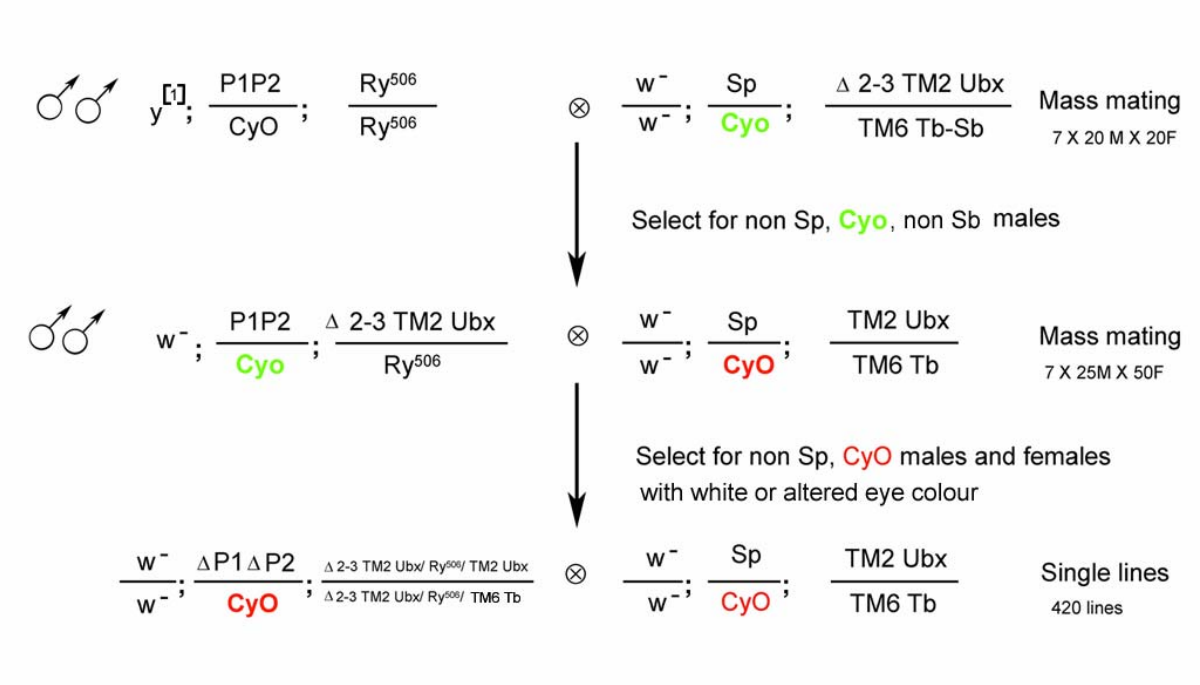

Fig. 3.3.2b- P element mobilization strategy for the "14101" insertion line.

1.	Total lines set up	420
2.	Lines survived as balanced stocks	300
3.	Total homozygous viable lines	60
4.	Lines with altered eye colour	5
5.	Lines that had altered band pattern in southern screening	5

Table 3.3.2b – Statistics of the P element mobilization experiment.

Flies were subjected to screening for deletions at the P2 insertion locus as well as the CG12933 locus by southern blotting (see Materials and Methods 2.3.2). Lines that showed any alterations in band patterns as compared to the wild type were selected. Initially 6 lines (8 flies each) were pooled together in one group prior to DNA isolation and the DNA

was blotted in a single lane. However, due to the probes that encompassed 10 kb from the 5' flanking region to the original insertion site, each group showed extra bands which might have arisen by small uninteresting deletions in the intron and hence this strategy was not very efficient in this case.

Since, the most interesting deletions would be expected to affect the exonic region of CG12933, the screening strategy was revised accordingly. An EcoNI/HindIII fragment that covered almost the entire CG12933 genomic region and about 6 kb region upstream to the annotated 1st exon of CG12933 was hybridized with 2 probes amplified by genomic PCR. Both probes recognized the same 8 kb EcoNI/HindIII region. Probe 1 was 1.9 kb probe that covered CG12933. Probe 2 was 1.5 kb probe and covered a region 5.4 kb upstream to CG12933.

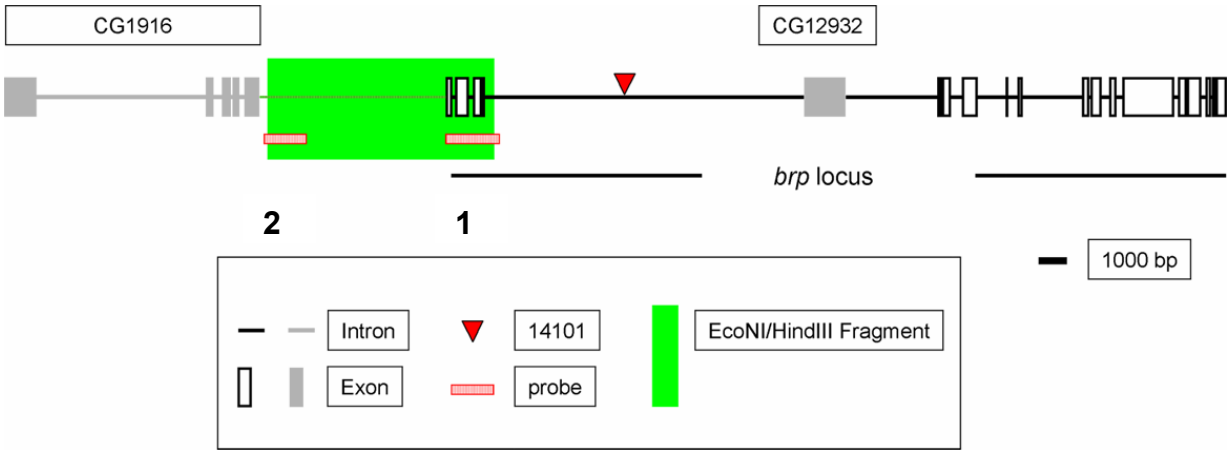

Fig. 3.3.2c – Southern blot screening strategy. Unrelated genes *wnt2* (CG1916) and CG12932 are shown in gray. The genomic region between *wnt2* and *brp* is marked by a dotted line.

These probes were chosen because the vital gene *wnt2* is in the 5' neighborhood of CG12933 and it was important to make sure that the deletions do not extend into this gene. In this strategy the blot was first probed with Probe 1 and then reprobed with probe 2 without the need to strip off the old label. If the deletion had exceeded the region covered by probe 1 but not by probe 2, extra bands would be expected on the blot when

probed with probe 2 (fig.3.3.2c). Based on the alteration in band patterns 5 lines were short listed for further analysis (fig. 3.3.2d).

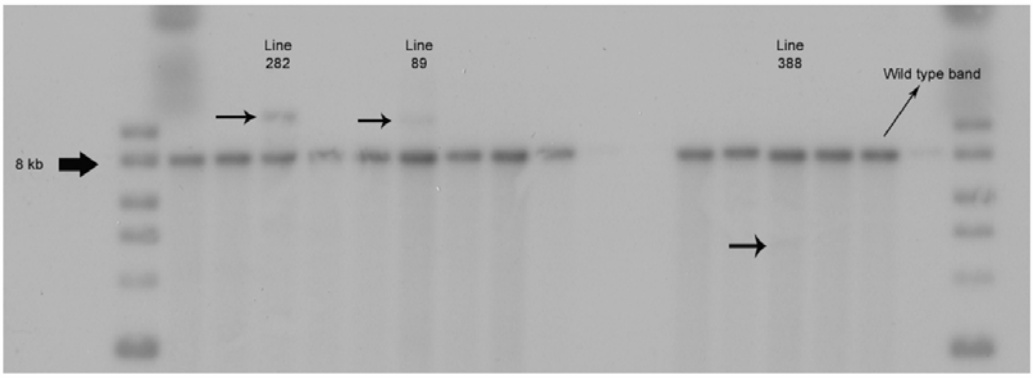

Fig. 3.3.2d- A representative southern blot (probed with probe 1 and 2) showing wild type and altered band patterns.

3.3.3 Pan-Neuronal Expression Of UAS-RNAi Results In BRP Specific Downregulation In The Brain

To determine if pan-neuronal expression of UAS-*brp* RNAi has effects specific to BRP expression levels brain sections were stained with CSP (MAB ab49), Synapsin (MAB 3C 11) and BRP (MAB nc82). As shown in figure 3.3.3a CSP and Synapsin levels appear to be normal in both wild type as well as UAS-*brp* RNAi X *elav* Gal4 offspring. While there is drastic reduction in BRP levels in UAS-*brp* RNAi X *elav* Gal4 offspring as seen with the MAB nc82 staining. It was also concluded that the overall brain structure looks normal as visualized by CSP and Synapsin stainings.

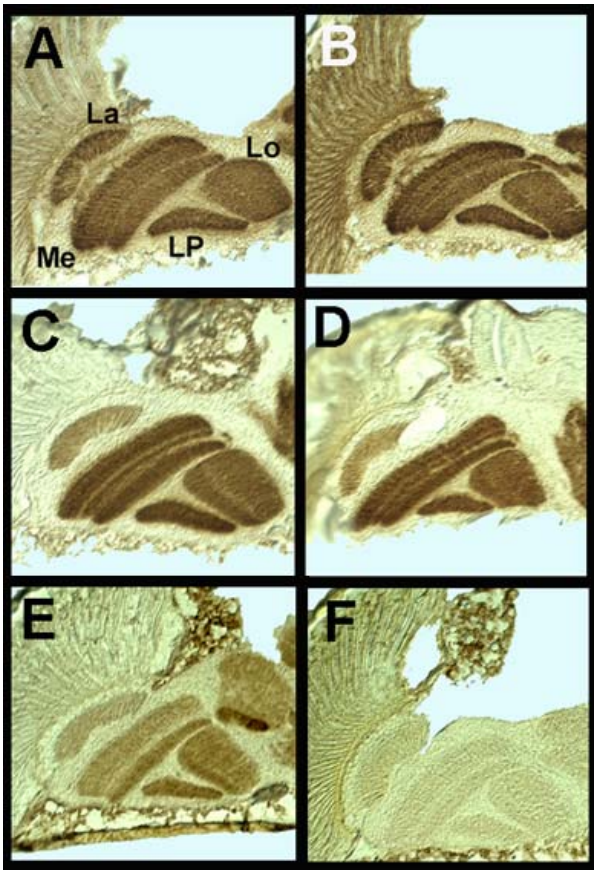

Fig. 3.3.3a- Pan-neuronal expression of UAS-*brp* RNAi. A, C & E- UAS-*brp* RNAi (C8) X w^{1118} offspring. B, D & F- UAS-*brp* RNAi (C8) X *elav* Gal4 offspring. A & B- MAB ab49 (1:100), C & D- MAB 3C11 (1:100), E & F – MAB nc82 (1:50) (La- Lamina, Me- Medula, Lo- Lobula, LP- Lobular plate).

3.3.4 Downregulation Of *brp* Adversely Affects Synaptic Function

Finally, a functional investigation of the BRP protein was undertaken.

Although the knockout of the *brp* gene was not yet completed it seemed important to gain first results on BRP function by characterizing the effects of pan-neuronal knockdown of the gene. As described above, 2 lines (B12 and C8) with effective RNAi transgenes under UAS control were generated by the collaborating group (S. Sigrist) and were kindly provided for structural and functional analysis.

UAS-RNAi *brp* (B12) is inserted on the X chromosome and UAS-RNAi *brp* (C8) is inserted on the 3rd chromosome. Pan-neuronal expression of B12 with *elav*-Gal4 results in late embryonic lethality. Pan neuronal expression of RNAi C8 with *elav* Gal4 does not give a lethal phenotype. However, the synaptic vesicle release at the NMJs of 3rd instar larvae is reduced by 50% (S. Sigrist, Wagh et al., submitted) while bouton morphology as well as number of active zones per bouton are not significantly affected. These flies survive to adulthood although protein levels are very low as verified by immunohistochemistry and western blot (fig. 3.3.4a). Adults show a peculiar phenotype. Flies lack any motivation to fly or walk. When thrown into the air flight is often short and unstable. Based on this phenotype the gene was named “bruchpilot” (German for crash pilot).

In order to investigate if the observed behavioral phenotype as well as the reduced BRP levels on western blot and immunostainings are significant physiologically also in adult synaptic transmission, ERG (Electroretinogram, Heisenberg, 1971) analysis was carried out.

