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CHARACTERIZATION AND LOCALIZATION OF CASKIN1 AND INTERSECTIN1 IN MAMMALIAN RETINA.

A thesis submitted to the Faculty of Medicine in fulfilment of the requirements for the degree of

Dr.med.

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Declaration

I hereby declare that the Dr. med. thesis entitled "CHRACTERIZATION AND LOCALIZATION OF CASKIN1 AND INTERSECTIN1 IN MAMMALIAN RETINA" is a presentation of my original research work. Where other sources of information have been used, they have been acknowledged. No portion of work contained in this thesis has been submitted in support of any application for any other degree or qualification.

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RIZWANA ANJUM

Supervisor:

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Dedicated to my parents,
my brothers and my sister.

CONTENTS

SUMMARY

The synapses are the major site of interneuronal communication in the nervous system. Synaptic transmission provides the neurons with highly focal and fast signalling capabilities. Conventional and Ribbon synapses are two types chemical synapses in the nervous system. Conventional synapses are found in various types of neurons. However, the ribbon synapses, a specialized subclass of chemical synapses, have a more restricted distribution. They are found in photoreceptors and bipolar cells of retina, cochlear and vestibular hair cells and in pinealocytes. Physiologically, ribbon synapses are characterized by the tonic neurotransmitter release mediated by continuous exocytosis of synaptic vesicle. The defining feature of the ribbon synapses is the presence of an electron dense presynaptic specialization called the synaptic ribbon which is anchored perpendicular to the presynaptic plasma membrane at the active zone.

The protein RIBEYE, identified by Schmitz *et al*., (2000), is the main component of the synaptic ribbons. RIBEYE consists of a unique amino terminal A-domain and a carboxy terminal B-domain which is identical to protein CtBP2. Identification of other interaction partners of RIBEYE can help us understand how the synaptic ribbons work on molecular level. In this concern, the main aim of my experimental work is focused on the characterization and localization of Caskin1 and Intersectin1 in the mammalian retina. Retina provides a simple approach to identify ribbon synapses in the outer plexiform layer as well as the conventional synapses present primarily in the inner plexiform layer.

Caskin1 contains protein-protein interaction domains and is demonstrated to be present only in the conventional synapses of brain. For my thesis work, Caskin1 was selected to study its interaction with RIBEYE, because Caskin1 contains putative interacting motifs with PxDLS consensus sequence (EBI interaction database). In my experimental work, I studied that Caskin1 is predominantly expressed in the inner plexiform layer of retina. Moreover, in the outer plexiform layer Caskin1 is expressed only specific to the cone photoreceptor synapses of the retina.

In the second part of my thesis work, I used five different Intersectin1 antibodies

raised against GST fusion proteins of Intersectin1. On the basis of mass YTH, Stelzl *et al*., 2005 found a preliminary evidence for an interaction between Munc119 and Intersectin1 by automated Yeast 2 hybrid interaction. In the present study, I analysed the physiological significance of this finding by localizing Intersectin1 in the retina. In my studies, I demonstrated the presence of Intersectin1-L (200 kDa) and Intersectin1-S (145kDa) in bovine retina. Munc119 is an essential interacting partner of RIBEYE. The co-immunoprecipitation experiment has shown an interaction between Munc119/Intersectin1. The binding of Intersectin1 to synaptic terminals in the outer plexiform layer is highly specific because the presynaptic Intersectin1 signals are completely blocked with Intersectin1-GST but not by control protein GST alone. Immunolabelling with Intersectin1 and RIBEYE shows co-localization of Intersectin1 with RIBEYE in outer plexiform layer of bovine retina. Surprisingly, one Intersectin1 antibody (Intersectin 229) labelled the synaptic ribbons in outer plexiform layer of retina. The same antibody has also expressed an additional lower molecular band (45kDa) in the western blot. In conclusion, the data obtained from this research work provides a better understanding of the morphological analysis of localization of Intersectin1 in the retina.

SUZAMMENFASSUNG

Synapsen stellen die Hauptkomponenten der interneuralen Kommunikation im Nervensystem dar. Synaptische Transmission verleiht den Neuronen die Fähigkeit zur stark fokussierten und schnellen Signalübermittlung. Konventionelle und Ribbon-Synapsen sind 2 Arten von chemischen Synapsen im Nervensystem. Konventionelle Synapsen findet man in vielen verschiedenen Arten von Neuronen, während die Ribbon-Synapsen, eine spezialisierte Unterart der chemischen Synapsen, eine begrenzte Verteilung haben. Man findet sie in den Photorezeptoren und Bipolarzellen der Retina, des Innenohrs, der vestibulären Haarzellen sowie in den Pinealocyten. Physiologisch werden Ribbon-Synapsen durch ihre tonische Freisetzung von Neurotransmittern charakterisiert, welche durch die kontinuierliche Exocytose von synaptischen Vesikeln herbeigeführt wird. Das besondere Merkmal der Ribbon-Synapsen ist das Vorhandensein einer elektronendichten präsynaptischen Spezialisierung, die als synaptischer Ribbon bezeichnet wird, welcher rechtwinkelig zur präsynaptischen Membran in der aktiven Zone der Synapse verankert ist.

Das Protein RIBEYE, identifiziert durch Schmitz *et al*., (2000), ist der Hauptbestandteil der synaptischen Ribbons. RIBEYE besteht aus einer amino-terminalen A-Domäne und einer carboxyterminalen B-Domäne, welche identisch mit dem Protein CtBp2 ist. Die Identifizierung der Interaktionspartner von RIBEYE kann uns helfen die Funktion des synaptischen Ribbons auf der molekularen Ebene zu verstehen.Vor diesem Hintergrund war das Hauptziel meiner experimentellen Arbeit die Charakterisierung und Lokalisation der Proteine Caskin1 und Interleukin 1 in der Retina von Säugetieren. Retina stellt einen einfachen Ansatz zur Identifizierung der Ribbon-Synapsen, welche in der äußeren plexiformen Schicht vorkommen, als auch der konventionellen Synapsen, welche primär in der inneren plexiformen Schicht vorkommen, dar.

Caskin1 enthält Protein-Protein Interaktionsdomänen und kommt soweit bisher bekannt im Gehirn vor. Da Caskin1 ein putatives Interaktionsmotiv mit der PxDLS Konsensussequenz (EBI interaction database) enthält, wurde es in meiner Arbeit ausgewählt um eine mögliche Interaktion mit RIBEYE zu untersuchen. In meiner

experimentellen Arbeit habe ich gezeigt, dass Caskin1 hauptsächlich in der inneren plexiformen Schicht der Retina exprimiert wird. Desweiteren wird es in der äußeren plexiformen Schicht der Retina spezifisch in den Zapfen exprimiert.

Im zweiten Teil meiner Arbeit habe ich fünf verschiedene Intersetin1 Antikörper gerichtet gegen GST-Intersectin1 Fusionsproteine genutzt. Auf der Grundlage des Quantitatven-Yeast-Two-Hybrid-Screens fanden Stelzl *et al*. (2005) einen ersten Hinweis auf eine Interaktion zwischen Munc 119 und Intersectin1 durch Interaktion im automatisierten Yeast 2 hybrid Screen. In der vorliegenden Studie habe ich durch Lokalisierung von Intersectin1 in der Retina die physiologische Signifikanz dieser Ergebnisse untersucht. In meinen Versuchen habe ich gezeigt, dass Intersectin1-L (200 kDa) und Intersectin1-S (145 kDa) in Rinder-Retina vorkommen. Munc119 ist ein essentieller Interaktionspartner von RIBEYE. Die Co-Immunoprecipitation zeigte eine Interaktion zwischen Munc119 und Intersectin1. Die Bindung des Intersectin1-Antikörpers an synaptischen Terminalen der äußeren plexiformen Schicht ist hoch spezifisch, da das präsynaptischen Intersectin1-GST komplett blockiert wird, aber nicht durch das Kontrollprotein GST allein. Immunfärbungen mit Intersectin1 und RIBEYE zeigen eine Co- Lokalisation der beiden Proteine in der äußeren plexiformen Schicht der Rinder-Retina. Überraschenderweise markierte ein Intersectin1-Antikörper (Intersectin 229) die synaptischen Ribbons in der äußeren plexiformen Schicht der Retina. Der gleiche Antikörper zeigte auch im Western-Blot eine zusätzliche tiefere Bande bei 45kDa.

Zusammengefasst tragen die durch diese Arbeit erlangten Daten zu einem besseren Verständnis der morphologischen Deutung und der Lokalisation von Intersectin1 in der Retina bei.

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CHAPTER 1
INTRODUCTION

The visual system is an extraordinary system in the quantity and quality of information it supplies about the world. The eye, a vital organ for vision, can detect a single photon and transmits its signal to higher brain centers which can extrapolate the signals conveyed from retina to build a precise image of the external environment. The retina offers an excellent source of material for detailed anatomical, physiological and pharmacological analyses of the neural mechanisms underlying basic information processing by the vertebrate brain.

1.1. STRUCTURE AND FUNCTION OF EYE:

The eye is the most important human sense organ as it receives the light and forms an image on the photosensitive layer called the retina. The eye is a fluid filled sphere enclosed by three layers or tunicae (Fig.1). The outer fibrous layer consists of sclera and cornea. The middle vascularised layer includes the iris, ciliary body and the choroid. The inner most nervous layer includes the retina which contains the photosensitive rod and cone cells and associated neurons. Once the light passes through the cornea and pupil, it travels to the lens which changes its accommodation by varying its convexity. After passing through the vitreous humor (a clear jelly-like substance), the light falls on the retina where photosensitive cells, the rods and the cones, are responsible for light detection. The rod cells respond to the condition of dim light. The cone cells can distinguish colour from wavelength of light being reflected off the viewed object. Each colour has its own unique wavelength. The rods and cones convert the light wavelength into electrical signals and send these signals to the inner retina and finally to the optic nerve. The optic nerve transmits the electrical signals to the lateral geniculate nucleus and the visual fields of the occipital lobe in brain where the signals are translated into what we perceive as light.

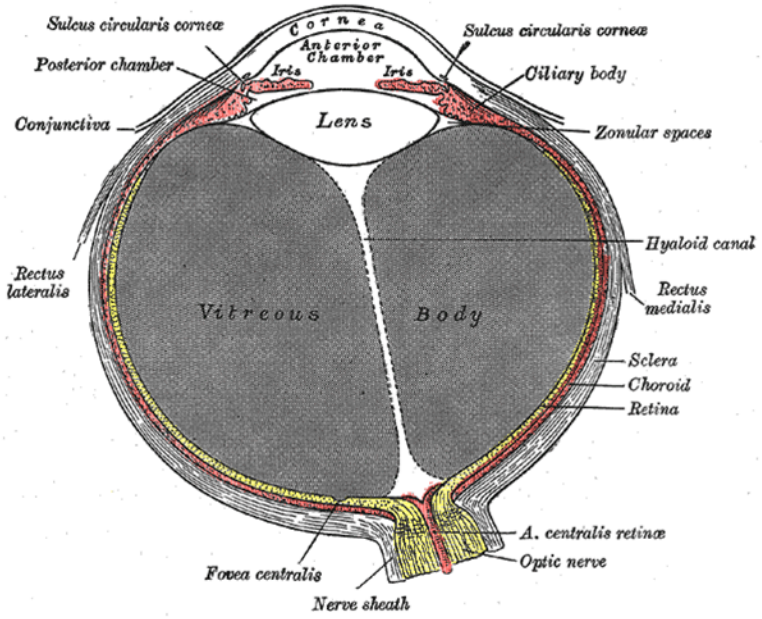

Figure 1. Section through the adult human eye: en.wikipedia.org/wiki/Uvea

1.2. The structural organisation of retina:

There is a complex network of higher order sensory neurons in the retina. A variety of visual signals e.g. brightness, darkness, contrast, colour and motion, all process side by side by these neurons in the retina (Wässle *et al*., 2004). The retina and the optic nerve develop as an out growth of the brain, therefore , retina or the neural part of the eye is a part of central nervous system (for review, see Purves *et al*., 2001). Similar to the other structures of central nervous system, a large number of different neurons are present in the retina (Masland, 2001). The eye is derived from three types of embryonic tissue: the neural tube (neuroectoderm), from which the proper retina and its associated pigment epithelium arise; the mesoderm of the head region, which produces the corneoscleral and uveal tunics; and the surface ectoderm, from which the lens develops. The mature mammalian retina consists of two distinct components: the retinal pigmented epithelium (RPE) and the neural retina, which is composed of neurons and glial cells.

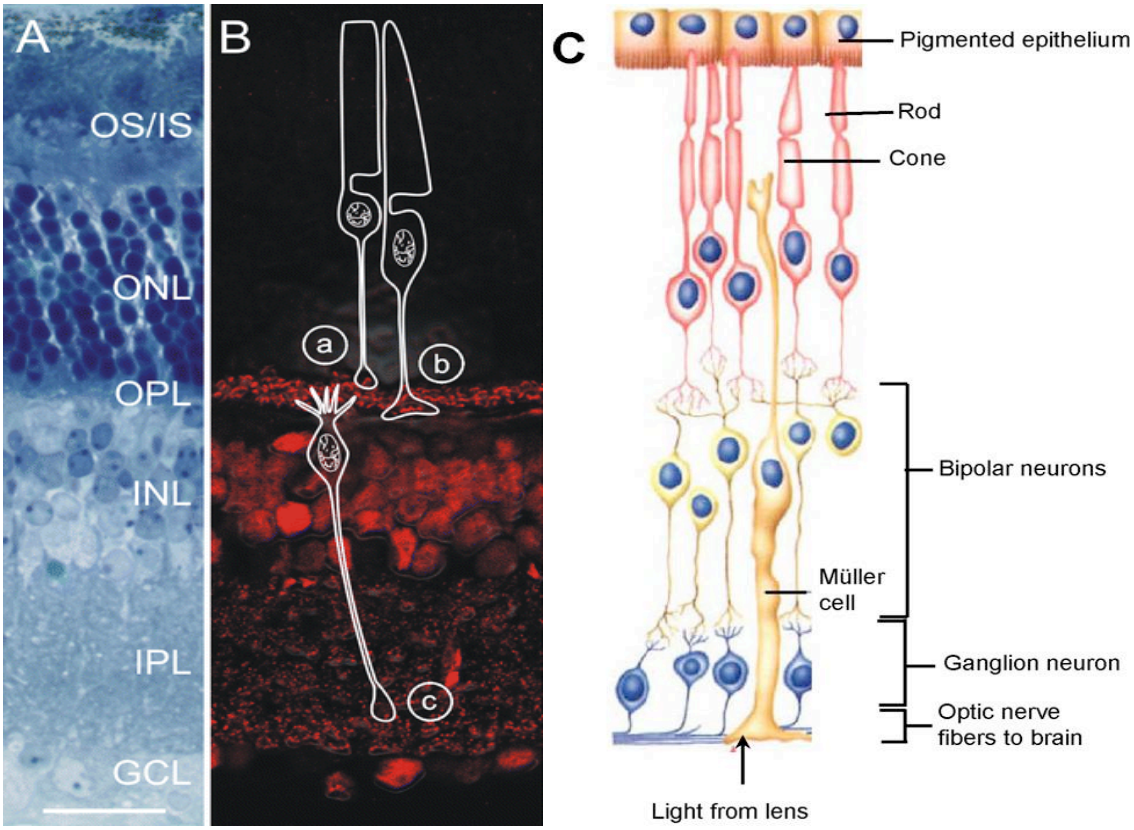

Figure 2. Ribbon synapses of the mammalian retina. A.. Toluidine blue-stained vertical cryostat section of a mammalian retina showing the various retinal layers (OS/IS outer and inner segments of the rod and cone photoreceptors, ONL outer nuclear layer containing the somata of the photoreceptors, OPL outer plexiform layer or first synaptic region, INL inner nuclear layer containing the somata of the second order neurons, i.e. horizontal, bipolar and amacrine cells, IPL inner plexiform layer or second synaptic region, GCL ganglion cell layer containing the somata of the ganglion cells and of displaced amacrine cells). B. Confocal laser scanning micrograph of a vertical cryostat section through the mouse retina stained with an antibody against CtBP2/RIBEYE, which labels the ribbon synapses in the two synaptic layers of the retina (cell somata in the INL and the GCL are also CtBP2-immunoreactive). The rod (cell a) and cone (cell b) photoreceptors make ribbon synaptic contacts onto postsynaptic bipolar cells in the OPL. The bipolar cells (cell c), in turn, transmit the signals at their ribbon synapses onto amacrine and ganglion cells in the IPL. Bar 20 µm (A, B) (adapted from tom Dieck *et al*., 2006). C. Diagrammatic representation of the retina (Purves *et al*., 2001).

The neural retina is a highly organized structure and is composed of ten clearly defined cellular layers (Fig. 2 A, C) which are

1. Inner limiting membrane
2. Nerve fibre layer
3. Ganglionic cell layer
4. Inner plexiform layer
5. Inner nuclear layer
6. Outer plexiform layer
7. Outer nuclear layer
8. External limiting membrane
9. Outer and inner segments of photoreceptors
10. Pigment epithelium

1.2.1. The retinal pigment epithelium:

The retinal pigment epithelium (RPE) is composed of a single layer of hexagonal cells that are densly packed with pigment granules and is situated just internal to the Bruch's membrane. This layer has a close anatomical and functional relation with the retina. It plays a central role in retinal physiology by forming the outer blood-retinal barrier and controls the transportation of ions and metabolites. The RPE cells are indispensible for the maintenance of neural retina as they participate in visual pigment regeneration, phagocytosis and digestion of photoreceptor wastes and maintenance of retinal adhesion.

1.2.2. Photoreceptors:

The photoreceptor cells in the vertebrates are specialized and highly photosensitive neurons that are responsible for the transduction of light into an electrical signal. The process of vision is initiated when the photoreceptors transmit the electrical signal to other neurons in the retina (Kwok *et al*., 2008). There are two types of photoreceptor cells: rods and cones. The rods are responsible for scoptopic or nocturnal vision. The cones are employed for photopic or diurnal colour vision and are much less sensitive to light than rods, but have a higher temporal resolution. Both of these photoreceptors consist of five basic principle subcellular regions: the outer segment where the process of phototransduction takes place. It contains the visual pigment. A thin cilium joins the outer segment to the inner segment and allows the passage of proteins and other molecules between the inner and outer segments. The inner segment is packed with the cell organelles involved in metabolic activities to meet the high energy demands associated with phototransduction of the cell. The cell body/soma contains the nucleus. The synaptic terminal transmits the light signal to the second order neurons: the bipolar and horizontal cells. An organized stack of more than 1000 discs which are separated by the plasma membrane is present in the outer segment of the rod photoreceptor cells. Identical organizations of closely packed membrane discs which are continuous with the plasma membranes are located in the outer segment of the cone photoreceptors (Kwok *et al*., 2008). There is a continuous process of regeneration in the outer segments. In this process, addition of new disc membranes occurs at the base of the outer segments. The old discs from the distal end are broken down and finally disappear

by phagocytic process carried out by the neighbouring retinal pigment epithelial cells (Young *et al*., 1967; Young *et al*., 1969). In this manner, a complete renewal of outer segment takes place within a period of 10 days (Kwok *et al*., 2008). Rhodopsin is an important visual pigment and is present in high concentration in the outer segment of rod photoreceptors (Liang *et al*., 2003; Nickell *et al*., 2007). Photo-activation of rhodopsin results in the activation of G-protein transducin. This results in activation of phosphodiesterase 6 which in turn causes hydrolysis of cGMP in the plasma membrane, and hyper-polarization of the cell (Hamer *et al*., 2005; Kiel *et al*., 2011). This phototransduction is followed by inactivation of rhodopsin and other components of the visual cascade, resynthesis of cGMP, regeneration of rhodopsin from 11-*cis*-retinal and opsin (Lamb and Pugh, 2004; Kwok *et al*., 2008). After these reactions, the rod cell goes back to its dark state. The outer segments of cone photoreceptors have almost similar photo-excitation and recovery process. However, different but related genes encode the participating proteins in the cone outer segment (Kwok *et al*., 2008).

For the initiation of transmitter release, both rod and cone photoreceptors utilize voltage-dependent L-type calcium channels and specialized ribbon synapses (Heidelberger *et al*., 2005). Transmission of signals from the ribbon synapses of photoreceptors takes place through their connection with the dendrites of the bipolar and horizontal cells in the outer plexiform layer. In the inner plexiform layer, signal is transmitted through the synaptic contacts between the bipolar cells, amacrine cells and ganglion cells (tom Dieck *et al*., 2006) and finally the higher centers in the brain receive that signal.

1.2.3. The retinal bipolar cells:

The bipolar cell, being the second order retinal neuron, occupies a pivotal position in the retina as a major neuron that bridges the gap between the two synaptic layers of retina. Their dendrites reside in the outer plexiform layer and the synaptic terminals are in the inner plexiform layer. The major task of the bipolar cells is to transmit spatially and temporally filtered signals from the OPL to five anatomically defined strata present in the second synaptic layer (IPL) of the retina (Ghosh *et al*., 2004). Ribbon synapses are also present in the bipolar cells. The bipolar cells respond to light stimulus by relatively slow changes of membrane potential and these responses are unusual in being

non-spiking (Heidelberger *et al*., 2005). There are two basic types of bipolar cells (Dowling &Werblin, 1969): ON bipolar cells and OFF Bipolar cells and the two types can be distinguished both their functions and morphology. From the anatomical studies, it is found out that mouse, cat, rat, rabbit and primate retinae have almost 10 different types of cone bipolar cell and one type of rod bipolar cells (Fig. 3) (Haverkamp *et al*., 2008)**.** A typical mammalian retina is known to have 9-10 different types of cone driven bipolar cells (Masland, 2001).