Offspring of the cross RNAi C8 X *elav* Gal4 showed unaffected receptor potential but absence of ON and OFF transients. (Figure 3.3.4a F). An even stronger effect can be expected when only photoreceptor synapses are targeted using *gmr* Gal4 and the RNAi B12 line. This was verified by immunohistochemistry which showed a strong reduction of staining in the lamina (fig. 3.3.4a H). Similar to pan neuronally expressed RNAi C8 animals, *gmr* Gal4 X B12 offspring also showed the presence of normal receptor potential and absence of ON and OFF transients (fig. 3.3.4a J), indicating loss of function at the

targeted synapses. The interpretation of this result is difficult, however, because the regular structure of the eye is disturbed in these flies (see Discussion).

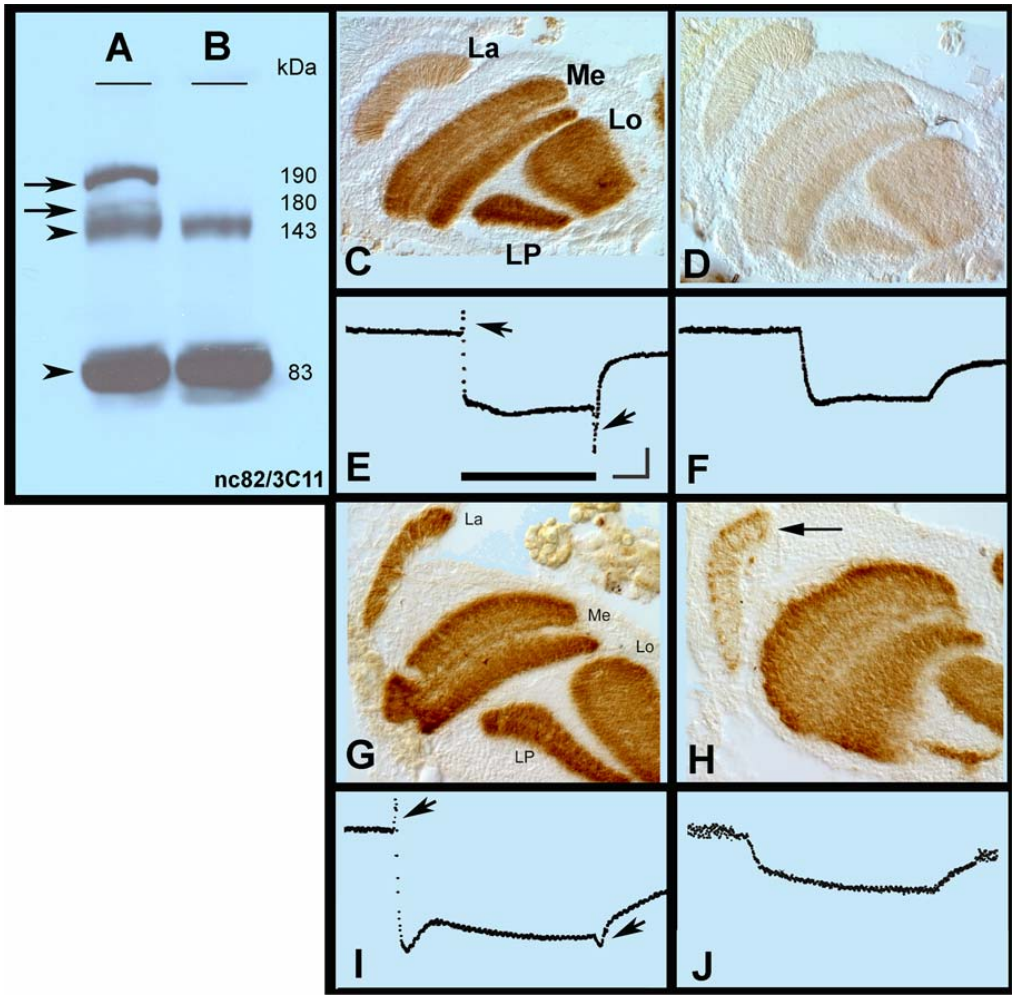

Fig. 3.3.4a –Functional analysis of the BRP protein. Western blot from offspring of w^{1118} X UAS-*brp* RNAi (C8) (lane A) and UAS-*brp* RNAi (C8) X *elav* Gal4 and (lane B) stained with MAB nc82 (arrows) and MAB 3C11 (Triangles). Pan neuronal (D) as well photoreceptor specific (H) down- regulation of BRP using *elav*-Gal4 (C8, D) and *gmr* Gal4 (B12, H) lines. Sections were stained with MAB nc82. C and G are respective controls for both experiments. E & F- Representative ERG profiles from the offspring of w^{1118} X UAS-*brp* RNAi (C8) and UAS-*brp* RNAi (C8) X *elav* Gal4 animals, respectively. I & J - Representative ERG profiles for the offspring of w^{1118} X *gmr* Gal4 and UAS-*brp* RNAi (B12) X *gmr* Gal4, respectively. Arrows mark the ON and OFF transients flanking the receptor potential. Scale 50 mV/200 ms for E,F & I and 10 mV/200ms for J. (La- Lamina, Me- Medula, Lo- Lobula, LP – Lobular plate).

3.3.5 BRP Downregulation Results In Various Behavioral Deficits

The ERG profile of F1 flies from the crosses *gmr* Gal4 X UAS-*brp* RNAi (B12) and *elav* Gal4 X UAS-*brp* RNAi (C8) strongly suggested a synaptic dysfunction at the lamina. Photoreceptor neurons R1-R6 which are known to be involved in visual motion detection (Heisenberg and Buchner, 1977) make their synaptic connections in the lamina. A strong downregulation of BRP in the photoreceptors using *gmr* Gal4 X UAS-*brp* RNAi (B12) offspring (fig. 3.3.4a G & H) as well as the defective ERG (fig. 3.3.4a I & J) of the same animals suggested that these flies might also show a defect in visual behavior. An optomotor behavior paradigm (Buchner, 1976) was used to test this speculation. Animals were fixed in the center of a rotating striped drum and subjected to a movement detection task at various light intensities. They could walk on a small styrofoam sphere whose rotations were monitored electronically. In this way the turning tendency of the animal can be assessed. At higher intensities the *gmr* Gal4 X UAS- *brp* RNAi (B12) offspring showed drastically diminished responses at all light intensities as compared to the controls. The *elav* Gal4 X UAS-*brp* RNAi (C8) offspring failed to walk (see below) and hence could not be tested in this assay. A representative comparison of a single *gmr* Gal4 X UAS-*brp* RNAi (B12) offspring and a control fly is shown in fig. 3.3.5a. The average response at the highest light intensity of 5 flies in each group (inset) demonstrate a constant phenotype. It is concluded that at high light intensities animals with retina specific *brp* knockdown are not absolutely blind but show severely reduced visual motion detection ability.

Initial qualitative observations of the offspring of *elav* Gal4 X UAS-*brp* RNA*i* (C8) animals had indicated that these animals lacked almost all walking activity. They also exhibited very unstable flight. In order to be able to quantify these peculiar observations a few more behavioral assays were carried out on these animals.

When tapped to the bottom of a vial wild type flies have the tendency to climb vertically up the walls which is termed the negative geotaxis. When tested for this ability (see Method 2.9 for the details of all behavioral assays) *elav* Gal4 X UAS-*brp* RNAi (C8) animals showed lack of any motivation to climb up (fig. 3.3.5b-A). These animals barely showed any movement when tested for walking ability (performed in red light, fig. 3.3.5b-B). Finally, the animals were also tested for their ability to fly using the flight tester assay

as described by Benzer (1973). About 45% of the mutant animals sank to the bottom of the paraffin coated cylinder indicating a severely affected ability to fly normally (fig. 3.3.5b-C & D).

Interestingly, a careful investigation also revealed that the flies do not seem to have a mechanistic problem with respect to the ability to move or fly. If left outside on the table they would stay motionless but if pushed continuously or dropped from a distance they would exhibit a normal ability to walk (though with a little limp at times) and fly. Preliminary observations in this respect suggest a motivational deficit in these animals (see Discussion).

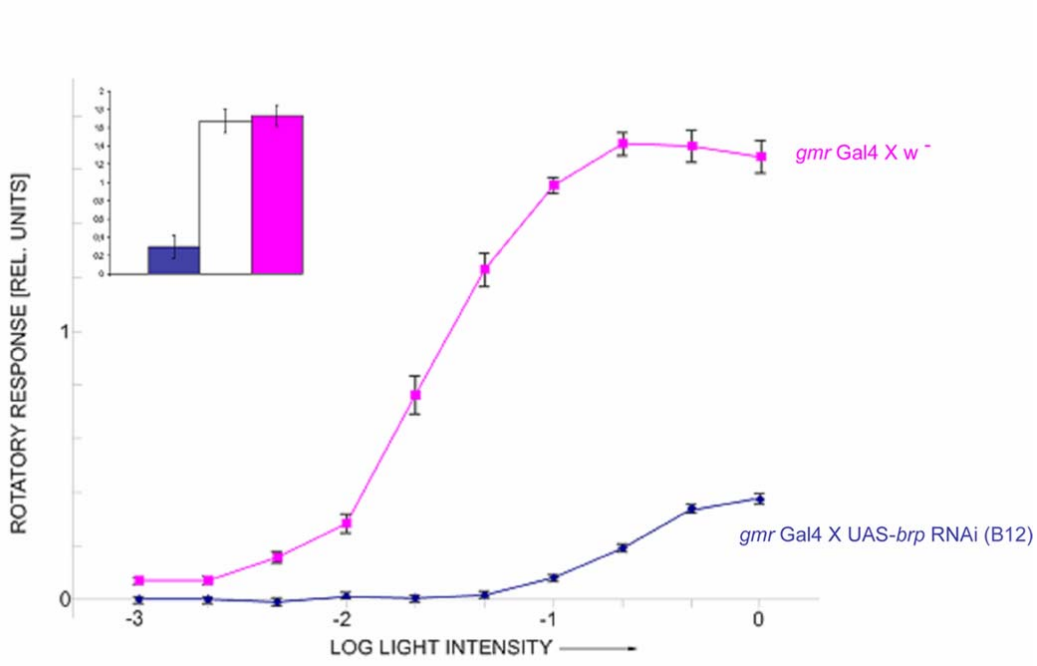

Fig. 3.3.5a – Optomotor response. The graph shows mean rotatory responses plotted against increasing light intensity. Inset- A bar diagram comparing the mean rotatory responses at the highest light intensity for offspring of *gmr* Gal4 X UAS brp RNAi (B12) (left column), w^{1118} X UAS-*brp* RNAi (B12) (middle column) and *gmr* Gal4 X w^{1118} (right column)(n = 5, kindly provided by E. Buchner).