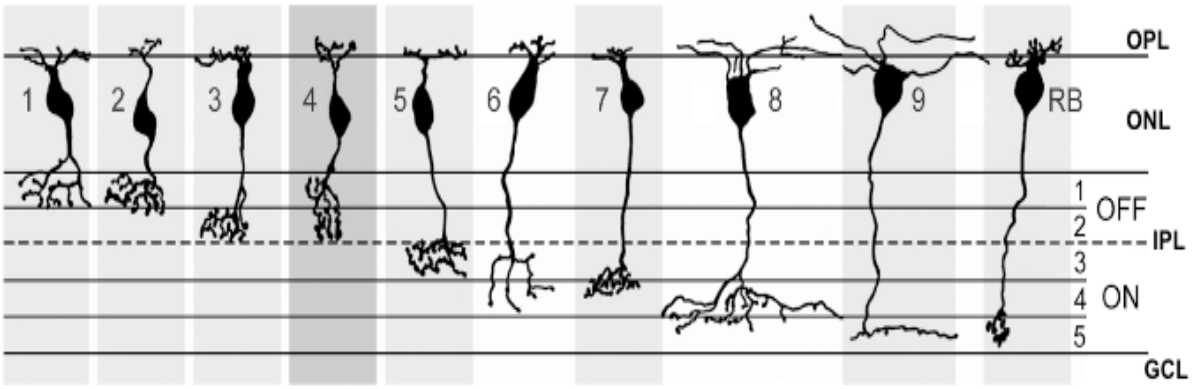

Figure 3. Schematic diagrams of different type bipolar cells of the mouse retina. The dashed horizontal lines dividing the IPL represent the border between the OFF- (upper) and the ON- (lower) sub layers. Bipolar cells with axons terminating above this line represent OFF-bipolar, those with axons terminating below this line represent ON-bipolar. Abbreviations: RB, rod bipolar cell; 1-9, cone bipolar cells (modified from Haverkamp *et al*., 2008).

1.2.4. The retinal horizontal and amacrine cells:

Retinal interneurons, i.e. amacrine cells and horizontal cells, have their cell bodies in the inner nuclear layer of the retina. The processes of amacrine cells are projected into the inner plexiform layer (Vigh *et al*., 2000). In the mammalian retina, AII amacrine cells receive synaptic signals from depolarizing and hyperpolarizing bipolar cells and are important relay stations for rod- mediated signals. In addition, they receive input signals from other amacrine cells (Pang *et al*., 2007). The interaction between horizontal cells and photoreceptor terminals, known as “lateral elements”, is post-synaptic to photoreceptor terminals (Hirano *et al*., 2011). These lateral interactions between receptors, horizontal cells and bipolar cells in the outer plexiform layer play a vital role in the visual system's sensitivity to luminance contrast over a broad range of light intensities. The neuronal circuits between photoreceptors, ganglionic cells and optic nerve play an organised functional role in neural processing that a message is transmitted to brain along the optic nerve.

1.2.5. The outer and inner limiting membranes:

The inner and outer limiting membranes are the architectural supports for the retina and are formed by the principal glial cells of the retina called the Müller cells. Müller cells bodies are located in the inner nuclear layer and they project irregularly thick and thin processes in either direction to the outer limiting membrane and inner limiting membrane. The inner limiting membrane is formed by the conical end feet of the Müller cells. The junctions forming the outer limiting membrane are between the Müller cells and other Müller cells and photoreceptor cells as zona adherens.

1.2.6. The outer nuclear layer (ONL):

This layer contains the cell bodies of rods and cone photoreceptors which are grouped parallel to each other and spread their light sensitive processes, the outer segments, in the direction of the retinal pigment epithelium.

1.2.7 The outer plexiform layer (OPL):

The OPL is the first synaptic layer in the retina which interconnects the dendrites of horizontal and bipolar cells with the terminals of photoreceptors forming the first level of intra-retinal information processing.

1.2.8. The inner nuclear layer (INL):

The inner nuclear layer contains the cell bodies of bipolar, amacrine and horizontal cells for the processing of signals. The neuronal cell bodies of supporting Müller glial cells are also located here.

1.2.9. The inner plexiform layer (IPL):

The inner plexiform layer contains the dendrites of the ganglion cells, amacrine cells and the axon terminals of bipolar cells and forms a dense plexus. The bipolar cells transmit the signals of photoreceptors from the outer retinal layers. These signals are modulated by synaptic interactions with amacrine and ganglion cells and finally transferred to the ganglion cells.

1.2.10. The ganglion cell layer (GCL):

This layer contains the ganglion cell bodies and it transmits the visual information via optic nerve to the lateral geniculate ganglion in the brain. Almost 10-15 different ganglion cells receive the signals from the bipolar cells (Masland, 2001).

1.2.11. The nerve fibre layer:

This nerve fibre layer contains axons of the ganglion cells and they receive their myelin sheaths after exit from the eyeball. The fibre density is lowest in the fovea. The optic nerve connects all axons of ganglion cells and this bundle of more than a million fibres (in humans) then passes information to the next relay station in the brain for sorting and integrating into additional information processing channels.

1.3. Synapses in mammalian retina:

The retina receives light signals over several orders of magnitude. The processing of visual signals requires a range of synapses with different kinetics in signal transmission. There are two main types of structurally and functionally suited synapses in the retina and these include conventional chemical synapses and the ribbon synapses (for review, see tom Dieck, 2006). These synapses in the retina are present in two layers that can be easily recognised morphologically: the thin outer plexiform layer (OPL) which contains the photoreceptor ribbon synapses and the more complex inner plexiform layer (IPL) containing mainly the conventional synapses (Ullrich *et al*., 1994).

1.4. Ribbon synapse of retina:

Information is encoded by the conventional neurons by involving a change in the rate of action potential which is supposed to limit the amount of information transfer (for review, see tom Dieck and Brandstätter, 2006). On the contrary, the ribbon synapse constitutes a distinct type of chemical synapses (for review, see Schmitz, 2009). The ribbon synapses present in the photoreceptors and bipolar cells of the retina and saccular and vestibular hair cells in the inner ear (Morgans, 2000; Schmitz, 2009) are capable of transmitting light and sound signals respectively , over a broad range of several orders of magnitude in intensity (for review, see tom Dieck., 2006). The ribbon synapses have presynaptic, sheet-like organelles called synaptic ribbons which consist

of a lamellar organization (Sterling *et al*., 2005). The synaptic ribbon is an electron-dense structure of considerable size. It is located perpendicular to the active zone of the plasma membrane where exocytosis of the synaptic vesicles takes place (Schaeffer *et al*., 1982; Schmitz *et al*., 2000). A thin stalk-like connection whose composition is not known, maintains the connection between the synaptic vesicles and the ribbon (Wagner, 1996).The synaptic ribbon is anchored along its base to archiform density. The archiform density is an electron dense structure, localized within a small evagination of the presynaptic membrane which is called synaptic ridge. The synaptic ridge contains clusters of L-type voltage gated Ca^{2+} channels (Schmitz., 2009).

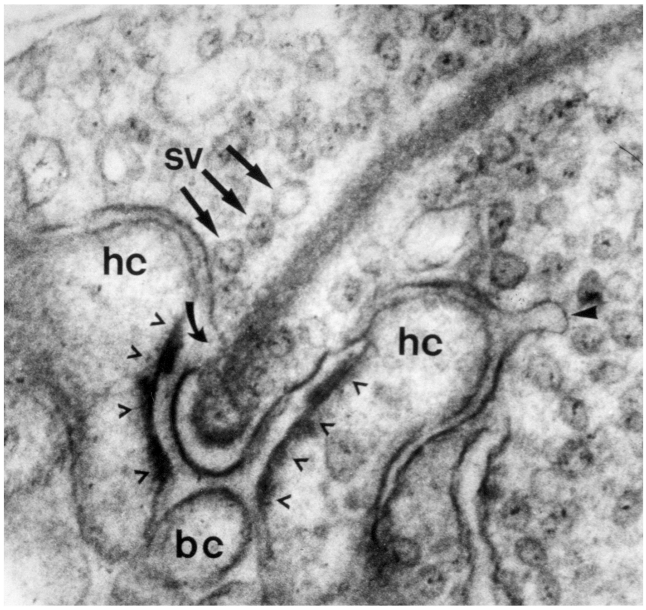

Figure 4. Electron Micrograph of the photoreceptor ribbon synapse. Abbreviations: sr, synaptic ribbon; sv, synaptic vesicle; bc, postsynaptic dendrites of bipolar cell; hc, postsynaptic dendrites of horizontal cells; black arrows-Synaptic vesicle; bold arrow head-endocytosis ; arrow head -post synaptic density (Schmitz, 2009).

1.5. Ultrastructural characteristics of ribbon synapses:

The shape of the ribbon and the number tethered vesicles differs among various types of photoreceptors and bipolar cells. In mammals, rod synapses contain a small terminal and a single large synaptic ribbon which consists of a total of almost ~770 synaptic vesicles (Sterling and Matthews 2005). The synaptic ribbon bends at four deep invaginating postsynaptic elements, dendrites of bipolar cells and processes of horizontal cells. As a result, the synaptic ribbon attains a horseshoe shape structure which can be easily seen at light microscopic level (tom Dieck *et al*., 2006). As compared to the rods, cone synapses contain more synaptic ribbons (10-12 ribbons per

terminals). The size of individual ribbons in the mammalian retina is shorter in cones than in rods, but the total ribbon surface and the number of vesicles that are tethered to ribbon is considerably larger in cones than in rods (for review, see Sterling and Matthews 2005; Heidelberger *et al*., 2005). Likewise, ribbon synapses are also present in retinal bipolar cells (Schmitz, 2009). In mammals, 30-40 ribbons are present in bipolar cells. For instance, a rod bipolar cell contains 30-40 ribbons (Sterling *et al*., 2005).

1.6. Functions of retinal ribbon synapses:

Ribbon synapses are considered as specialized chemical synapses because they are capable of maintaining rapid exocytosis of synaptic vesicles for a long period of time (for review, see Fuchs, 2005; Heidelberger *et al*., 2005; Prescott and Zenisek, 2005; Sterling and Matthews, 2005; Nouvian *et al*., 2006; Singer, 2007). This could only be achieved by a very rapid transportation of synaptic vesicles to the active zone of ribbon in a manner that resembles a conveyor belt (see review, Sterling & Matthews, 2005; tom Dieck *et al*., 2006). The ribbon has the capacity to accommodate a large reservoir of primed releasable vesicles. A small rapidly releasable pool is formed by the vesicles that are in closest contact with the presynaptic plasma membrane at the base of the ribbon. However, the rest of the vesicles tethered to the ribbon make the large slower releasable pool (von Gersdorff *et al*., 1996; Heidelberger *et al*., 2002, 2005; Parson and Sterling 2003). High Ca^{2+} stimulation at ribbon synapse, triggers release of the entire pool of vesicles tethered to the ribbon on a millisecond frame (von Gersdorff *et al*., 1996). Then, the primed vesicles move down the ribbon in a rapid way and reach the active zone and are readily available for the fusion for exocytosis (for review, see Heidelberger *et al*., 2005).

1.7. Molecular composition of retinal ribbon synapses:

RIBEYE is identified as a major component specific for the synaptic ribbons (Schmitz *et al*., 2009). In addition to RIBEYE, the photoreceptor ribbons also contain clusters of CtBP1/BARS (tom Dieck *et al*., 2005). Other protein recognized to be present in synaptic ribbons of ribbon synapses include kinesin isoform KIF3A (Muresan *et al*., 1999), the cytomatrix protein RIM1 and Piccolo. The protein RIM2, Bassoon,

CAST/ELKS and L-type Ca^{2+} channel-1 subunit are also found to be localized in the active zone of synaptic ribbons. Bassoon, a cytomatrix protein, is responsible to anchor the synaptic ribbon at the base of active zone (tom Dieck *et al*., 2005). Synaptic vesicle protein SV2B appears to be present at all ribbon synapses in the retina. However, SV2A is present in the terminals of cones but not in rods (Wang *et al*., 2003). Detailed analysis of the distribution of different presynaptic proteins in ribbon synapses have demonstrated that they possess the same proteins in general, which are present in the conventional synapses (for review, see Sterling and Matthews, 2005; Schmitz, 2009). However, minor differences were observed, as for example, the use of syntaxin3b instead of syntaxin 1 for fusion and of L-type Ca^{2+} channels instead of N-, P/Q-, or R-type channels in some but not all species (for review, see Sterling and Matthews, 2005).

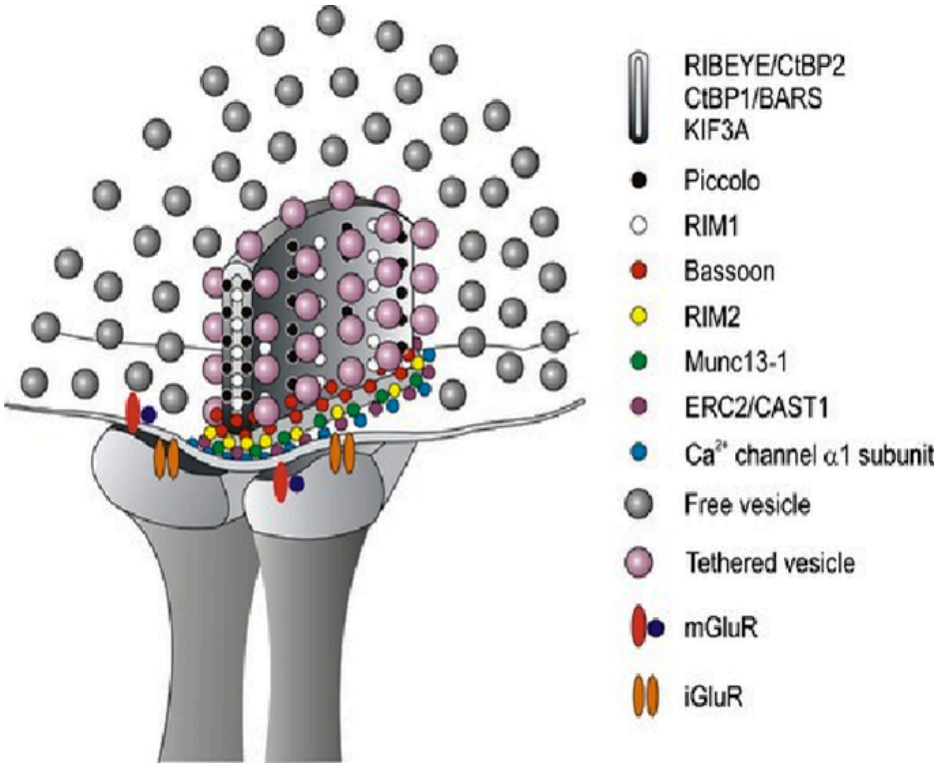

Figure 5. Representation of a retinal ribbon synapse. The differential distribution of CAZ proteins defines two presynaptic compartments at the ribbon synapse. The ribbon-associated compartment includes RIBEYE/CtBP2, CtBP1/BARS, KIF3A, Piccolo and RIM1. The active zone compartment includes RIM2, Munc13-1, ERC2/CAST1, and a L-type calcium channel α1 subunit. At the photoreceptor ribbon synapse, Bassoon localizes at the border between the two compartments. Various types of postsynaptic and/or presynaptic metabotropic (mGluR) and ionotropic (iGluR) glutamate receptors mediate the action of glutamate, which is released at the retinal ribbon synapses (S. tom Dieck *et al*., 2006).

1.8. RIBEYE the major component of synaptic ribbons:

1.8.1. Structural organization of RIBEYE:

RIBEYE, a novel protein, is identified as a main element of the synaptic ribbons of retina (Schmitz *et al*., 2000). RIBEYE consists of two domains which include: an N-terminal A domain (563 aa) and a C-terminal B domain (425 aa). The A domain is not significantly homologous to any currently described protein and it consists of a relative abundance of serine and proline residues. On the other hand, the RIBEYE(B) domain bears resemblance to CtBP2 sequence except the 20 N-terminal amino acids of CtBP2 (Schmitz *et al*., 2000). There is absence of RIBEYE(A)-domain in D. melanogaster C-elegans and other lower vertebrates and invertebrates. This supports the idea that RIBEYE and retinal synaptic ribbons are an evolutionary characteristic of vertebrates (Schmitz *et al*., 2000). Several studies have demonstrated that RIBEYE is the major component of synaptic ribbons (Schmitz *et al*., 2000; Zenisek *et al*., 2004; Wan *et al*., 2005; Magupalli *et al*., 2008). Hence, it is supposed that RIBEYE has a vital influence on the function of synaptic ribbons.

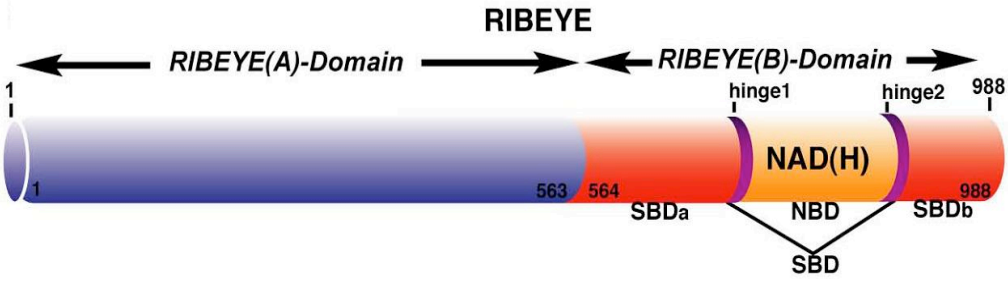

Figure 6. **Schematic structure of RIBEYE**. RIBEYE contains of a large amino-terminal A-domain and a carboxyterminal B-domain. The B-domain of RIBEYE contains the NADH-binding subdomain (NBD, depicted in yellow) and the substrate-binding subdomain (SBD, denoted in red).

1.8.2. Structure of RIBEYE (B) domain:

There are two main globular sub-domains contained by RIBEYE(B)-domain. These include a NADH-binding sub-domain (NBD) and a substrate-binding sub-domain (SBD) (Fig. 6). The dinucleotide-binding domain bears an evolutionarily conserved structure and it forms the core homology domain among these proteins (Chinnadurai, 2003). The B-domain of RIBEYE is known to bind nicotinamide adenine dinuleotide (NAD^+ or NADH [NAD (H)]) with high affinity. It belongs to the family of D- isomer-

specific 2-hydroxy acid dehydrogenases (Schmitz *et al*., 2000). It is demonstrated that CtBPs, CtBP1, CtBP2 and RIBEYE(B)-domain (Kumar *et al*., 2002; Nardini *et al*., 2003, Magupalli *et al*., 2008) homodimerize through the dinucleotide-binding domain. This results in the formation of an extensively large hydrophobic dimerization interface. From the topographical and functional aspects, the docking site of RIBEYE(A) for RIBEYE(B) is different from that of RIBEYE(B) homodimerization interface. Moreover, it is negatively regulated by nicotinamide adenine dinucleotide (Magupalli *et al*., 2008). In addition, the nicotinamide adenine dinucleotide coenzyme (NAD and NADP) plays not only a central pivotal role in metabolism as a carrier of reducing equivalent, but is also equally vital for cellular signalling (for review, see Chinnadurai, 2002 and 2003). The homology of RIBEYE(B) domain/ CtBP2 to NAD^+ domain-dependent 2-hydroxyacid dehydrogenases has functional importance, and the domain may serve as an enzyme participating in synaptic vesicle priming on synaptic ribbon and in transcriptional repression (Schmitz *et al*., 2000; Schwarz *et al*., 2011).

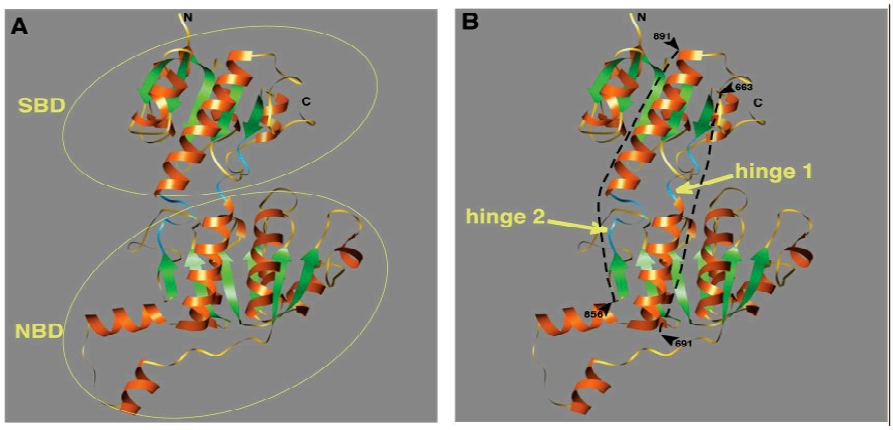

Figure 7. Predicated structure of RIBEYE(B) domain using homology model of CtBP2. (A, B) Structure model of the B-domain of RIBEYE based on the crystal structure of tCtBP1 (Kumar *et al*., 2002; Nardini *et al*., 2003; see also Magupalli *et al*., 2008; Alpadi *et al*., 2008). The structure model covers large parts of the B-domain (RE(B)575-905). The B-domain of RIBEYE consists of a NAD(H)-binding subdomain (NBD) and a substrate-binding subdomain (SBD) which are connected by two flexible hinge regions, hinge 1 and hinge 2 (colored in blue).

1.8.3. Functional role of RIBEYE in the ribbon synapses:

The RIBEYE(A)-domain seems to have a predominantly structural position. But the B-domain is important for the cytoplasmic face of the synaptic ribbon. A possible model for the function of RIBEYE is proposed by Schmitz *et al*., 2000. According to this model, the N-terminal A-domain is responsible primarily for the formation of

aggregates of assembled ribbons. But it is not sufficient for RIBEYE alone to organize the ribbons, therefore, at least one additional protein, as an inner core component, is needed in this mode. The presence of such a protein component is suggested by the finding of a second unique protein in the biochemically purified ribbon fraction (Schmitz *et al*., 2000).