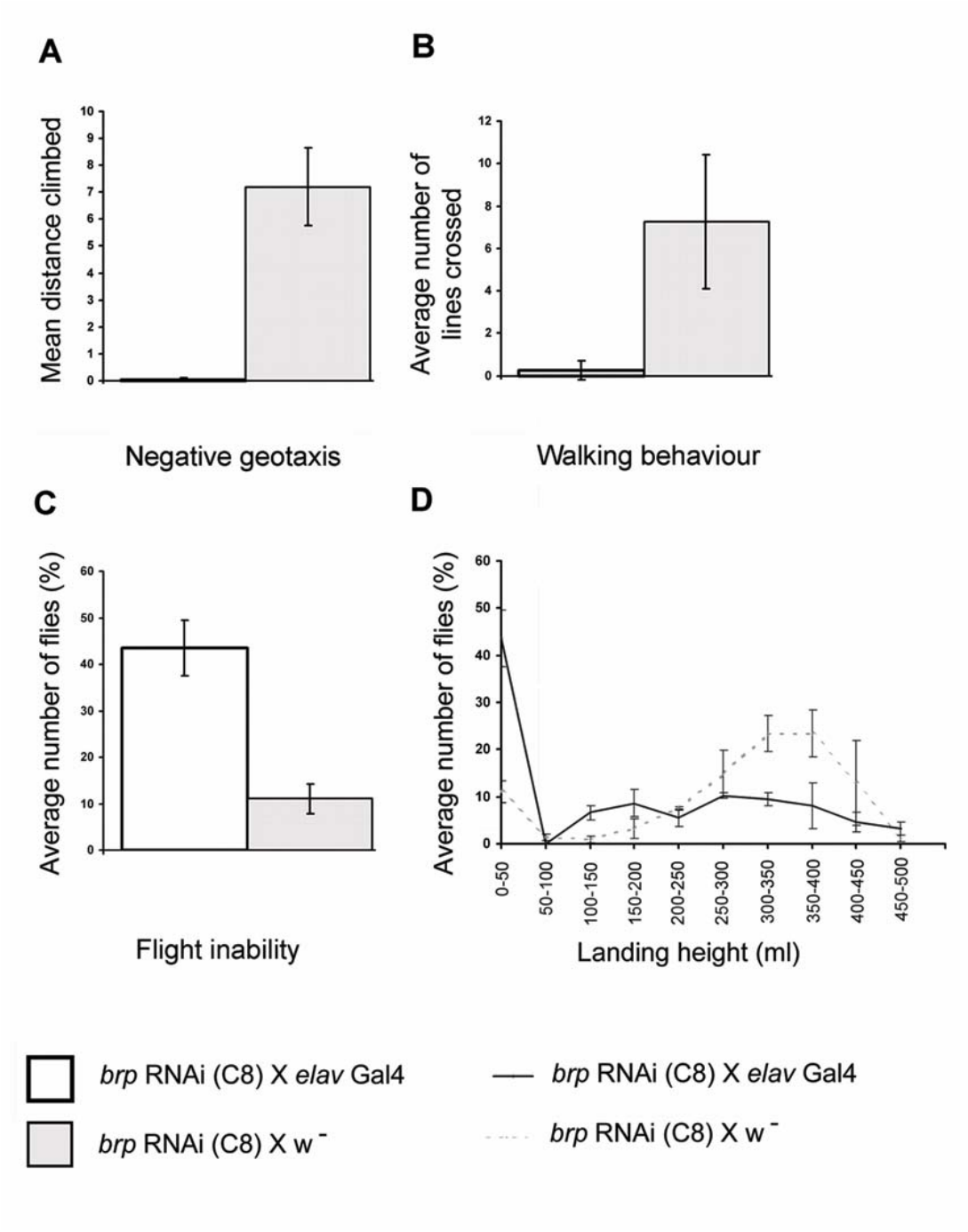

Fig. 3.3.5b- Locomotor activity and flying ability of the UAS-*brp* RNAi X *elav* Gal4 offspring. A- Negative geotaxis (n=5), B- Walking behavior (n= 8), C- Flight tester – total number of flies at the bottom of the cylinder (n= 3) and D- Flight tester curve- entire distribution (n= 3) (note: B was performed in red light).

3.3.6. BRP Downregulation Results In Loss Of Synaptic Ribbons

After observing the functional defects at the photoreceptor synapses, an ultrastructural study was undertaken in order to investigate whether there are structural changes associated with the dysfunctional synapses. The *gmr* Gal4 X UAS-*brp* RNAi (B12) as well as the *elav* Gal4 X UAS-*brp* RNAi (C8) offspring were fixed in modified Karnovsky's fixative and processed for EM (see the Method 2.8). Photoreceptor synapses were identified with the help of capitate projections (Stark and Carlson, 1986). In the *gmr* Gal4 X UAS *brp* RNAi (B12) offspring cartridge assembly did not appear very well preserved as compared to their wild type counterparts. T-bars were very rare and sometimes structures similar to T-bars were observed floating in the presynapses (fig. 3.3.6a). In the *elav* Gal4 X UAS-*brp* RNAi (C8) offspring cartridge assembly appeared well preserved, however T-bars were totally absent. A quantitation of T-bars from a total of 15 cartridges evaluated in 3 individual sections of the RNAi expressing animals showed no recognizable T-bars as opposed to 11 clear T-bars in 15 cartridges of 3 wild type sections. In many instances cartridges with homogeneous distribution of synaptic vesicles were observed in the sections from the RNAi animals. It was concluded that normal BRP expression is crucial for the formation and or maintainance of the synaptic ribbons.

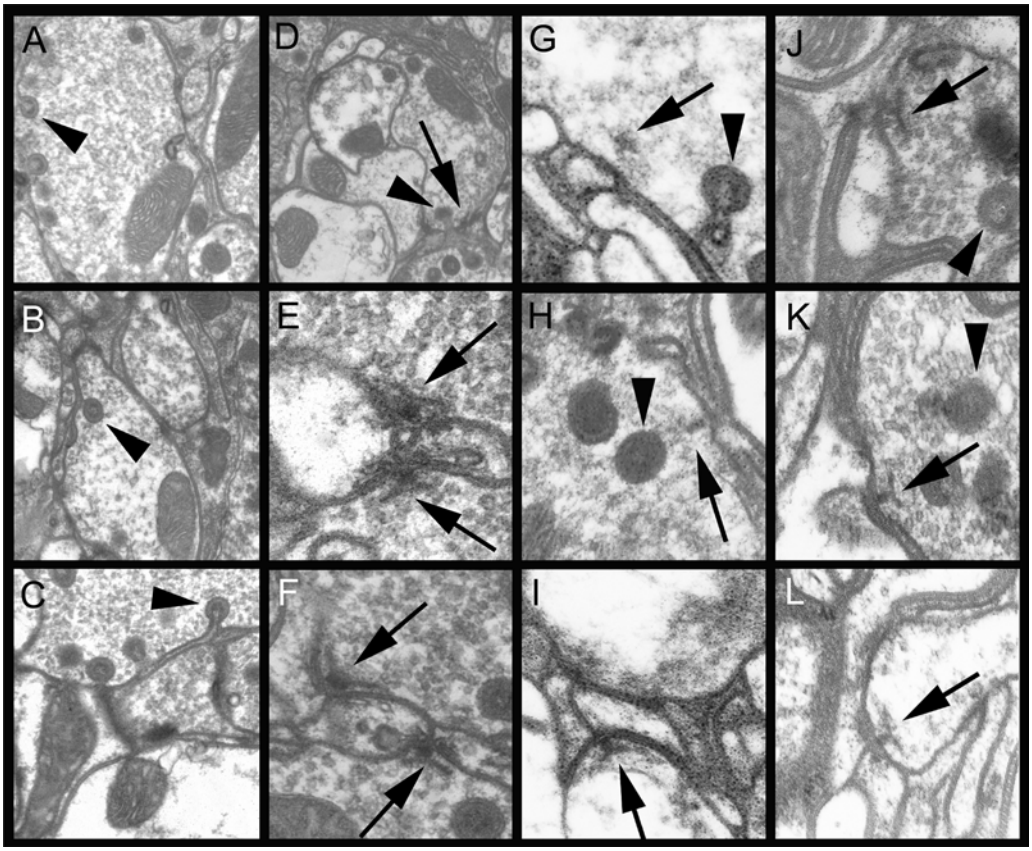

Fig. 3.3.6a- Ultrastructural analysis of the *brp* RNAi animals. A comparison of the synapses in the lamina of F1 offspring of crosses UAS-*brp* RNAi (C8) X *elav* Gal4 (A-C), UAS-*brp* RNAi (C8) X w^{1118} (D-F), UAS-*brp* RNAi (B12) X *gmr* Gal4 (G-I) and *gmr* Gal4 X w^{1118} (J-L). T-bars are indicated by arrows and the capitate projections of the presynaptic photoreceptor terminals are indicated by triangles. The T-bar containing profile in panel I does not have capitate projections and thus presumably belongs to a lamina or medulla interneuron which does not express *gmr* Gal4 and hence lacks RNAi expression (in collaboration with E. Asan).

C h a p t e r 4: D I S C U S S I O N

4.1 Identification And Subcellular Localization Of The MAB nc82 Antigen

4.1.1 Limitations To The Subcellular Localization Of BRP By Immunoelectron Microscopy And Possible Alternatives

The distinctive feature of MAB nc82 stainings first recognized at larval neuromuscular junctions was that the synaptic boutons were not filled with label as observed with other synaptic antibodies (e.g. against CSP, Synaptotagmin, Syntaxin) but displayed discrete spots apparently associated with their plasma membrane (figure 3.1.1b). Active zone molecules show a punctuate staining complementing peri-active zone markers such as Dynamin and also exhibit a tight association with the post-synaptic density which contains Glutamate receptors and P21 activated kinase (PAK). Both these localization patterns are observed in double stainings with MAB nc82 and antisera against Dynamin, Glutamate receptor and PAK. These experiments identified the spots labelled by MAB nc82 as active zones. Small spots at high density are also observed in high resolution confocal images of adult neuropil (not shown), while synaptic vesicle proteins such as Synaptotagmin or CSP show a rather homogeneous distribution under these conditions. This suggests that active zone binding is a general feature of MAB nc82 not restricted to the larval neuromuscular junction. So far the sub-cellular localization of the nc82 antigen at the presynaptic active zones has essentially been determined by light microscopic analysis. First attempts to localize the nc82 antigen at the CAZ by immunoelectron microscopy did not succeed. Glutaraldehyde (GA) is required for EM fixation in order to preserve the ultrastructure. GA cross links proteins and already 0.03% GA in the fixative destroys the nc82 epitope during the process (data not shown). The use of tags like GFP for subcellular localization of various proteins by immunoelectron microscopy is well established (Wucherpfennig et al., 2003). Animals with a photoreceptor-specific expression of BRP-GFP (offspring of the cross UAS-*brp-gfp* (S. Sigrist) X *rh1*-Gal4) were used to standardize fixation conditions for immunoelectron microscopy using anti-GFP antibody on frozen 10 μm sections (fig. 4.1.1a). Incorporation of 0.05% GA resulted in highly reduced but still visible GFP staining. However, whether ultrastructure of the retina is sufficiently preserved with these

fixation conditions for the identification of active zones and T-bars remains to be seen. GFP label disappeared in these experiments when higher GA concentrations (0.2%) were employed. The use of a different GFP antiserum that may recognize GFP after fixation at higher GA concentrations also remains to be explored.

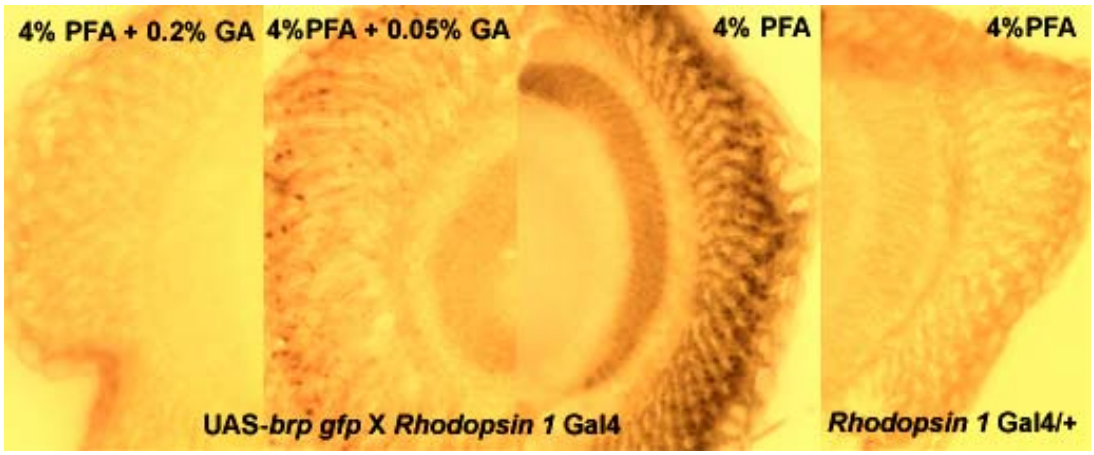

Fig. 4.1.1a- Effect of GA concentration on the antigenicity of the GFP antigen. Anti-GFP stained frozen sections of flies expressing BRP exclusively in photoreceptor cells (left and middle panels) and a control fly (no GFP expression, right panel). The flies were fixed in 4% PFA + 0%, 0.05% or 0.2% gluteraldehyde added as indicated.

4.1.2 Recognition Of Another CAZ Protein By MAB nc82 Due To A Possible Cross Reaction

Apart from the two high molecular weight bands a weaker band at about 60 kDa had been observed transiently on nc82 immunoblots (see figure 3.1.2a). Resent work by the Bellen group (personal communication), has shown that recombinantly expressed protein dNMNAT (nicotinamide mononucleotide adenyltransferase, coded by CG13645; 96B11) can also be recognized by MAB nc82 at about 60 kDa. This molecule has a putative nucleotidyltransferase activity (EC:2.7.7.-) possibly involved in coenzyme metabolism, catalyzing the last step of NAD biosynthesis, and recently it is also thought to be a structural component of the T-bars. RNAi knockdown experiments as well as ectopic expression of BRP in non-neuronal tissue have convincingly shown the antigen specificity of MAB nc82. MAB nc82 staining persists in dNMNAT null mutants further supporting its antigenic specificity. It might be possible that BRP and dNMNAT share epitopes and hence MAB nc82 is able to cross react with both.