RIBEYE being a scaffold protein consists of ideal properties which could explain the assembly of synaptic ribbons from RIBEYE subunits (Magupalli *et al*., 2008). In addition, a multistep process is involved in the assembly of synaptic ribbons from RIBEYE. This multistep process includes the synaptic spheres, spherical synaptic ribbon like structures (Schmitz *et al*., 2000). The RIBEYE(B) domain serves as an enzyme and this domain can bind with NAD^+ with high affinity. This points towards the fact, that its homology with NAD^+-dependent dehydrogenases is functionally relevant (Schmitz *et al*., 2000; Schwarz *et al*., 2011). Interestingly CtBP1, a close homolog of CtBP2, was also proposed to participate in membrane traffic as "BARS" (brefeldin A-ADP ribosylated substrate). It has a unique role in membrane fission in the Golgi complex because it functions as a lysophosphatidic acid coenzyme A acyltransferase (Weigert *et al*., 1999). The structural relationship of CtBPs with NAD^+ dependent dehydrogenases goes well with the argument that CtBP is ADP ribosylated in an NAD^+–dependent reaction which occurs in parallel with GAPDH (another NAD^+-dependent dehydrogenase) (Di Girolamo *et al*., 1995). Because a little chemical similarity is found between the reaction mechanisms of acyltransferases and dehydrogenases, questions are raised regarding the precise enzymatic role of CtBP1 in Golgi membrane traffic (Schmitz *et al*., 2000). In transcription, CtBPs do not function by directly binding DNA. In fact, specific DNA binding proteins are needed for this interaction (Schaeper *et al*., 1995; Turner and Crossley, 1998). The binding sequences for CtBPs bear a consensus sequence which is characterized by a PXDLS motif. It is proposed that B-domain of RIBEYE (which is identical with CtBP2) is displayed on the surface of ribbons and RIBEYE interacts with a target sequence containing the consensus motif PXDLS of CtBPs (Schmitz *et al*., 2000). On the basis of this model, it is suggested that this interaction of the target sequence protein in the synaptic vesicle may be involved in docking and/or translocation of vesicles and an unknown enzymatic reaction of the B-

domain may be involved in priming (Schmitz *et al*., 2000). This model would give an explanation for how ribbons are evolved and how do they work. In this concern, identification of the binding partners for the B domain on the ribbon surface and the role of NAD binding in their function will give a valuable insight about how this domain might perform this proposed function (Schmitz *et al*., 2000). In this connection, Munc 119 a mammalian ortholog of C. elegans protein unc119 is identified as a RIBEYE interacting protein at photoreceptor ribbon synapses and NADH binding domain of RIBEYE is found to be responsible for the interaction with Munc119 (Alpadi *et al*., 2008).

1.9. Munc119-a novel photoreceptor protein:

Munc119, also known as Retina Gene 4 protein (RG4), is highly enriched synaptic protein at photoreceptor cells. It is the mammalian ortholog of Caenorhabditis elegans proteins Unc-119 (Maduro *et al*., 1995). Munc119/HRG4 has been present in the photoreceptor synapses in the outer plexiform layer of the retina, (Higashide *et al*., 1996), and also in the inner segments of the photoreceptors to a certain degree. It is predominantly associated with synaptic vesicles (Higashide *et al*., 1998). A characteristic developmental pattern regarding the expression of HRG4/Munc119 in both rod and cone photoreceptors has been found which shows a relationship with the maturation of the photoreceptors in the rat retina. This supports the fact that Munc119 may be a functionally important protein for the photoreceptors (Higashide *et al*., 1999). In the developing rat retina, the gene for Munc119 is significantly expressed around post natal day 5. This correlates with the time when the differentiation begins to takes place with the formation of the outer plexiform layer, outer nuclear layer, inner and our segments (Higashide *et al*., 1996). Afterward, the expression level is rapidly increased through the rest of the period of photoreceptor maturation. It attained the maximum level by post natal day 23, when the retina was fully developed. Finally, it remained constant thereafter and this gene is expressed by both rods and cone photoreceptors. This developmental pattern of expression is identical to other photoreceptor specific genes such as rhodopsin. Thus, highlighting that HRG4/RRG4 may play a significant role in mature photoreceptors (Higashide *et al*., 1996).

1.9.1. Molecular structure of Munc119:

Munc119 contains 240 amino acids and shows a two domain structure. There is a proximal N-terminal terminal, containing 77 amino acids, rich in proline and glycine, forming turns. It is moderately conserved (67%) between human and rat. The Carboxy terminal consists of 163 amino acid residues and α- helices, β sheets and turns. It bears a significant sequence homology to Prenyl binding protein PrBP/δ (previously designated as δ-subunit of photoreceptor cGMP dependent Phosphodiesterase (PDE6). It is 100 % conserved between the human and rat species (Higashide *et al*., 1996, 1998).

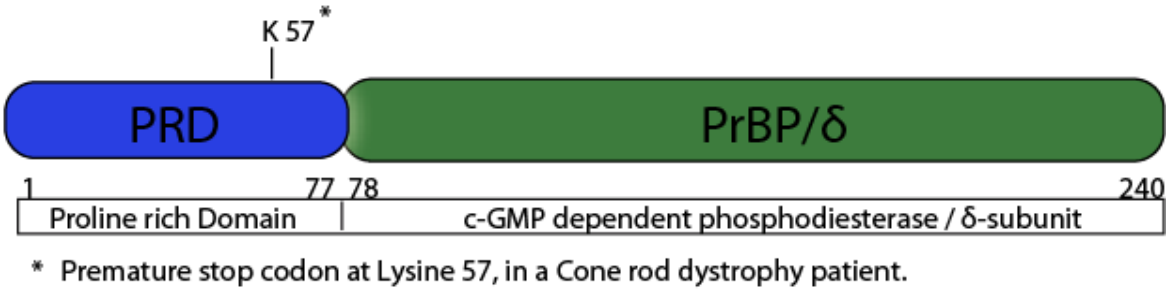

Figure 8. Schematic domain structure of Munc119. The proline-rich domain (PRD, aa1-77) is shaded in blue, the PrBP/_-homology domain of Munc119 (aa78-240) in green. The lysine K57 indicates the site of a premature stop mutation that causes cone-rod-dystrophy in a human patient (Higashide et al., 1998).

The C-terminal PrBP/δ homology HRG4 bears homology to C.elegans protein UNC119. Disorganised neural architecture and paralysis in the worm was observed when there is loss of C.elegans protein UNC119 (Maduro & Pilgrim, 1995). UNC119 has also been found to be essential for normal development of zebrafish nervous system (Manning *et al*., 2004). An interaction between HRG4 and ARF-like protein 2 (ARL-2) by yeast two hybrid strategy has been detected. (Kobayashi *et al*., 2003). HRG4 is a synaptic protein enriched in the photoreceptors. It could be associated with synaptic vesicles which are present in the cytoplasm and on the pre-synaptic membrane during docking and exocytosis (Higashide *et al*., 1998).

1.9.2. Important role of Munc119 in photoreceptor synaptic transmission:

Munc119, one of the abundant proteins in the retina, is consistent with its functional importance in the retina (Wistow *et al*., 2002). On the basis of the homology of HRG4 with UNC119, it could be suggested that HRG4 may play an important role in synaptic

vesicle cycle. In addition, Munc119 also shares several molecular features which are in common with a number of known synaptic proteins that take part in endocytosis and exocytosis (Higashide *et al*., 1998). Munc119 also contains proline rich sequence and the proline-rich regions in proteins are important for protein-protein interactions, most of which also include phosphorylation (Williamson *et al*., 1994).

On the basis of novel structural composition, UNC 119 is a lipid binding protein found to be necessary for proper trafficking of G-protein which is a subunit in mammalian photoreceptors and C.elegans sensory neurons (Zhang *et al*., 2011). It is demonstrated that the visual G protein, Transducin undergoes translocations in both direction, between the outer segment (OS) and the inner segment (IS) of rod photoreceptors and this translocation is dependent on light (Gopalakrishna *et al*., 2011). In this regard, UNC119 has occupies a main position as a protein needed for Transducin transport in darkness (Zhang *et al*., 2011). UNC 119 was reported to interact with the N- terminus of GTP-bound $G\alpha_{t1}$ (rod transducin-α subunit) in an acylation-dependent manner. Importantly, there is a disturbance of the return of rod G protein transducin (Gt) to the outer segment in dark in UNC119 knock out mice (Zhang *et al*., 2011). Since, the GTPase activity of Gαt1 (rod transducin-α subunit) was found to be inhibited by UNC119, therefore it could be concluded that the return of transducin to outer segment in darkness is based on diffusion of stable UNC119-$G\alpha_{t1}$ GTP complex (Zhang *et al*., 2011). The interaction of human UNC119 (HRG4) with transducin is dependent on the N-acylation, however, it does not require the GTP-bound form of $G\alpha_{t1}$ (Gopalakrishna *et al*., 2011).

The essential function of Munc 119 for synaptic transmission at photoreceptor synapses and for vision has been demonstrated in a cone-rod dystrophy patient with a premature termination codon mutation. This truncation mutation resulted in Munc119 that deletes the PrBP/δ homology domain. Severe synaptic degeneration has been detected in a transgenic mouse model of this human mutation (Kobayashi *et al*., 2000). Likewise, a knock out mouse model of UNC119 (MRG4) has also demonstrated a different dysfunction at distal IS/OS regions. This dysfunction has been characterized by a slow and progressive degeneration of retina (Ishiba *et al*., 2007). The recent proteomic has

reported to observe a decrease in UNC119 expression as one of the possible factors that contribute to retinal degeneration in a transgenic mouse model (Posokhova *et al*., 2011).

It is demonstrated that Munc119 interacts with CaBP4 (Haeseleer *et al*., 2008). Munc 119 could activate Src-type signalling kinases in the photoreceptor synapses and this is recently noticed for Munc119 in various cells of the immune system (Cen *et al.*, 2003; Gorskka *et al*., 2004). Munc119 has high homology to PrBP/δ which binds and dissociates prenylated proteins from membranes (Zhang *et al*., 2004, 2007).This enzymatic activity plays an important role in intercellular membrane and protein trafficking (Zhang *et al*., 2004, 2007). In photoreceptors, the trafficking role of PrBP/δ predominantly takes place in the inner and outer segments. It is suggested that Munc119 performs same tasks in the terminals of photoreceptor ribbon synapses (Alpadi *et al*., 2008). Munc119 has been identified as a RIBEYE interacting protein at photoreceptor ribbon synapses (Alpadi *et al*., 2008). A unique RIBEYE protein is characterized to be a major component of synaptic ribbons (Sterling *et al*., 2005; Heidelberger *et al*., 2005; tom Dieck *et al.,* 2006). The PrBP/δ homology domain of Munc119 is essential for interaction with the NADH binding region of RIBEYE(B) domain. But RIBEYE-Munc119 interaction does not depend on NADH binding. Munc119 is a synaptic ribbon associated component and it could be recruited to synaptic ribbons via its interaction with RIBEYE (Alpadi *et al.*, 2008).