4.1.3 BRP Isoforms And Possibility Of A Post-Translational Modification

Mass spectrometry analysis determined the two spots detected by MAB nc82 in 2D gels as proteins encoded by the same genetic locus thus identifying them as isoforms. These spots have the same isoelectric point but a difference of at least 10 kDa corresponding to about 100 amino acids. However, while there is no indication of possible splice variants that may code for these two proteins, a 300 bp difference could escape detection in an 11 kb signal (see northern blots 3.2.4). A possibility of massive post-translational modification like glycosylation or palmitoylation cannot be excluded but proteolytic cleavage seems more likely to be responsible for this difference in molecular weight.

4.1.4 BRP Immunoprecipitation And Methods To Identify Interaction Partners

Bioinformatic structure analysis and BLAST searches strongly predict the presence of coiled-coil domains for the BRP primary sequence. A middle portion of the protein has a significant homology to a myosin heavy chain domain, indicating its similarity to a large family of structural proteins. This information was particularly useful while standardizing the solubilization conditions for the native protein.

Initial attempts to solubilize BRP in the absence of ionic detergents like SDS did not succeed. Antigen-antibody reactions may be inhibited in the presence of ionic detergents and hence it was difficult to immunoprecipitate the nc82 antigen. Structural proteins like myosin can be easily solubilized in the presence of high salt concentrations. Since BRP shares significant homology with myosin heavy chain, similar buffer conditions were used for its solubilization.

However, these conditions are far more drastic than usual physiological conditions in the *milieu interior* of a neuron. High ionic strength may promote strong hydrophobic interactions amongst proteins which may not interact under physiological conditions otherwise. This would increase the difficulty in the identification of true interaction partners of BRP. One possibility would be to achieve maximum separation amongst the co-precipitated proteins by using a 2D PAGE followed by identification of each visible spot and further validation of the interaction *in vivo*. An attempt to restore the physiological conditions prior to elution of the protein by reducing the salt concentration during

consecutive washing steps may also help to reduce the false/forced protein-protein interactions.

4.1.5 High Degree Of Conservation For BRP Amongst Dipteran Insects

A remarkable degree of conservation is observed at the amino acid level between *Drosophila* BRP and its mosquito homologue even though both species have diverged millions of years ago. Between more related species like *Drosophila melanogaster and Drosophila pseudoobscura* even intronic sequences seem well preserved. A recent analysis shows a high level of conservation of this protein in honeybee *Apis melifera* (Order: hymenoptera). The nc82 antibody clearly identifies the protein from *Anopheles gambie* (fig.3.2.3b) and *Musca domestica* (data not shown). These data are in agreement with the bioinformatic analysis. Its high degree of conservation suggests an important function for BRP at the insect synapses. BRP is not conserved to the same extent amongst other invertebrates (e.g. *C. elegance* see discussion below).

4.1.6 Localization Of The nc82 Antigen In The C-Terminal Part Of The BRP Protein

Identification by MAB nc82 of the protein recombinantly expressed in *E. coli* from cDNA (AT09405) leads to two conclusions

1. The cDNA obtained from BDGP (AT09405) is a part of the locus that encodes BRP.
2. The nc82 epitope has not yet been mapped but since the polypeptide encoded by the AT09405 cDNA is recognized by MAB nc82 the epitope is localized on the amino acid sequence encoded by CG30337 (AT09405 essentially represents this ORF). Later analysis of the *brp* gene structure revealed that the ORF CG30337 is responsible for encoding the C-terminal part of the BRP protein and hence the nc82 epitope is present in the C-terminal region.

4.2 Characterization Of The Gene Coding For The nc82 Antigen

4.2.1 Search For Connectivity Between Neighbouring Annotated ORFs Using Bioinformatics And RT-PCR Analysis

Prior to the present experimental analysis only bioinformatic searches were used for determining the genetic structure of the region containing the *brp* gene. However, annotations of ORFs by gene prediction programs vary for different organisms. Sometimes, two ORFs annotated as two separate genes in one organism could be indicated as exonic stretches separated by a large intron, representing a single ORF in another species. Initially it was thought that BRP could be encoded by a single large locus in *Anopheles* (ENSANGP0000004221) which during evolution split into CG30337 and CG30336 as two independent genes in *Drosophila*. However, MAB nc82 recognizes 2 proteins at 180 and 190 kDa in head homogenate from *Anopheles* identical to what has been observed in *Drosophila*. This made the speculation about a split of the *brp* gene during evolution unlikely.

Addition of CG30336 as part of the *brp* locus significantly increased the molecular weight of the computed protein (173.86 kDa). However, the actual observed molecular weights (190 and 180 kDa respectively) indicated a possibility of other neighbouring ORFs being a part of the *brp* locus. The cDNA AT09405 contains a poly A sequence at its 3' end which suggested that this end of the cDNA is likely to be complete. Therefore ORFs annotated in the region upstream of CG30336 were investigated further. Gene structure analysis using RT-PCR was carried out using adult as well as larval (S. Sigrist) RNA. RT-PCR products obtained using both RNA pools did not show any significant difference in the sequences (see Appendix 2). This may indicate that there is no temporal change in the splicing of the *brp* mRNA with respect to at least the most abundant isoforms. Attempts to obtain clear nc82 signals from larval brains on immunoblots have not been successful so far. Therefore, precise information on the biochemical nature of the larval BRP and its isoforms remains elusive. In situ hybridization (S. Sigrist, Wagh et al., submitted) clearly shows that the onset of embryonic neuronal differentiation and expression of *brp* mRNA occur simultaneously.

Attempts to connect CG12932 to the other annotated exons of the region by RT-PCR did not succeed. The protein encoded by CG12932 is conserved in *Anopheles*. Interestingly, the corresponding ORF in mosquito is also situated within the *Anopheles brp* locus at a

position similar to that in the fly. The CG12932 ORF seems to have no conserved domains and the standard protein analysis software tools could not predict any information about its subcellular localization, homology with vertebrate proteins and putative function. Nonetheless, for the clarification of the gene structure and the functional analysis of the *brp* gene it was important to obtain additional evidence whether CG12932 could be included as a part of the *brp* locus (see northern blots below).

Conceptual translation of the composite cDNA sequence extended from CG30337 in 5' direction by RT-PCR revealed a single ORF limited 5' by a TGA stop codon four triplets upstream of the 1st ATG start codon as annotated for the CG12933 ORF. By linking cDNA AT09405 and appropriate RT-PCR fragments, the composite cDNA containing the exons of CG12933, CG30336, CG30337 and two new exons was synthesized by the Sigrist group. After adding a GFP tag this composite cDNA was inserted into the pUAST vector and transformed into w^{1118} Drosophila germline. Comparison of anti-GFP and anti-BRP (MAB nc82) staining verified the correct targeting of the transgene for synaptic active zones and, by ectopic expression once more the identity of BRP and the nc82 antigen (see Wagh et al, submitted).

4.2.2 Presence Of Coiled-Coil Domains And Primary Indications Regarding The Function Of BRP At The CAZ

Bioinformatic programs specially designed to detect coiled-coil (CC) motifs in the BRP protein sequence predict 11 CC motifs with probability 1 (Coilscan, Husar). These domains are spread out all over the protein sequence. The largest of the predicted domains are present in the middle of the sequence. Coiled-coil motifs are characterized by a heptad repeat pattern in which 1st and 4th residues are hydrophobic while residues in the 5th and 7th position are predominantly charged or polar. The CC motif has been identified in a variety of proteins associated with the cytoskeleton, the Golgi, the centromeres, the nuclear matrix and the chromatin (Rose et al., 2004). These motifs play a crucial role in attaching functional proteins to the solid scaffold of the cell. This may confer an adapter like function upon BRP.

Interestingly, *C. elegance* CAST (CeCAST) shows more structural similarity to vertebrate CAST/ERC with respect to chain length, distribution of CC domains and conservation of the PDZ domain binding domain IWA at the C-terminus than BRP.

Amino acid identity amongst insect BRP and vertebrate ERC family proteins is limited to the first five CC domains (figure 3.2.3a).

4.2.3 Indications Of Large Untranslated Regions For The Larger brp Transcript And Possibility Of A Smaller BRP Isoform Not Detectable By MAB nc82

The northern blot was repeated twice (data not shown). The 11 kb band was highly reproducible and generated the strongest signal on the x-ray film. The cDNA which was obtained by combining RT PCR products (comprising of CG12933, CG30336 and CG30337) is 5.5 kb long. The discrepancy between the transcript size observed on the blot and the size of the cDNA can be explained by certain assumptions. All mRNAs have leader and trailer sequences that are not translated (5' or 3' UTR). An untranslated region of 5.5 kb may be unusual but is not impossible. In the case of the *brp* gene the assumption of a long 3' UTR would mean that the poly A tail of the cDNA ATO9405 was artificially generated by internal priming during reverse transcription, a speculation not supported by the sequence surrounding the polyadenylation site. Genes like *elav* and *fne* (*found in neurons*) have been reported to have 3' UTRs of upto 6 kb (Brody et al., 2002). A role of the 6 kb 3' UTR for *elav* has been demonstrated in normal *elav* functioning. In general, 3' UTRs act *in cis* to regulate mRNA translation, stability, and localization through association with regulatory proteins or with antisense RNA. 3' UTRs also play a role *in trans* in myoblast growth and differentiation (Samson, 2003). Little is known about the regulation of BRP expression, however the presence of a possibly long UTR may point towards a similar mode as postulated for other neuronal proteins like ELAV and FNE. Alternative assumptions like long 5' UTRs (van der Velden and Thomas, 1999) or incomplete splicing may also help to explain the large northern signal.

Reprobing of the blot with a probe specific for CG12932 did not give a signal with similarity to the *brp* transcripts. An exposure time of 21 days was required to obtain a faint but visible signal using this probe. Like the RT-PCR data this again suggests that CG12932 is an unrelated but conserved locus. The faint signal suggests that the encoded protein probably is not abundantly expressed in the adult brain.

The approximate molecular weight of at least one of the isoforms as seen in western blots matches with the computed molecular mass of the protein encoded by the ORF of the composite *brp* cDNA. Hence, it is unlikely that the cDNA is still lacking a significant additional coding region.

The smaller 2 kb band obtained in the northern blots with *brp* full length cDNA would be expected to code for a protein of upto about 66 kDa molecular weight (assuming it does not have large UTRs). However, no such protein seems is reproducibly detectable on an nc82 immunoblot. (The transient 60 kDa band has been regarded as a possible cross reaction with dNMNAT, as discussed above. From the bacterial expression of a partial cDNA it is clear that the nc82 epitope is present in the C-terminal region of the BRP protein. Failure to detect a smaller product with MAB nc82 may indicate that the 2 kb splice variant is transcribed from the 5' region of the *brp* gene. The weakness of the corresponding signal on the northern blot may reflect its lesser abundance as compared to its larger variant.

4.3 Functional Investigation Of The Bruchpilot Protein

4.3.1 Tissue Specific RNAi As An Alternative To Classical Mutagenesis

A spatio-temporally controlled downregulation of BRP in the brain could be achieved by creating UAS-*brp* RNAi animals. The exact mechanism of RNA interference is not completely understood. However, the extent of downregulation of the protein seems to vary depending upon which domain is targeted. Also, expression of a transgene is regulated to a considerable extent by its point of insertion in the genome. Only two independent insertions were obtained with our own RNAi constructs. Both produced no obvious phenotype when expressed pan–neuronally and were not further characterized since the collaborating group had already obtained highly effective RNAi lines. Thus no information is available on the reason why the DW1 and DW2 RNAi lines were ineffective. Two constructs were generated by the Sigrist group. Series B of RNAi construct targeted the 5th CC domain of the BRP protein encoded by CG30336 while series C construct targeted the 6th CC domain encoded by CG30337. Both these RNAi lines effect a severe, specific downregulation of BRP when Gal4 is present in the cell. Pan-neuronal expression of RNAi B12 with *elav* Gal4 causes lethality at a late embryonic stage. Pan neuronal expression of RNAi C8 with elav Gal4 does not cause lethality. However, adults have severe phenotypes and show drastic downregulation of BRP on the immunoblot as well as on MAB nc82 stained brain sections. RNAi B12 can be effectively used to study BRP function at retinal synapses by expressing Gal4 in the retina only. With the *gmr* Gal4 driver both lines show downregulation of BRP to a similar degree.

4.3.2 Unresolved Aspects Of P Element Mobilization And Screening Due To Complexity Of The *brp* locus And Multiple P Insertions In The Original P Element Stock

Several P element lines were available that could have been used to create deletions in the *brp* gene (refer to table 3.3.2a in the results). Deletions in the 3' regions usually result in truncated products which may or may not be functional and hence could not guarantee a null mutation. Deletions in the 5' region are more likely to result in functionally null phenotypes. Amongst available stocks line 14101 is the only one where a P element is situated within the *brp* locus near the 5' region and therefore it was used for the mobilization experiment.

The *brp* locus spans more than 22 kb of the genomic region. The presence of the presumably unrelated gene CG12932 within the large 4th intron of the *brp* gene imposes constrains on creating deletions affecting only the *brp* gene. A larger deletion extending far upstream to the first exon of the *brp* locus could result in a deletion of some part of the *Wnt2* gene, making interpretation of a possible phenotype difficult. Line 14101 has 2 insertions on the same chromosome separated by 100 kb (not shown in the sketch). Insertion P1 is in a genomic region containing the genes *wunen* and *wunen* 2 (abbreviated as *wun* and *wun2* respectively). These genes are very similar and encode products with phosphatidate phosphatase activity (EC: 3.1.3.4). The proteins are integral to the membrane and are expressed in the embryo (ectoderm, embryonic central nervous system, hindgut primordium and posterior midgut primordium). Loss-of-function mutations for both these genes have been isolated which seem to affect the germ cells and are female sterile. In studies on *wun* function, *wun* and *wun2* appear to act redundantly (flybase). The P1 insertion has not been characterised well. Work done in parallel with our experiments in the collaborating group (S. Sigrist, personal communication) suggests that in the 14101 line in fact part of the P element itself and several kb between *wun* and *wun2* exons are deleted. This will make a remobilization of P1 impossible. Interestingly, the 14101 P insertion line is lethal at a late pupal stage in homozygous condition. Due to the deletion in the *wun* gene region it becomes essential to determine if this deletion or P2 alone or both in co-operation could cause this lethal phenotype. Since *wun* phenotypes are well studied and the functional redundancy of both genes is established (Flybase), it is unlikely that the deletion in the *wun* region would result in lethality. Because further

mobilization and excision of P1 is not possible, the fact that the jump out mutagenesis produced 60 homozygous viable lines means that the P2 insertion in line 14101 causes the lethality which can be reversed by a precise jump-out. P2 is inserted in the large 16 kb 4th intron of the *brp* locus, 5 kb downstream to the 4th *brp* exon and 7 kb upstream to the CG12932 locus. Both P elements of the 14101 line are quoted as being the P (SUPorP) transposable elements (Roseman et al, 1995). P element P (SUPorP) is a transposon created by cloning a suppressor of hairy wing *Su (Hw)* binding region of the *gypsy* retrotransposon between the P element inverted repeats.

Su (Hw) naturally accumulates in all cells during development and is capable of binding to the *gypsy* element. If inserted between an enhancer and a promoter of a certain gene the P (SUPorP) element can inactivate the enhancer (Roseman et al, 1995). This may result in a drastic downregulation of the expression of the affected gene and could mimic a null phenotype. Attempts to verify a downregulation of BRP by gene dosage immunoblots on heterozygous flies from the line 14101 using MAB nc82 were inconclusive however (data not shown).

Also, no protein with an altered molecular weight was observed on MAB nc82 stained immunoblots from these animals which could have indicated formation of a possibly dominant negative BRP protein due to the interference of this insertion.

This of course, does not rule out the possibility of a product that does not incorporate the epitope for MAB nc82. An immunoblot from homozygous late pupae or larval brains might have been a definitive approach to this problem. However, larval western blots with MAB nc82 have been unsuccessful (Jeblic, R., F2 laboratory course, 2003; Erbguth, K., F2 laboratory course, 2004) and pupal blots were not attempted. A real time PCR analysis of CG12933, CG30336 and CG30337 from homozygous larvae from line 14101 (S. Sigrist) indicated that CG12933 transcription is upregulated by 400% indicating a compensatory upregulation in response to the defective transcription of the entire gene. Whether these transcripts are translated is not known.

An analysis of the total jump out lines shows that about 80% of the total lines are homozygous lethal. Imprecise jump out events occur with a very low probability. If the information available about the P1 insertion is correct then all of the jump out events scored so far for white eye colour must be due to P2 mobilization. It has also been observed that imprecise excision could be of two kinds. 1. Either the P element mobilizes completely excising in addition some part of the adjacent genomic region or 2. Excising partially including the w^+ gene leaving some part of the P element still inserted in the

original insertion site. The second type of excision is experimentally supported in the current study. A fraction of the homozygous lethal lines were analysed by PCR for the presence of the P2 insertion at its original locus. About 26% of the total lines analysed tested positive for the presence of P2 element fragments (data not shown). This may suggest that in these lines the *brp* gene structure is not restored which could cause lethality similar to that of the original P insertion in line 14101. Homozygous viable lines were not analysed. They are assumed to represent precise jump-out events or small inconsequential deletions.

So far the available facts for the P2 insertion are confusing. The data suggest that the P2 insertion in the original line 14101 represents already a lethal mutation for the *brp* locus. However, since the upstream regulatory region and the CG12933 ORF are not affected, a small BRP fragment could still be made that might have some function during development.

To create BRP null mutants has been attempted by our collaborating group (S. Sigrist) using trans-mobilization of the P-elements present in lines 14101 and 13948 thereby deleting the entire region between these two insertions (refer to the table 3.3.2a and figure 3.3.2a indicating relative positions of the different P element lines flanking *brp* locus). This approach has been particularly useful in creating a large C-terminal deletion in the *brp* gene. This line is denoted as brp^{69}. However, CG12932 has also been deleted in these mutants. These animals have been found to be homozygous lethal at late larval stages, similar to the line 14101 and the upstream regulatory region and ORF CG12933 again remain unaffected.

4.3.3 The Role Of BRP In Normal Synaptic Function

The ERG is one of the few robust physiological assays available to test functionality of identified synapses in adult flies. The ERG monitors the extracellular electrical activity at the compound eye and lamina in response to light. It consists of two superimposed components: a negative-going sustained component corresponding to photoreceptor depolarization, and a positive light on- and negative light off-component resulting from neurotransmission-dependent hyper- polarization and depolarization, respectively, of second order neurons in the lamina that are postsynaptic to the photoreceptor cells. These so called ON and OFF transients (fig 2.8a in Material and Methods) depend on the synaptic release of histamine from the photoreceptor terminals in response to light (Burg et al., 1993). Thus, mutants that in the ERG show the light-induced depolarization of the photoreceptor cells but specifically lack the ON/OFF transients, likely have a specific defect in synaptic transmission. Exactly this is the case for *brp* RNAi (C8) X *elav*-Gal4 and *brp* RNAi (B12) x *gmr*-Gal4 offspring. Thus, it can be concluded that BRP is needed within the presynaptic photoreceptor neurons to allow efficient synaptic transmission at the histaminergic photoreceptor synapses in the lamina.

Photoreceptors R1 to R6 are responsible for motion detection. A defect in their synaptic contacts would therefore be expected to result in motion-blind flies. However, the optomotor behavior (figure-3.3.5a) clearly indicates that flies with photoreceptor-specific *brp* knockdown are able to detect motion although with a strongly diminished efficiency.

Even though the ERG represents a robust assay to study synaptic function, it is a relatively crude test. It accounts for extracellular electrical activity and the trace represents a cumulative potential between the different electrode (in contact with the ommatidia) and the ground electrode (in the thorax or in the head capsule). Loss of ON and OFF transients clearly indicates a loss or disturbance of synaptic function at the lamina. However, an asynchronous transmitter release from defective synapses could desynchronize the ON and OFF currents thereby eliminating them from the cumulative trace. An assumption that residual levels of BRP at the synapses are able to keep them functional at least partially is supported by the observed residual optomotor behavior. Similar studies could not be conducted with *elav* Gal4 X UAS *brp* RNAi (C8) offspring as the animals are unmotivated to move even in the presence of normal light and hence are unsuitable for this paradigm.

Finally, *elav* Gal4 X UAS-*brp* RNAi (C8) offspring were tested for their ability to walk and fly using simple behavioral assays (see method 2.9 for the assays). It was observed that the RNAi expressing animals initiate very little movement on their own. The name "Bruchpilot" was given the protein expressed in these animals on the basis of preliminary observations that they exhibit instable flight. This observation was quantitatively verified using the flight tester as described by Benzer (1973). About 45% of the RNAi expressing animals sank to the bottom of the cylinder and the rest were distributed unevenly mainly in the lower half of the cylinder indicating a severely impaired flying ability (fig.3.3.5b-C & D).

However, these animals have a normal appearance with respect to the body size, wings and leg structure and position. Interestingly, it was observed that if these animals are pushed continuously they exhibit normal walking. If dropped from a distance several times they also exhibit normal flying ability. This may point towards a motivational deficit in these animals rather than to a mechanical inability imposed by defective synapses. Further investigation is necessary in order to clarify this point.

In parallel to these experiments investigation of BRP function in larvae was carried out by the collaborating group (S. Sigrist). Larval offspring of the UAS-*brp* RNAi (C8) X *elav* Gal4 were analyzed in two-electrode voltage clamp (TEVC) recordings (S. Sigrist, Wagh et al., Submitted). Amplitudes of spontaneous miniature currents (mEJCs) were indistinguishable between RNAi larvae and wild-type controls. Thus, consistent with normal receptor field size and shape, BRP reduction does not affect the response of individual postsynaptic glutamate receptor fields, nor does it seem to influence glutamate content of synaptic vesicles. However, amplitudes of evoked junctional currents (EJCs) were reduced by 40% with very high statistical significance. Since miniature current amplitudes are unaffected, quantal content - the number of vesicles that fuse per stimulus – must be decreased. Because the number of postsynaptic receptor fields is not altered in *brp* RNAi larvae, the number of vesicles that fuse per synapse is obviously decreased after reducing BRP. This drop could be caused by a decreased release probability per vesicle, or a decrease in the number of vesicles available for release per synapse or both. We thus can conclude that wild-type BRP levels are necessary for normal evoked release at the glutamatergic synapse of the NMJ, while spontaneous release, postsynaptic sensitivity or basic neuronal morphology apparently are not affected by inhibition of BRP expression.

4.3.5 BRP And Formation Of Sub-Synaptic Structures

Interestingly, the *gmr* Gal4 X UAS *brp* RNAi (B12) offspring showed a strong lamina specific *brp* downregulation as documented by immunohistochemistry on adult brain sections, exhibited a loss of ON and OFF transients in ERG traces and also showed significant ultrastructural defects as well as major loss of synaptic ribbons (T-bars). *Rh1* Gal4 (*Rhodopsin 1*) is another strong photoreceptor specific Gal4 driver. Neither UAS-*brp* RNAi (B12) X *Rh1* Gal4 nor UAS-*brp* RNAi (C8) X *Rh1* Gal4 offspring showed significant reduction in the *brp* levels at the photoreceptor synapses (data not shown). The experiment was performed also at higher temperature (29^0C) in order to boost the driver expression. However, this modification too had little effect on *brp* expression levels. The *gmr* Gal4 X UAS-*brp* RNAi (B12) offspring, on the other hand, even showed a rough eye phenotype which results from a general structural defect. Expression of *gmr* Gal4 starts early in development while *Rhodopsin 1* Gal4 expression begins in the late pupal stage. An important point could be conceived from this set of experiments. *Brp* expression may be crucial in the developing nervous system for the formation and/or maintainance of newly formed synaptic connections. An inducible expression system (e.g. Gal 80) may be used to probe this speculation further. A careful analysis of the involvement of normal *brp* expression in the different developmental stages may throw some light on the developmental significance of this protein.

Notably, these ultra-structural abnormalities were not observed in RNAi (C8) X *elav* Gal4 offspring. *Elav* Gal4 expression also begins early during the development but the adult animals do not show a rough eye phenotype. At an ultra-structural level the cartridge assembly appears to be well preserved. However, similar to the *gmr* Gal4 driven RNAi offspring these flies also show absence of the T-bars.

Expression of the *gmr* -Gal4 on its own exhibits a rough eye phenotype in homozygous condition at 25^0C and even in heterozygous condition at 29^0C. Hence, the general structural deficits observed in the RNAi flies driven by the *gmr*-Gal4 driver could be regarded as the property of the driver itself.