1.10. Caskin1, a scaffold protein:

Caskin1 (CASK interacting protein 1) is a novel multidomain protein. On the basis of the presence of multiple domains in Caskin1, it was suggested that Caskin1 act as an adaptor protein (Tabuchi *et al*., 2002). This is further supported by the observation that adaptor proteins usually contain multiple domains and are capable of forming molecular scaffolds resulting in large signalling networks by forming links between proteins (Tsunoda and Zuker, 1999; Pawson and Nash, 2000). Moreover, adaptor proteins play a vital role at the synapses, where they participate as scaffolds of presynaptic and postsynaptic signalling machines (for review, see Garner *et al*., 2000; Sheng and Sala, 2001).

A strong interaction has been found between Caskin1 and CASK protein (Tabuchi *et al*., 2002). CASK is a member of the family of membrane associated guanylate kinase (MAGUK) proteins. CASK is evolutionary conserved in Caenorhabditis elegans. In contrast, Caskin1 homolog was not detected in C. elegans. There are two isoform of Caskin which include Caskin 1 and Caskin 2. The two Caskins resemble each other and each Caskin isoform is highly conserved evolutionary (Tabuchi *et al*., 2002). In humans, Caskin2 homolog is also expressed. There is a similar overall domain organization for both Caskins but no interaction has been found between Caskin2 and CASK (Tabuchi *et al*., 2002).

1.10.1. Molecular structure of Caskin1:

Based on databank searches, it observed that Caskin1 and its isoform Caskin2, both are composed of two major regions. These include an N-terminal half and a C-terminal half. The N-terminal half demonstrates a multidomain organization which consists of six ankyrin repeats, a single SH3 domain, and two sterile α motifs-SAM domains (Fig. 9) (Tabuchi *et al*., 2002). The three dimensional structure of these domains has also been demonstrated (Li *et al*., 2005; Mayer *et al*., 2001; Kim *et al*., 2003; Stafford *et al*., 2011). On the other hand, the C-terminal half is composed of a long proline rich sequence and a unique C-terminal domain (CTD). It is already proposed that Proline is incompatible with repetitive secondary structural elements (Williamson et al., 1994) and is found to be highly enriched in IDPs (intrinsically disordered proteins) (Tompa *et al*., 2002). Hence, it could be assumed that the C-terminus (amino acids 603- 1430) of Caskin1 might be intrinsically disordered (Balázs *et al*., 2009). Although the N-terminal halves of Caskin1 and 2 resemble each other but the C-terminal regions exhibit considerable divergence. Moreover, they still demonstrate identical patches that are usually organised around proline residues (Tabuchi *et al*, 2002).

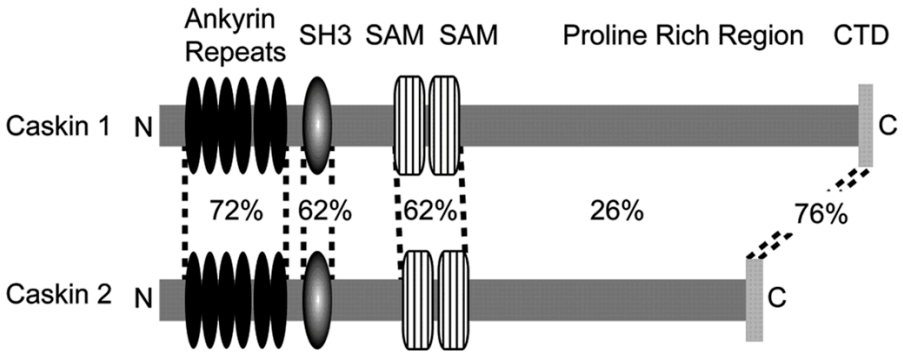

Figure 9. Domain structures of Caskins. The domain structures of Caskin1 and 2 are shown schematically. Numbers between the Caskin1 and 2 structures indicate percentage identity between the various domains. Tabuchi K *et al*. J. Neurosci. 2002; 22:4264-4273.

The structure of Caskins is similar to that of SHANKs which is a family of postsynaptic scaffolding proteins. SHANKs interact with the guanylate kinase-associated protein, glutamate receptor-interacting protein homer and Cortactin (for review, see Sheng and Sala, 2001). It is found that SHANKs and Caskins both are composed of N-terminal ankyrin repeats. The N-terminal is followed by a SH3 domain and a large C-terminal which consists of a proline-rich sequence. On the basis of the similarity in domain structure between Caskins and SHANKs, it could be proposed that they bear convergent evolution and may perform an identical function (Gundelfinger *et al*., 2006; Baron *et al*., 2006). However, SHANKs are characterized by the presence of a PDZ domain that follows SH3 domain. This PDZ domain is not present in Caskins. Furthermore, in SHANKs, there is a single SAM domain located at the end of the C terminus, whereas two SAM domains are localized in the middle of the Caskins (Fig. 9). A small sequence identity has been noticed among the ankyrin repeats, SH3 and SAM domains of Caskins and SHANKs (Tabuchi *et al*., 2002). Moreover, this identical domain structure of Caskins and SHANKs does not show an evolutionary connection but instead point toward a similar scaffolding function (Tabuchi *et al*., 2002).

1.10.2. Tissue distribution of Caskin1:

Caskin1 is expressed only in brain. It has been demonstrated that Caskin1 was located mainly in the neuropil. It was found to be specifically enriched in synaptic areas. This pattern of distribution is similar to that of synaptic vesicle proteins (Tabuchi *et al*., 2002).

1.10.3. Interactions of Caskin1:

Caskin1 is specific in the vertebrate, but CASK and the rest of other CASK interacting proteins are evolutionary conserved in invertebrates (Tabuchi *et al*., 2002). It has been demonstrated that there is an interaction between the proline-rich region of Caskin1 and the adaptor protein Ab1-interactor-2 (Abi2) (Balázs *et al*., 2009). Abi2 is an adaptor protein identified by its binding with Ab1 tyrosine kinase (Dai & Pendergast *et al*., 1995). Moreover, the C-terminal part of Caskin1 protein contains a long proline rich region and it is stated that because this proline rich region is required in protein-protein interactions (Dyson and Wright, 2002). Interestingly, an interaction between Caskin1 and Synaptotagmin has been determined through a yeast two hybrid assay (Balázs *et al*., 2009). It is already proved that Liprin occupies a pivotal position in presynaptic scaffolding (for review, see Spangler & Hoogenraad 2007). Recently, it is documented that Caskin1 competes with liprin in order to bind to LAR (leukocyte common antigen related receptor protein), and motor axon guidance in Drosophila is entirely based on the presence of LAR (Weng *et al*., 2011). On the basis of this study it could be proposed that Caskin1, as a neuronal adaptor protein, might be required for axon growth and guidance (Weng *et al*., 2011).Caskin1 directly competes with Mint1 for binding with CASK protein (Tabuchi *et al*., 2002).

A close relationship has been identified between Caskin1 expression and maternal ingestion of ethanol. In these experiments it was found that there is a considerable decrease in the level of Caskin1 mRNA in rats who were exposed to ethanol during the intrauterine life (Middleton *et al*., 2009). Similarly, another interesting study has demonstrated that Caskin1 may play a significant role in infantile myoclonic epilepsy (Ala *et al*., 2008).

Cerebral ischemia or stroke is a major cause of death in the world but this acute neurological injury still lacks an effective and absolute therapy. Hence it is very important in stroke research, to discover major targets and to explain their mechanism and precise role during the temporal evolution of ischemia/ reperfusion (I/R) injury. Very recently, several proteins (Caskin1, Shank3) that are important in stroke are discovered (Datta *et al*., 2011). It is demonstrated that during the first 24 hours of cerebral I/R (ischemia/reperfusion) injury, a generalized downward trend has been

observed for the synapse related proteins (Caskin1, Shank) and this could be referred towards a compromised synaptic function. A direct association has been found between the down regulated proteins like Caskin1 and the synaptic scaffolding protein, CASK. Caskin1 and Shank3 both were located in the post synaptic density and their domain structure also bears a close resemblance. Therefore, on this observation, the down regulation observed in the current model could be justified (Tabuchi *et al*., 2002; Datta *et al*., 2011). A generalized decrease in the normal synaptic function might be related with the down regulation of these proteins (Caskin1, Shank3) at 24 hours post ischemia/ reperfusion (I/R) injury (Datta *et al*., 2011). The B-domain of RIBEYE is identical to CtBP2 (Schmitz *et al*., 2000), and it is suggested that RIBEYE interacts with other proteins through this target sequence containing the consensus motif PxDLS of CtBP2 (Schmitz *et al*., 2000). Caskin1 contains short motifs with the consensus PxDLS (EBI interaction database). Therefore, it could be possible that Caskin1 might interact with RIBEYE through this motif with the consensus PxDLS.

1.11. Intersectin 1:

Stelzl *et al*., (2005) demonstrated interaction between Munc119 and Intersectin by automated yeast two hybrid interaction. Munc119 is an interacting partner of Ribbon protein RIBEYE (Alpadi *et al*., 2008). Based on this, in the present study, I analysed the distribution of Intersectin1 in mammalian retina. Intersectins are considered as adaptor/scaffold proteins on the basis of the presence of a unique structural organization which consists of multiple domains. Adaptor/scaffold proteins are responsible for the regulation of various cellular activities which include proliferation, differentiation, cell cycle control, cell survival and migration (Pawson and Scott, 1997; Szmkiewicz *et al*., 2004; Zeke *et al*., 2009). In this regard, the function of Intersectin proteins as adaptors have been analysed in different cell types and organisms (Tsyba *et al*., 2011). There are two Intersectin genes in humans. These genes are located on human chromosome 21(q22.1-q22.2) and 2(pter-p25.1) respectively. The domain structure of Intersectin1 and Intersectin 2 is identical (Pucharcos *et al*., 2000). Both have long and short isoforms. These isoforms are produced as a result of alternative splicing (Guipponi *et al*., 1998; Pucharcos *et al*., 2001). Intersectin1 and Intersectin2 genes of vertebrates bear very similar organization (Pucharcos *et al*., 1999, 2001). They are composed of more

than 40 exons in vertebrates, but orthologous Intersectin genes of nematodes (C.elegans) and arthopods (D. melanogaster) contain 8 and 11 exons, respectively. In addition, in vertebrates most of the exon boundaries are conserved between Intersectin1 and Intersectin2. For the paralogous genes, the mechanisms of generation of the two major spliced variants are similar (Pucharcos *et al*., 2001). This predicts that, the long isoform is generated as a result of alternative splicing of exon 30 and it appeared earlier in evolutions before gene duplication. However, no conservation between Intersectin1 and Intersectin2 has been found for the remaining familiar alternative splicing events are (Pucharcos *et al*., 2001; Tsyba *et al*., 2004). Intersectin1 transcripts which contain exon 20 are specific for the neurons and their expression is regulated during development (Tsyba *et al*., 2004, 2008). There is an increase in the expression of $^{+}$ exon 20 variant of Intersectin1 during development of fetal brain. In contrast to this, the level of the transcript lacking exon 20 follows a corresponding decline. It is found that the ratio of Intersectin1 isoforms with and without exon 25 and 26 (the SH3 domain), is variable in fetal and adult brain (Pucharcos *et al*., 2001; Tsyba *et al*., 2004).