4.3.6 Comparative Molecular Architecture Of Active Zones And Future Experiments To Investigate BRP Function

The current study raises many important questions which will need prominent attention in the future. The N-terminal sequence of BRP is homologus to the vertebrate ERC/CAST. BRP is twice as large in size and almost completely composed of coil-coil motifs. Proteins of the ERC family show a conserved PDZ binding domain IWA at the C-terminus (Deguchi-Tawarada et al., 2004). This domain is necessary for interaction with RIM. An IWA domain does not exist in BRP. An extensive search for this domain in the intronic/genomic regions surrounding *brp* locus was negative. No other protein in the flybase showed homology to the C-terminus of vertebrate ERC/CAST. *Drosophila* has a single locus coding for the fly homologue of RIM. The characteristic C2 and PDZ domains of *Drosophila* (dm) RIM are highly conserved suggesting a similar function for this molecule at the fly CAZ. It remains to be seen if BRP and dm RIM can physically interact in the absence of a canonical PDZ binding motif. Since BRP can be effectively immunoprecipitated, such a study may be possible. Antibodies against dmRIM have not been published to date. However, according to the annotation (Flybase) dm RIM appears to be a very large protein (2469 aa). One strategy to investigate such an interaction would be to carry out a mass-spectrometric analysis of high molecular weight bands that co-precipitate with BRP.

It might be worthwhile to obtain double knockouts for the *Rim* and *brp* genes and observe the phenotype by various methods. The publicly available *Rim* mutant $Rim^{EY05246}$ has been reported to be viable and fertile while a strong *brp* knockdown (as in the RNAi (B12) X *elav* Gal4 offspring) shows embryonic lethality (S. Sigrist, Personal communication). It might be interesting to look at the CAZ structure assembly and possible physiological phenotype in homozygous embryos of such double mutants.

The multidomain CAZ organizers Bassoon and Piccolo do not have sequence homology to any known or computed fly (or invertebrate) proteins. These are very large proteins (400 and 550 kDa respectively) with CC motifs, C2 domains and Piccolo-Bassoon homology domains (Ziv and Garner, 2004). Bassoon has been shown to interact with ERC/CAST in mammals. Whether at all some other proteins which could be functional homologues of these two proteins are present in the fly is not known. Nor has the ability

of BRP to interact with other proteins been determined. Immunoprecipitation studies would probably answer the question of interaction partners very effectively.

At least in vertebrates proteins of the ERC family seem to link several active zone molecules together. A recent study at the photoreceptor ribbon synapse has shown that Bassoon mutants have a floating ribbon phenotype (Dick et al, 2003). A major component of the synaptic ribbons is a protein called RIBEYE (Schmitz et al., 2000). Evolutionarily, RIBEYE belongs to the C-terminal binding proteins (CtBP) which act as transcriptional repressors in bacteria. They have assumed different functions during evolution (Schmitz et al., 2000). These proteins have two domains. The B domain has a strong affinity for NAD^+ and the A domain is responsible for oligomerization. RIBEYE probably constitutes the central building block of the electron dense photoreceptor synaptic ribbon complex. Bassoon has been shown to physically interact with RIBEYE on one hand and to ERC on the other (see fig. 4.3.5a for a model). The floating ribbon phenotype clearly suggests an adapter function for Bassoon. Motor protein KIF3A, Piccolo, RIM1 and synapsins are also shown to be present at the synaptic ribbons (tom Dieck et al., 2005), while immunolocalization suggests that the neuronal isoform of ERC is present at the base of the ribbon as a component of the CAZ.

Drosophila active zones are well characterized at the ultrastructural level. However, their molecular architecture is almost completely unknown (see Introduction). The only active zone molecule so far described in the fly is the N-type calcium channel cacophony. A recent review by Zhai and Bellen (2005) nicely points out the similarities in active zone architecture at different synapses across species. In that light it seems possible that *Drosophila* active zones are very similar with respect to structure and function to the vertebrate active zones. However, molecular details for the fly active zone are scarce. Although a CtBP homologous protein exists in the fly (encoded by a gene in 87D8-9) its presence at the T-bars has not been investigated. This may be another interesting question to address while studying the components of *Drosophila* CAZ. The presence of motor proteins at the T–shaped ribbons of insect synapses also has not been investigated so far. Synaptic ribbons seem to have an accessory function of tethering synaptic vesicles which thus are added to the RRP. Synapses of retinal or auditory receptor cells show a sustained, tonic release of vesicles upon stimulation and probably require a constant supply of SVs to the readily releasable pool. Such synapses also have prominent synaptic

ribbons (see Introduction). Interestingly, T-bars seem to be present in most of the synapses as seen in adult brain including the giant fiber giant fiber system (Blagburn et al., 1999) as well as larval NMJs. Similar to the Bassoon knock-out phenotype, where floating synaptic ribbons are observed, the *Drosophila wit* mutants show a floating T-bar phenotype and impairment of sustained release (Aberle et al., 2002, Marques et al., 2002). *Wit* codes for a bone morphogenic protein and may have a function in the formation and assembly of active zones. At any rate, it underscores the accessory function of synaptic ribbons across different species.

With the limited information that has been accumulated so far it appears that BRP certainly plays an important role in the maintainance of structure and function of the synapses. The Sigrist group has shown that downregulation of BRP results in a significant reduction of evoked release at the larval NMJs. Dysfunctional retinal synapses in the adult brain of BRP knockdown flies as seen by ERG and ultrastructural analysis also support this conclusion. At the ultrastructural level these flies show the absence of well formed T-bars and sometimes the presence of floating structure that could be remnants of T-bars at the presynapses. These observations suggest that BRP might have a Bassoon-like adapter function at the active zone. However, while mice mutant for Bassoon are viable with a propensity to develop spontaneous epileptic seizures, a drastic reduction in BRP level by strong RNAi effects as well as initial data obtained from *brp* hypomorphic mutants such as brp^{69} or brp^{P14101} show that partial absence of BRP results in lethality during larval developmental stages. This could mean that BRP has more than one role to play in normal functioning of synapses. Knock down studies at the photoreceptor synapses using *gmr* and *Rhodopsin* Gal4 drivers also highlight an interesting developmental aspect for BRP. Downregulation of BRP after synapse formation is completed does not show any dramatic decrease or abnormality at the photoreceptor synapses. This could mean that BRP expression is necessary throughout synaptic development. The fact that *brp* mRNA expression is temporally correlated with neuronal differentiation early in the development (S. Sigrist, Wagh et al., submitted) supports this possibility even further.

If at all BRP is able to function as an adapter, it remains to be seen which components of the CAZ and synaptic ribbons interact with it. What signals confer upon CAZ proteins the property to localize at the active zones is an open question. Specialized vesicles that carry

pre-assembled active zone components to the CAZ (Piccolo-Bassoon transport vesicles, PTVs) have been shown to be present in vertebrates (Zhai et al., 2001). Whether this also holds true for invertebrate CAZ remains to be seen. GFP linked to BRP may be used to mark these vesicles such that their biogenesis and transport could be studied further with live imaging. BRP is an excellent presynaptic AZ marker and probably can be used to trace nascent synaptic contacts marked by CAZ assembly and hence can be effectively used to study the process of synaptogenesis and synaptic plasticity.

Finally, the targeting of BRP to active zones may be exploited to improve functional calcium imaging (Fiala et al., 2002). The CAZ is shown to be in direct contact with calcium channels. If it is possible to identify the domain that is responsible for targeting of BRP to the CAZ, a fusion of this domain with DNA-encoded calcium sensors could allow calcium sensing specifically at the active zone. This would be a valuable enhancement of a novel physiological tool to study the process of evoked release in detail and to analyse real neuronal networks in intact brains.

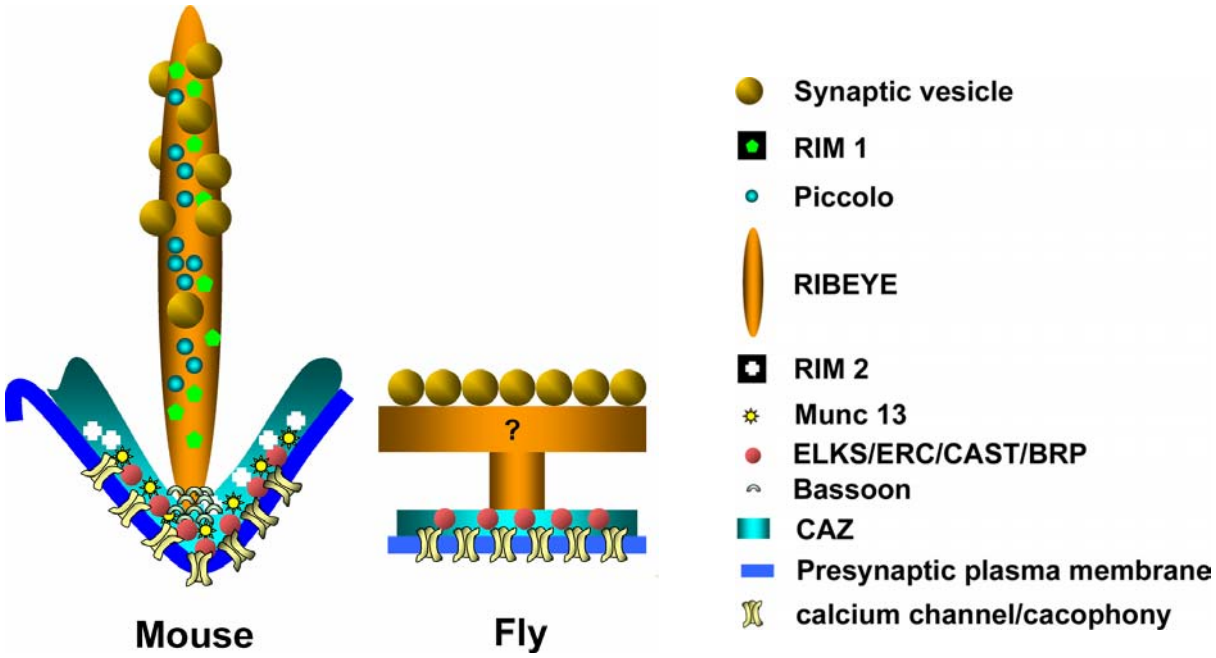

Fig. 4.3.5a Comparison of the known molecular components of a vertebrate photoreceptor synaptic ribbon complex (tom Dieck et al., 2005) with a *Drosophila* T-bar (not drawn to scale).

B I B L I O G R A P H Y

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A P P E N D I X 1

Primer Sequences. (Note: Restriction Sites Are Indicated By Red Underlined Letters).

Primer	Sequence 5'-3'
RT PCR	
36.5 RTP	GGA CAA CCA GGT GGA TAT GG
36.3 RTP	ATC TTG TAG TCG GCC ACC TC
E-37.5	CCA AAC CGA AAC CCG AAA ACA GTC
E-37.3	GGT GCG CTC CAT CTC CTC CTT
36-E-37.5	CTA TGA ACC CAT ATG CAT AAA ACA CAT AC
36-E-37.3	AGT CTC GCG CTC CTT CTG C
Missing E- 37.5	ACA ACC TTT GGC AGG ACC AC
Missing E-37. 3	CGA CTG CAG GTT GTC GTA GT
36 – Missing E. 5	GGC CGA CTA CAA GAT CAA GC
36 – Missing E. 3	GGG CTC GAT CCA GTT CCT
36 – nstd X1-550.5	CTG TAC CTT GTT CCT TTC CAA CCA
36 – nstd X3-550.3	CTC GGA GCT GCT GTG GTG
36 X1- 591. 5	GCA TTA CCA TGC GTG GCA AC
36 ? ME – 204. 5	CGC CAT AGA AGC CCA AAT AAA ATG
36 ? ME – 204. 3	GTT GCG AAT ACG GGT GAC TTG
GSP1. 3	TGT CCG TTT GTA GCT TGT CG
GSP2(Bgl II). 3	GGC GAT AGA TCT CGT TCT GG
GSP3.3	GTC GGG GAA ACT GGT CAT AAT G
GSP4.3	AAC GCT CCA GAT CGT GTT TCA GCC G
Ex. 1 (Eco RV). 5	CTA AGA TAT CGC ATT ACC ATG CGT GGC AAC
Bgl II E1. 5	GAA GAT CTG CAT TAC CAT GCG TGG CAA
Mlu I E3. 3	TCC AGA CGC GTC TGT AGC TTC TCC A
Li Ex1. 5	ATG GGC AGT CCA TAC TAC CG
Li Ex2. 3	TCC CGA ATG GGT ATG AAC TCG
932.3	GTC TGC AGG TGA TTC TGA TGC
932.5	CGA GTG GTG TCC AGT TAC CC