1.11.1. Isoforms of Intersectin1:

There are two isoforms of Intersectin/EHSH1 protein which include the long Isoform (Intersectin/EHSH1-L) and the short Isoform (Intersectin/EHSH1-S) (Ning *et al*., 2008). These isoforms are formed by alternative splicing of exon 30 that results in the termination codon for the short isoform (Guipponi *et al*., 1998, Pucharcos *et al*., 2001; Sengar *et al*., 1999). In the vertebrates, a longer isoform (Intersectin-1) is produced when the Intersectin gene undergoes alternative splicing. The neurons contain mainly this long isoform (Hussain *et al*., 1999; Ma *et al*., 2003; Tsyba *et al*., 2004; Yu *et al*., 2008).

It has been analysed that after birth, there is an increase in the ratio of mRNA of Intersectin/EHSH1-L to Intersectin/EHSH1-S expression in the brain. Hence, it could be concluded that the expressions of these two isoforms of Intersectin are precisely regulated in every cell or tissue (O'Bryan *et al*., 2001). Studies conducted on the brains taken from rats of different age groups have demonstrated that Intersectin/EHSH1 appeared during embryogenesis and then it is permanently present for the rest of the life. However, as the developmental process is further continued, there is a significantly

prominent change in the ratio of EHSH1-L to that of EHSH1-S isoforms. The long isoform expression is increased rapidly after birth. It is followed by a decline after postnatal day 18. On the other hand, the expression of short isoform gradually becomes less prominent after postnatal day 4 (Okamoto *et al*., 1999). This observation could be well supported with the fact that there is an increase in the activity of synapses after birth , therefore , the expression of proteins which are associated with the endocytosis and exocytosis is also raised (Ning *et al*., 2008). Accordingly, it could be suggested that Intersectin-L isoform, enriched in neuron could participate in neuronal endocytosis, exocytosis and neurotransmitter release. Furthermore, the short isoform Intersectin-S which is predominantly present in glial cells, could be responsible for regulation of intracellular signalling process and performs an important function in cell division and proliferation (Verkhratsky *et al*.,2006, Bessis *et al*., 2007).

1.11.2. Distribution of Intersectin1:

Various experimental studies have documented presence of Intersectin1 in neurons. Likewise, the Intersectin1 has been identified in the nervous system of C. elegans not only during the larva stages but in adult worms as well (Rose *et al*., 2007). Moreover, the research data has described that high levels of Dap 160 are present in the central and peripheral neurons of Drosophila larval during the entire process of development (Tomancak *et al*., 2004; Marie *et al*., 2004). An Increased expression of Intersectin1 mRNA has been found out in the nervous system of mouse embryos during intrauterine development (Reymond *et al*., 2002). It has been observed that Intersectin1/EHSH1 is highly enriched in the mammalian tissues (Guipponi *et al*., 1998; Okamoto *et al*., 1999; Sengar *et al*., 1999). Intersectin1/EHSH1-L is predominantly present in neurons of brain (Pucharcos *et al*., 2000, 2001; Guipponi *et al*., 1998), specifically in the neurons of somatodendritic region of cortex, hippocampus, globus pallidus, subthalamic nucleus and substantia nigra (Ma *et al*., 2003; Ning *et al*.,2008). In contrast, Intersectin/EHSH1-S is reported to be found mainly in astrocytes and microglia (Ning *et al*., 2008). Additionally, this short isoform of Intersectin1/EHSH1 is also detected in different other types of cells which include endocrine tissues of the pancreas, adrenal, thyroid and pituitary (Pucharcos *et al*., 2001; Ning *et al*., 2008).

It is documented through studies carried out on the mammalian epithelial cell and rat hippocampus neurons, that Intersectin1 is localized in the cytoplasm and mainly in the perinuclear zone of Golgi like organelles (Hussain *et al*., 1999; Predescu *et al*., 2003; Ma *et al*., 2003; Pucharcos *et al*., 2000). Similarly, it was also observed that the Intersectin1 is enriched predominantly in CCPs (clathrin coated pits) at the plasma membrane (Hussain *et al*., 1999).

1.11.3. Structural model of Intersectin1:

Intersectin1/EHSH1 is highly conserved during evolution. It is composed of multiple domains (Ning *et al*, 2008). At the N-terminus, Intersectin1/EHSH1 is formed by two EH domains. After the EH domains, there is a central KLERQ domain.

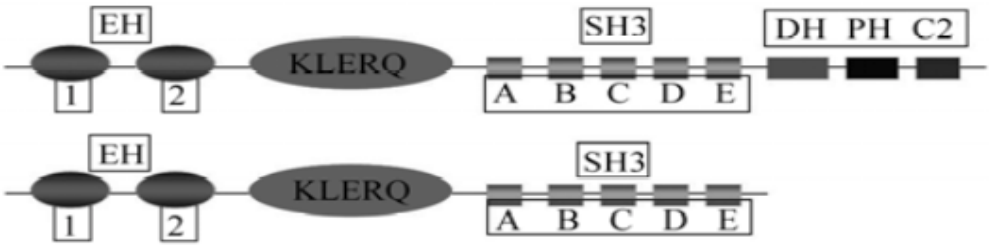

Figure 10. Domain structures of two isoforms of Intersectin1: The top is the Intersectin1-L isoform, and the bottom is Intersectin1-S isoform.

This central domain is formed of a charged alpha-helix. Moreover, this domain is completely made up of lysine (11%), leucine (12%), glutamate (20%), arginine (13%) and glutamine (15%). The other C terminus of Intersectin/EHSH1 is characterized by the presence of five SH3 domains (SH3A-E) (Okamoto *et al*; 1999). In addition to these, three additional C-terminals domains in the long isoform (EHSH1-L) have also been revealed. These include a Dbl homology domain (DH), a Pleckstrin homology domain (PH) and a C2 domain (Guipponi *et al*., 1998, Sengar *et al*., 1999).

1.11.4. Role of Intersectin1 in endocytosis and exocytosis:

There has been sufficient data that could support the fact that proteins of Intersectin family are involved in endocytosis. Intersectin1 is composed of EH and SH3 domains and the presence of these domains is recognised well in the proteins involved in endocytosis (Tsyba *et al*., 2011). The EH domain at the N-terminal of Intersectin1 is found to interact with the endocytic accessory protein epsin (Yamabhai *et al*., 1998).

The Epsin1 binds with EH domain of Intersectin1 and is found in the presynaptic as well as postsynaptic sites (Yao *et al*., 2003). Furthermore the interaction between EH domains of Intersectin1 and stonin2 has also been recognised. Stonin2 is an endocytic sorting adaptor for identifying the synaptic vesicle cargo (Martina *et al*., 2001; Kelly and Phillips, 2005). Moreover, the EH domain of Intersectin1 also interacts with SCAMP1 which is known well as a secretory carrier membrane protein (Fernández-Chacón *et al*., 2000). As far as the CCR (central coiled region) of intersectin1 is concerned, it is observed that it binds with high affinity with synaptosome associated proteins (SNAP-25 and SNAP-23) (Okamoto *et al*., 1999). CCR is reported to form heterodimers with the scaffolding adaptor Eps15 (Seneger *et al*., 1999; Koh *et al*., 2007). Intersectin1 plays a key role as an active member of presynaptic endocytic complex associated with Synaptotagmin (Khanna *et al*., 2006). It is studied that the SH3 domains of Intersectin1 can interact with Dynamin1 which participates in vesicle fission. The potential interacting partners of SH3 domain of Intersectin1 include a Synaptojanin 1, a synaptic protein synapsin, a membrane deforming protein SGIP1 and the AP2-binding protein connecdenn (Roos and Kelly, 1998; Yamabhai *et al*., 1998; Okamoto *et al*., 1999; Evergren *et al*., 2007; Dergai *et al*., 2010; Allaire *et al*., 2006). Recently, it is identified that Intersectin1 through its SH3 domain interacts with SHIP2 (SH2 domain containing inositol 5-phosphatase2) and this interaction resulted in the recruitment of SHIP to CCPs (clathrin coated pits) (Xie *et al*., 2008; Nakatsu *et al*., 2010). Evidence highlighting the participation of Intersectin1 in endocytosis is obtained from the study which demonstrated inhibition of clathrin-mediated endocytosis (CME) in response to over expression or knockdown of Intersectin1 (Sengar *et al*., 1999; Pucharcos *et al*., 2000; Martin *et al*., 2006; Thomas *et al*., 2009). It is also identified that the PH domain of Intersectin/EHSH1 interacts with phosophoinositides (Snyder *et al*., 2001). Moreover, the C2-domain is found to take part in phospholipid binding through Ca^{2+} dependent and Ca^{2+} independent pathways (Rizo and Südhof, 1998). Interestingly, Intersectins are also known to participate in caveolae endocytosis which is considered to be a vital process in carrying out transcytosis of proteins in endothelial cells (Predescu *et al*., 2003; Klein *et al*., 2009). Moreover, Intersectin1 long isoform has been recognized to carry out actin cytoskeleton rearrangements which are needed by neuroendocrine cells to perform exocytosis process (Malacombe *et al*., 2006). The DH

domain of long isoform of Intersectin1 exhibits GEF (guanine nucleotide exchange factor) activity. This DH domain of Intersectin1 is responsible to catalyse the release of GDP from Rho GTPases as well as its activation by binding with GTP (Rossman *et al*., 2005). Various studies have demonstrated that Intersectin1 acts as a vital connection between endocytosis and intracellular signal transduction. It has been identified that the SH3 domain of Intersectin1 interacts with Sos1 (a guanine nucleotide exchange factor for Ras) and results in the formation of protein complexes. In this way stimulation of Ras is carried out by Intersectin1 (Tong *et al*., 2000; Mohney *et al*., 2003). The importance of Intersectin1 in synaptic vesicle recycling has been analysed. Intersectin1 is involved in recycling of synaptic vesicles in Lamprey giant synapses (Evergren *et al*., 2007). Very recently, it is suggested that Intersectin1 form homo and heterodimers. Moreover, Intersectin1 is shown to interact with c components of Arf6 GTPase and Rab5 GTPase pathways (Wong *et al*., 2012).

1.12. Working hypothesis:

Caskin1 and Intersectin1 are widely distributed in the conventional synapses of the central nervous system. However, the presence of these proteins in the retina has not yet been demonstrated. The main aim of the current study was to demonstrate the morphological analysis of the distribution of Caskin1 and Intersectin1 proteins in the retina that would help in a better understanding of the structure and composition of the retinal synapses.