Kpn I LieX1. 5	TTG GTA CCA TGG GCA GTC CAT ACT AC
Oligo dT Kpn I	CGG CGC GGT ACC TTT TTT TTT TTT TTT TTT TTT TTT TTT
FW1951. 5	CGT CCA GAA GCT GGA GTC A
REV3049. 3	GCT GCG TAT CCA TGA GCT TC
W462. 5	ATC AAT TCT CCG ACG AGC TG
Rev2178.3	CTG GAT CTC CTT TTG CAT GG
FW4.5	ACT CAA GGC CAA GCT GGA G
Rev1558.3	AAA CGA TCG ACC TCC TTC AGT
RNAi	
gDNA-cDNA construct	
RNAi gDNA. 5	GGG GTA CCC ATC TGG CCA AGC A
RNAi gDNA. 3	CGG GAT CCA TCT GGC CGG GAT AAA TG
RNAi cDNA. 5	GCT CTA GAC ATC TGG CCA AGC AGC A
RNAi cDNA. 3	CGG GAT CCC CTG GTT GGA CAT CTT G
cDNA-cDNA construct	
RNAi cDNA. 5a	CGG AAT TCA CTC AAG GCC AAG CTG GA
RNAi cDNA. 5b	GCT CTA GAA CTC AAG GCC AAG CTG GA
RNAi cDNA. 3ab	CGG GAT CCC TGC AGC TTA CGC AGA T
GFP.5	CGG GAT CCT GAC CCT GAA GTT CAT CT
GFP.3	CGG GAT CCG TCT TGT AGT TGC CGT C
RNAi B12 (S. Sigrist)	
TR961Bs	GAC TGA ATT CCA GGA GCG TAA TCG CCT CAC CAG
TR996Bas	CTG ATC TAG AGC CCG CAG CTT GAT CTT GTA GTC GG
RNAi C8 (S. Sigrist)	
TR963Cs	GAC TGA ATT CAG CTA CAG ACG GAC ATT CGG CGT GC
TR997Cas	CAC TTC TAG ACG GAT CGC TGC TTT TCC TGA TTG GC
Bacterial expression of the AT09405 cDNA	
pET 9405.5	GGA TTC ATG CGA CTC AAG GCC AAG
pET 9405.3	AAG CTT ATC CTG CTG TTG CTG TTG CTG
pET 9405.3a	AAG CTT TCG CAT TGC ATT TAC ATG GTG TCA T
pET 9405.5b	AAT TGG ATC CAT GCG ACT CAA GGC CAA G

pET 9405.3b	ACA TAA GCT TTC GCA TTG CAT TTA CAT GGT GTC AT
Northern blot	
CG12932 probe	
932p.5	TTG AAC GAG GCA CAC AAG TC
932p.3	CGG CAG TTT CGG GTA ATC TA
Southern blot	
P1 probe	
P1, 5' FWD99668.5	CGC TGA CAA GGT GTT ATG GA
p1, 5' rev 104462.3	GAC GGA GCA GTA AAG GCT TG
P1, 3' FWD99668.5	AAG CCT TTA CTG CTC CGT CA
p1, 3' rev 104462.3	TTT TGT GGG GAG TGT CCT TC
P2 probe	
P2, 5' FWD203061.5	GAC AGT GGA GGC AGT CAA CA
p2, 5' rev 207760.3	TGC CAT TAG ACT GTG CCA AAA C
P2, 3' FWD207189.5	ATT CGA GTT TGA AGG CAA GCG
p2, 3' rev 211849.3	GTT ATT GCT TTC CCT TTG GTC CT
Probe 1 (P2)	
201011.5	CGT TCC GTC ATC CTC ATT TGG
202963.3	GAA AAG TGT CAG GCT CTG TGG
Probe 2 (P2)	
194228.5	CGG GAG CAT ATC GCT AGA AC
195777.3	CCC ACC TTC CGT CAC TTC ATT
Other primers for P element insertion analysis	
Flanking P1.5	GAA TGA ACG AGC GAC ACA GA
Flanking p1.3	TGA ATA ATC CTC GGG AGT GC
Flanking P2.5	CAC CCG CAC ACA TAA AGT TG
Flanking p2.3	TGC CAT TAG ACT GTG CCA AAA
A 12933 4KB.5	GTT GCA TCC GGT AGA CCA GT
A 12933 4KB.3	AAT TAA GCG ACA ACG GCA AC
A 12933 2KB.3	TAC CCG CTA CCC TGT TTG TC
A 12933 2KB.5	ACA AAC AGG GTA GCG GGT AG

3’ 14101- 207760.3	TGC CAT TAG ACT GTG CCA AA
5’ 14101- 206218.5	ATG GAG CAC GGA AGA TAT GG

A P P E N D I X 2

The *brp* cDNA Sequence (RNAi Target Domains Are Highlighted)

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  51 ATGGGCAGTC CATACTACCG CGACATGGAT GAGCCAACCA GTCCGGCCGG
 101 AGCGGGTCAC CATCGCAGCC GGAGCGCCAG CAGACCACCG ATGGCCCATG
 151 CCATGGACTA TCCAAGAACC CGCTACCAAT CGCTGGATCG CGGTGGACTC
 201 GTGGATCCCC ACGATCGCGA GTTCATACCC ATTCGGGAGC CCCGCGACCG
 251 CTCCAGGGAC AGATCCCTCG AACGGGGCCT GTACCTAGAG GACGAGCTCT
 301 ATGGCAGATC AGCGCGTCAG AGCCCCAGCG CCATGGGTGG ATACAATACG
 351 GGCATGGGCC CCACATCGGA TCGAGCTTAT TTGGGCGACC TGCAGCATCA
 401 GAACACCGAC CTGCAGCGGG AGCTGGGGGA CCTGAAGCGG GAACTGGAGC
 451 TGACTAACCA GAAGCTGGGC AGCTCGATGC ACAGCATCAA GACATTCTGG
 501 TCGCCGGAGC TCAAGAAGGA GCGAGCACTG CGCAAGGAGG AGAGCGCCAA
 551 GTACAGTCTG ATCAACGATC AGTTGAAGCT GCTCAGCACG GAGAACCAGA
 601 AACAAGCCAT GTTGGTGCGC CAGCTGGAGG AGGAGCTGCG CCTGCGAATG
 651 CGACAGCCCA ACCTGGAGAT GCAGCAGCAG ATGGAGGCGA TCTACGCGGA
 701 GAACGACCAC TTGCAGCGGG AGATCAGCAT CCTGCGCGAG ACGATCAAGG
 751 ATCTAGAGTG CCGGGTGGAG ACCCAGAAGC AAACACTGAT TGCCCGCGAC
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1151 GGAGCGTAAT CGCCTCACCA GCGAGCTTAC CGAGCTCAAG GACCACATGG
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3151 ACGAGAAGAG CCAGGTGGAG CTGCGCAAGC TGCAGGACAC GGACACCTTC

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5401 CTGTCTACTA TTTTGTATAC TACCGATGCC GATACCAATA CCAACTATGC

5451 AGTATTTCTA CGACTCTCAC ACACACTATG TACACTCTTT ACACACACAG

5501 ATAAACACAG GGACACACAC ACACATTTGT AAATGACACC ATGTAAATGC

5551 AATGCGAAAT GCATATTATT TGATAAAAAA AAAAAAAAAA AAA

RT- PCR Sequences And The DWc RNAi Domain.

RT-PCR sequences mapped on the genomic DNA (Primer sequences are marked in **bold** *italics*, sequences that are annotated as cDNA (Flybase) but not confirmed by sequencing in our group are marked in **bold** magenta. Sequences that are a part of the cDNA are highlighted in yellow and sequences that are confirmed by sequencing but are not part of the cDNA are highlitened in gray. The DWc RNAi target domain is highlitened in teal.

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CG30337

Missing E-37.3 36-E-37.3

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Amino Acid Sequence Of The BRP Protein (C-C Domains As Determined By Coilscan (HUSAR) Are Highlightened)

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 151  SPELKKERAP RKEESAKYSL INDQLKLLST ENQKQAMLVR QLEEELRLRM  2
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1251  RLHDRLEKAE AEREALRQAN RSGGAGAAPH PQLEKHVQKL ESDVKQLAME  8
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A P P E N D I X 3

Reagents

Nr.	Method	Reagents
1.	**3. Nucleic acid detection** **3A. Agarose gel electrophoresis**	**TBE (10 X)** 890 mM Tris 890 mM Borate 0.5 M EDTA pH: 8.0
		Ethidium bromide Solution 1 g of EtBr dissolved in 10 ml dH_2O or NH_4Ac Stored at 4 ^{0}C.
		6X loading dye/Stop buffer 10mM Tris HCl (pH 7.6) 0.03% Bromophenol Blue 0.03% Xylene Cyanol FF 60% Glycerol 60mM EDTA
2.	**3B. Southern blots**	**Depurination solution** 0.25 M HCL
		Denaturation solution 1.5 M NaCl 0.5 M NaOH
		Neutralization solution 1M NaCl 0.5 M Tris.Cl pH : 7 .0
		20X SSC 3 M NaCl 0.3 M Na_3 Citrate.$2H_2O$ pH: 7.0

3.	**3C. Northern blot**	**10X and 1X MOPS** 0.4 M MOPS, pH -7.00 0.1 M NaAc 10 mM EDTA
		12.3 M (37%) Formaldehyde, pH – 4.0
		Deionized formamide
		Formaldehyde loading buffer 1mM EDTA, pH - 8.0 0.25% (w/v) Bromophenolbleu 0.25% (w/v) Xylenecyanol 50% (v/v) Glycine
		0.5 M NH_4Ac (For EtBr staining and destaining)
		20 X SSC (See reagents in southern blot).
		Churchbuffer 15% deionized Formamide 200 mM $NaPO_4$, pH – 7.2 1 mM EDTA 7% SDS and 1% (w/v) BSA

4.	**4. Cloning** **TA cloning using TOPO cloning kit**	**S. O. C medium:** 2% Tryptone 0.5% Yeast extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM Glucose (dextrose)
		NZY^+ Medium 10% NZ amine (Caesin hydrolysate) 5% Yeast extract 5% NaCl pH: 7.5 Autoclaved and freshly supplimented with 12.5 mM $MgCl_2$ 12.5 mM $MgSO_4$ 10 mM Glucose Filter sterilized
		X gal 2% X Gal in DMF. Stored at –20 ^{0}C protected from light
		IPTG 20% Stock in dH_2O. Filter sterilzed.
		LB (1L) 10 g Tryptone 10 g NaCl 5 g Yeast extract pH : 7.5 dH_2O to 1L Autoclaved and store at + 4 ^{0}C after opening.

<table>
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<td>
LB + Agar + Antibiotic (1L)
10 g Tryptone
10 g NaCl
5 g Yeast extract
pH : 7.5
15 g Agar
dH_2O to 1L
Aprropriate antibiotic was added to the plates after allowing then to cool down sufficiently, prior to pouring..
</td>
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<td>5.</td>
<td>5. Protein analysis
5A. SDS PAGE</td>
<td>
<table>
<tr><th>SDS PAGE Running gel</th><th>10%</th><th>7.5%</th><th>5%</th></tr>
<tr><td>30% acrylamide/bis acrylamide (29:1)</td><td>5 ml</td><td>3.75 ml</td><td>2.5 ml</td></tr>
<tr><td>1.88 M Tris/HCl (pH:8.8)</td><td>3.0 ml</td><td>3.0 ml</td><td>3.0 ml</td></tr>
<tr><td>dH2O</td><td>4 ml</td><td>5.25 ml</td><td>6.5 ml</td></tr>
<tr><td>0.5% SDS-solution</td><td>3.0 ml</td><td>3.0 ml</td><td>3.0 ml</td></tr>
<tr><td>10% APS</td><td>150 ul</td><td>150 ul</td><td>150 ul</td></tr>
<tr><td>TEMED</td><td>25 ul</td><td>25 ul</td><td>25 ul</td></tr>
</table>
<table>
<tr><th>SDS PAGE Stacking gel</th><th>2.5%</th></tr>
<tr><td>30% Acrylamide/bis acrylamide (29:1)</td><td>2.0 ml</td></tr>
<tr><td>0.635 M Tris/HCl (pH 6.8)</td><td>2.4 ml</td></tr>
<tr><td>dH2O</td><td>5.2 ml</td></tr>
<tr><td>0.5% SDS - Solution</td><td>2.4 ml</td></tr>
<tr><td>10% APS</td><td>140 ul</td></tr>
<tr><td>TEMED</td><td>24 ul</td></tr>
</table>
</td>
</tr>
</table>

		SDS running buffer (5x) 30 g Tris 144 g Glycine 5 g SDS adjust pH to 8.3 . Volume 1 liter
		Laemmli Sample buffer (2x) 125 mM Tris (pH: 6.8) 6% Glycerin (86%) 2% SDS 0.025% Bromophenol bleu 5% 2- Mercaptoethanol Make up volume with dH2O
6.	**5B. Protein staining and processing in gel**	**Coomassie stain** 1% Coomassie brialliant blue 40% methanol 20% acetic acid **Silver staining** **Solution 1** 0.3% Sodium thiosulphate 0.15% Potasium ferricyanide 0.05% Sodium carbonate **Solution 2** 0.1% $AgNO_3$ **Solution 3** 2.5% Sodium carbonate **Solution 4** 2.5% Sodium carbonate 100ul/100ml Formaldehyde

7.	5C. 2D PAGE for separation of the nc82 antigen from fly head homogenate	**I1 (Lysis buffer 1)** 9.5 M Urea 0.5% SDS 5% Beta mercaptoethanol 2% Ampholines pH 2-11
		I2 (Lysis buffer 2) 9.5 M Urea 5% NP40 (w/w) 5% Beta mercaptoethanol 2% Ampholines pH 2-11
		J (Overlay solution) 6M Urea 5% (w/w) NP40 1% Ampholine pH 2-11 (Solutions I1, I2 and J were stored at -20 ^{0}C).
		K (30% Acrylamide –BAA solution) 28.38 g Acrylamide 1.62 g Bis-acrylamide 100 ml dH_2O
		L 10% Nonindet P40 10 g NP40 in 100 ml dH_2O
		M (Anode electrophoresis buffer) 10 mM H_3PO_4
		N (Kathode elctrophoresis buffer) 20 mM NaOH

		O (SDS sample buffer for 2nd dimention) 60 mM Tris-Cl pH: 6.8 60 mM Tris-Cl pH: 6.8 2%SDS 5% Betamercaptoethanol 10% Glycerin
		Rod gels 5.5 g Urea, 1.33 ml Acrylamide solution (K) 2 ml 10% NP40 solution (L) 2 ml H_2O, 0.5 ml Ampholine(s) pH 2-11 (Serva, Heidelberg) 30 ul 10% APS 20 ul TEMED Solution was heated to dissolve urea, Ampholines, APS and TEMED were added after the soltuion was allowed to cool down, allowed to polymerise overnight and overlayed with water prior to loading .
8.	**6. Protein detection, purification and localization** **6A. Western blots**	**Western blot transfer buffer** 20 mM Tris pH- 8.0 150 mM Glycine 10% Methanol pH: 8.3
9.	**6C. Immunoprecipitation**	**Buffer A** 0.6 M NaCl 15 mM Tris.HCl (pH 7.4) 15 mM Beta mercapto ethanol

		Buffer B 0.6 M KCl 15 mM Tris.HCl (pH 7.4) 1 mM Dithiothretol (DTT)
10.	**6D. Immunohistochemistry**	**PBST** 137 mM NaCl 2.7 mM KCl 1/15 M Na_2HPO_4 - 20 ml 1/15 M KH_2PO_4 - 5 ml 0.3% Triton X 100 pH: 7.3
		Sodium phosphate buffer 1/15 M Na_2HPO_4 - 20 ml 1/15 M KH_2PO_4 - 5 ml pH: 7.3
11.	**6E. Fixes for electron microscopy**	**Karnovsky's fixative** 2.5% formaldehyde (USB?, Sigma) 2.5% gluteraldehyde (Roth) 0.1M Na-cacodylate (Sigma) 7 mM $CaCl_2$ (MERCK)
12.	**8. Microinjections and transgenic flies**	**Injection oil**
		Injection buffer 5 mM KCl

<table>
<tr><td></td><td></td><td>0.1 mM Na-phosphate pH: 6.8
0.2</td></tr>
<tr><td></td><td></td><td>7% Sodium Hypochlorite</td></tr>
<tr><td>13.</td><td>9. Fly genetics</td><td>Composition of the fly medium
<table>
<tr><td>Water</td><td>39</td><td>Liters</td></tr>
<tr><td>Yeast</td><td>675</td><td>Grams</td></tr>
<tr><td>Soy flour</td><td>390</td><td>Grams</td></tr>
<tr><td>Yellow cornmeal</td><td>2,850</td><td>Grams</td></tr>
<tr><td>Light malt extract (dehydrated)</td><td>1,800</td><td>Grams</td></tr>
<tr><td>Agar</td><td>225</td><td>Grams</td></tr>
<tr><td>Light corn syrup</td><td>3</td><td>Liters</td></tr>
<tr><td>Propionic acid</td><td>188</td><td>Milliliters</td></tr>
</table></td></tr>
</table>

EXPLAINATION OF TERMS

Acronym	Explanation of the term
2-D	2 dimensional
3-D	3 dimensional
A	Adenosine
awd	*Abnormal wing disc*
AZ	Active zone
BDGP	Berkley *Drosophila* Genome Project
BMP	Bone morphogen protein
BPB	Bromo-phenol blue
BRP	Bruchpilot
Ca^{2+}	Calcium $^{2+}$
CAPS	Ca^{2+}-dependent activator protein
CASK	CAMKII domain containing MAGU Kinase
CAST	Cytomatrix associated structural protein
CAZ	Cytomatrix at the active zone
CC	Coiled-coil
cDNA	Copy DNA
CG	Celera Genomics
CLIP-190	Cytoplasmic linker protein 190
CNS	Central nervous system
Cs	Canton-S
CSP	Cysteine String Protein
CtBP	C-terminal binding protein
C-terminus	Carboxyl terminus
CyO	Curly of Oyster
DAB	Diaminobenzidine
Dap 160	Dynamin associated protein 160
dCTP	Deoxycytidine tri-phosphate
DEPC	Diethyl pyrocarbonate
DGluRIIC	*Drosophila* Glutamate receptor Subunit IIC
Dlar	*Drosophila* leucocyte antigen related like

Dlg	Disk Large
DNA	Deoxyribonucleic acid
dNMNAT	*Drosophila* nicotinamide mononucleotide adenyltransferase
dNTP	Deoxyribonuncleotide tri-phosphate
DPAK	*Drosophila* P21 associated kinase
DTT	Di-thio thretol
ECL	Enhanced chemiluminescence
EJC	Excitatory junction currents
ELKS	ELKS epsilon like
Eps-15	Epidermal growth factor receptor substrate 15
ERC	ELKS-Rab6 interacting protein-CAST
ERG	Electroretinogram
ERM	Ezrin/Radixin/Moesin
EtBr	Ethidium bromide
EtOH	Ethanol
fne	*Found in neurons*
GA	Glutaraldehyde
GABA	Gamma-aminobutyric acid
GDI	GDP-dissociation inhibitor
GDP	Guanosine di-phosphate
GFP	Green Fluorescent Protein
GTP	Guanosine tri-phosphate
HCl	Hydrochloric acid
IWA	Isoluecine-Tryptophan-Alanine
kb	Kilobase
kDa	Kilodalton
KIF3A	Kinase like protein KiF3A
KO	Knockout
LIN_2	Liquid Nitrogen
LVA	Larval lamp
MAB	Monoclonal antibody
mEJCs	Mean excitatory junction currents
MHC	Myosin heavy chain

MINT	Munc-18 interacting molecule
mRNA	Messenger RNA
MTOR	Megator
Munc	Mouse uncoordinated
NAD^+	Nicotine adenosine dinucleotide
NaPPi	Sodium pyrophosphate
NEPHGE	Non equilibrium pH gradient electrophoresis
NMJ	Neuromuscular junction
NSF	N-ethylmaleimide sensitive factor
NT	Neurotransmitter
N-terminus	Amino terminus
ORF	Open reading frame
PAK	P21 associated kinase
PDZ	PSD-95-Dlg-ZO-1
PKA	Protein Kinase A
PMSF	Phenyl methyl sufonyl fluoride
PSD	Post-synaptic density
PSD95	Post-synaptic density-95
PTV	Piccolo-Bassoon transport vesicle
Rh1	*Rhodopsin1*
RIM	Rab Interacting Molecule
RIMBP	RIM binding protein
RNA	Ribonucleic acid
RNAi	RNA interference
RPTP	Receptor protein tyrosine phosphatases
RRP	Readily releasable pool
RT	Reverse transcription/Room temperature
RT-PCR	Reverse transcription (followed by) polymerise chain reaction
Sap47	Synapse-associated protein 47
SAP90	Synapse-associated protein 90
SAP97	Synapse-associated protein 97
SDS	Sodium dodesyl sulphate
SDS-PAGE	SDS- Polyacrylamide gel electrophoresis

SLO-1	Slow poke
SM	Sec1/Munc18 like proteins
SNAP-25	Synaptosomal associated protein-25
SNARE	Soluble N-ethylmaleimide sensitive factor attachment protein receptor
SSC	Sodium chloride – Sodium isocitrate buffer
SV	Synaptic vesicle
TEVC	Two electrode voltage clamp
T-SNARE	Target- SNARE
UAS	Upstream activating sequence
ug	Microgram
um	micrometer
UTR	Untranslated region
UV	Ultra violate
VAMP	Vesicle associated membrane protein
V-SNARE	Vesicle- SNARE
Wit	Wishful thinking
WT	Wild type
wun	*Wunen*
ZIP	Zipper

Acknowledgements

I wish to thank my supervisor Prof. Erich Buchner for giving me the opportunity to work with him and for his excellent guidance.

I thank Dr. Stephan Sigrist, Dr. Tobias Rasse, Dr. Carolin Wichman and members of the Sigrist lab for their collaboration and co-operation on the Bruchpilot project.

I thank Prof. Dabauvalle and members of her lab for the excellent help they have provided for the 2-D PAGE experiments.

I also thank Prof. Esther Asan and her group for their collaboration on the electron microscopy.

I thank Dr. Burkhard Poeck and Dr. Matthias Porsch for providing useful guidance for molecular biological experiments. I thank Prof. Heisenberg, Prof. Gert Pflugfelder, Dr. Roland Strauss, Dr. Henrike Scholz, Dr. Andre' Fiala, Dr. Andrea Wizenmann, Isabell Schwenkert, Jens Rister and Pavel Masek for their insightful discussions and all the help from time to time.

I also thank the F2 students Roland Jeblick and Karen Erbguth and Diplom student Jittiporn Chaisaingmongkol for their excellent work and enthusiasm.

I am grateful to all my colleagues in the Buchner group for their constant help, assistance and also for providing a cheerful working environment.

I am also thankful to Franco Bertolucci, Dr. Marcus Mronz, Dr. Simon Pick, Johannes Schindelin, Arnim Jennet and the rest of the crew from the Heisenberg group for their invaluable help and interesting discussions.

I also thank all the technical staff for their assistance.

I am grateful to Sigi for her affection and help. I wish to thank my parents and sister for providing me with an opportunity to pursue my goals and my wife Saloni for her constant inspiration, support and troubleshooting.

Dhananjay Anil Wagh

Wuerzburg, 22.07.2005

Publikationen

Wagh DA, Rasse TM, Asan E, Hofbauer A, Schwenkert I, Dürrbeck H, Buchner S, Dabauvalle MC, Gang Q, Wichmann C, Kittel R, Sigrist SJ, Buchner E. (2005, submitted) *Bruchpilot*, a protein with homology to ELKS/CAST/ERC, is required for structural integrity and function of synaptic active zones in *Drosophila*.

Wagh D, Rasse TM, Hofbauer A, Schwenkert I, Dürrbeck H, Buchner S, Dabauvalle MC, Sigrist SJ, Buchner E, et al. (2004) nc82 antigen : Analysis of Gene structure, sub-synaptic localization and developmental expression in *Drosophila*. (Abstract and oral presentation, Neurofly 2004, Neuchâtel, Switzerland).

Wagh, DA, Jatzke S, Huber S, Dürrbeck H, Dabauvalle MC, Asan E, Leibold C, Hofbauer A , Buchner S, Buchner E (2003) Identification and characterization of the nc82 antigen, an active zone protein at the presynaptic terminal of dipteran insects. (Abstract and poster, 29th Goettingen neurobiology confernce, Goettingen, Germany, pp. 982).

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Würzburg, den 22.07.05 **Dhananjay Wagh**

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst habe und keine anderen Hilfsmittel als die angegebenen angewandt wurden. Alle aus der Literatur entnommenen Stellen und Abbildungen sind als solche kenntlich gemacht.

Die Dissertation wurde weder vollständig noch teilweise einer anderen Fakultät vorgelegt.

Ich habe außer dem durch die beigefügte Urkunde belegten Universitätsdiplom keine anderen akademischen Grade erworben oder zu erwerben versucht.

Würzburg, den 22.07.05

Dhananjay Anil Wagh