CHAPTER 2

MATERIALS AND METHODS

2.1. Antibodies used for immunolabeling:

Antibody	Source	Dilution used	Secondary antibody	Dilution used
Intersectin1/EHSH1 227 (Okamoto *et al*., 1999)	Rabbit polyclonal Prof. Thomas C. Südhof (Stanford)	1:500	Goat anti-rabbit GAR Cy3 (ZYMED) Cat. No: 81-6115	1:1000
Intersectin1/EHSH1 229 (Okamoto *et al*., 1999)	Rabbit polyclonal Prof. Thomas C. Südhof (Stanford)	1.500	Goat anti-rabbit GAR Cy3 (ZYMED) Cat. No: 81-6115	1:1000
Intersectin1/EHSH1 750 (Okamoto et al., 1999)	Rabbit polyclonal Prof. Thomas C. Südhof (Stanford)	1.500	Goat anti-rabbit GAR Cy3 (ZYMED) Cat. No: 81-6115	1:1000
U2656 (Schmitz *et al*.,2000)	Rabbit polyclonal	1:500	Goat anti-rabbit GAR Cy3 (ZYMED) Cat. No: 81-6115	1:1000
RIBEYE/CtBP2 (BDTransduction Laboratories) Cat.No: 612044	Mouse monoclonal	1:500	GAM Cy2 (Jackson Immuno Research) Cat. No:115-096-146	1:1000
Intersectin1 (EH-HOM) Final Immune serum (raised against GST-EH-HOM fusion protein)	Rabbit polyclonal (Lab- Made)	1:500	Goat anti-rabbit GAR Cy3 (ZYMED) Cat. No: 81-6115	1:1000
Caskin (SY SY) Cat. No: 185-002	Rabbit Polyclonal	1:500	Goat anti-rabbit GAR Cy3 (ZYMED) Cat. No: 81-6115	1:1000

Lecithin PNA Conjugates	Molecular Probes	1:200		
Caskin1control peptide(raised against 1416 aa-1430 aa of rat Caskin1)	SYSY Synaptic Systems	5µg		
Goat anti-Rabbit Gold Conjugate (10nm)	SIGMA; Brada et al 1984	1:100		
Synaptophysin(SIGMA) Cat. No: S5768	Mouse monoclonal		Alexa 488 CAM (JacksonImmuno Research) Cat. No: 115-096-146	1:1000
LPH Control Peptide	BioGenes	15µg		

2.2. Antibodies used for western blot analysis:

Antibody	Source	Dilution used	Secondary antibody	Dilution used
Intersectin1/EHSH1 227 (Okamoto *et al*., 1999)	Rabbit polyclonal Prof. Thomas C. Südhof (Stanford)	1:2000	Goat anti-rabbit (GAR)-POX (SIGMA) Cat. No:A6154	1:10,000
Intersectin1/EHSH1 229 (Okamoto *et al*., 1999)	Rabbit polyclonal Prof. Thomas C. Südhof (Stanford)	1:2000	Goat anti-rabbit (GAR)-POX (SIGMA) Cat. No:A6154	1:10,000
Intersectin1/EHSH1 750 (Okamoto *et al*., 1999)	Rabbit polyclonal Prof. Thomas C. Südhof (Stanford)	1:2000	Goat anti-rabbit (GAR)-POX (SIGMA) Cat. No:A6154	1:10,000

Intersectin1 (EH-HOM) Final Immune serum (raised against GST-EH-HOM fusion protein)	Rabbit polyclonal Lab. made	1:2000	Goat anti-rabbit (GAR)-POX (SIGMA) Cat. No:A6154	1:10,000
Anti-GST	Mouse Monoclonal (SIGMA)	1:5000	Goat anti-mouse (GAM)-POX (SIGMA) Cat. No:A3673	1:10,000
Munc119(V2T2, 120IT) (Alpadi *et al*., 2008)	Rabbit polyclonal Lab. made	1:2000	Goat anti-rabbit (GAR)-POX (SIGMA) Cat. No:A6154	1:10,000
Caskin (SY SY) Cat. No: 185-002	Rabbit polyclonal	1:2000	Goat anti-rabbit (GAR)-POX (SIGMA) Cat. No:A6154	1:10,000
Anti-Synptophysin	Mouse Monoclonal (SIGMA) Cat. No: S5768	1:2000	Goat anti-mouse (GAM)-POX (SIGMA) Cat. No:A3673	1:10,000

2.3. Reagents and Chemicals:

BSA (bovine serum albumin)	Sigma
EDTA	Roth
Sepharose A Beads	Sigma
Sodium chloride	Roth
NPG (n-Propylgallate)	Sigma
Ponceau-S	Roth
Tris	Roth
Triton X-100	Fluka
Beta-Mercaptoethanol	Roth

Uranylacetate	Merck
Glutaraldehyde	Sigma
Bradford-Reagent Roti®-Quant	Carl Roth
Wide range protein standard Roti® Mark	Roth

2.4. Buffers and Media:

ECL-Solution	ECL-I: Tris 1M pH 8.5 10 ml Luminol stock 1 ml Para-hydroxy Coumarin Acid (PCA) 440 µl. Make up to 100 ml with dd H2O ECL-II : Tris 1M pH 8.5 10 ml H2O2 64µl Make up to 100 ml with dd H2O
Loading buffer	10 µl 100mM EDTA 490 µl dd H2O 500 µl Glycerol
Blocking buffer for immuno cyto-chemistry	0.5% Bovine serum albumin in PBS (1x)
Lysis Buffer for (Coimmunoprecipitation)	150mM Tris HCl PH7.9 200mM NaCl 1.5mM EDTA 1.5% Triton x-100
5 X PBS	40 g NaCl 1 g KCl 7.2 g Na2HPO 41.2 g KH2PO4 Make up to 1 litre with dd H2O

Polyacrylamide gel 10%	**Resolving Gel:** 1.5 ml dd H2O 1.9 ml 1 M Tris pH 8.8 2.5 ml 30% Acryl amide 75µl 10% SDS, 1.5 ml 50% Glycero l5 µl TEMED 38 µl 10% APS **Stacking Gel:** 4.73 ml dd H2O 1.88 ml 1 M Tris pH 8.8 0.75 ml 30% Acryl amide 75µl 10% SDS 7.5µl TEMED 56.3 µl 10% APS
Ponceau S-stain	30 g Trichloroacetic acid 5 g Ponceau S Make up to 1 litre with dd H2O
SDS-PAGE-Electrophoresis buffer	3.03 g Tris 14.4 g Glycine 1.0 g SDS Make up to 1 litre with ddH20
SDS-loading buffer 4 x	1.6 g SDS 4 ml β-Mercaptoethanol 2 ml Glycerol 2 ml 1M Tris pH 74 mg Bromo phenol blue2 ml of ddH2O
Stripping Buffer	1% SDS in PBS(1x) 1ml ß-Mercaptoethanol
Transfer Buffer (Western Blot)	Tris 15.125 g Glycine 72.05 g Methanol 1 litre Make up to 5 litres with dd H2O
PMSF-Stock solution	40mM in 100% Isopropanol

2.5. Laboratory Instruments:

Fluorescence Microscope Axiovert 200M Camera Axiocam MRm	Carl Zeis
Chemidoc XRS System	Bio-Rad
pH Meter	Inolab
SDS-PAGE electrophoresis apparatus	Amersham Biosciences
Power Supply EPS 301	Amersham Biosciences
Rotator	NeoLab
Ultracentrifuge	Beckmann
Microtome-cryostat, Cryo-Star HM560MV SW40 rotor	Microm Int. GmbH, Walldorf Beckman
Western Blot Transfer apparatus	HOEFER Scientific Instruments
Transmission Electron Microscope	FEI; Tecnai G2
Ultra Turrax	IKA RW16 Basic
Vortex	VWR International

2.6. Immunolabeling experiments:

For immunolabeling experiments, I used cryosections from bovine retinas. The bovine eyes were obtained from a local slaughter house. The bovine eyes were available 30 minutes after post mortem and worked up immediately.

2.7. Cryopreservation of bovine retina/ Flash freezing of retinal tisssue:

The bovine eye was cut open at the equator of the eye. After removing the lens and vitreous, retina was peeled off gently and optic nerve was cut off. The specimen of retina was immersed into liquid nitrogen cooled isopentane for 30 seconds and then transferred into liquid nitrogen (-196ºC). This rapid freezing prevents formation of ice crystals during flash freezing process. The frozen specimen was kept in a pre-labelled aluminium foil package and stored at -80ºC before final use for preparing cryosections.

2.8. Preparation of cryostat sections of bovine retina:

The key instrument used for making cryosections is the Cryostat with a microtome. The frozen bovine retina was embedded in a tissue embedding medium (NEG-50, Thermo Scientific). After cutting off the excessive medium with a blade, the block of medium containing frozen retina was fixed on a specimen holder designed specially for this purpose.

Then, the cryosections of 10µm thicknesses were cut with microtome and thawed on glass slides. The slides with cryosections of bovine retina were heated on a heating plate for 10 minutes at 60ºC. Finally; these heat fixed sections were kept at -20ºC till used for immunolabeling experiments.

2.9. Single immunolabeling of cryostat sections from bovine retina:

Immunolabeling experiment was performed as previously described (Schmitz *et al*., 20000, 2006; Alpadi *et al*., 2008) using a Zeiss inverted Axiovert 200M microscope (Carl Zeiss) equipped for conventional epiflorescence microscopy. The tissue sections were heated on a hot plate at 60ºC for 10 minutes and then transferred to a wet chamber to cool down. The unspecific binding sites of the sections were blocked with 0.5% solution of BSA in PBS (phosphate buffered-saline) for 1 hour at room temperature. Incubation of the primary antibody (Intersectin1/EHSH1-227,750, 229, (Intersectin1-EH-HOM) and Caskin1) was done at 1:500 dilutions in blocking buffer at 4ºC overnight. After overnight incubation, the sections were washed with PBS (1x) three to four times with gentle shaking on the shaker. Then secondary antibody GAR Cy3 was applied (1:1000 in blocking buffer) for 1 hour at room temperature. The sections were

again washed with PBS (1x) for 3 to 4 times. Afterwards, without letting the sections drying out, the immunolabelled sections were carefully embedded by adding 20µl of antifade NPG (containing 1.5% w/v n-propylgallate in 60% glycerol in PBS). Finally the sections were covered with cover slips without capturing the air bubbles beneath them.

For negative controls, the whole procedure of immunolabelling was same as described above. However, the primary antibody was omitted and incubation of sections was carried out only with secondary antibody Goat anti rabbit (GAR) Cy3 (1:1000 dilution in blocking buffer).

2.10. Double immunolabeling of cryostat sections from bovine retina:

The protocol for double immunolabelling is same as that for single immunolabelling experiment (described above). However, the primary antibodies Intersectin1/EHSH1 (227,229,750), Intersectin1/EHSH1-EH-HOM and Caskin1 were used with antibody against Ribeye and Synaptophysin (both serving as presynaptic markers), in dilution 1:500 in blocking buffer. For secondary antibodies, I used Goat anti-rabbit (GAR) Cy3 and Goat anti-mouse (GAM) Cy2 but to detect anti-Synaptophysin, Alexa488 Chicken anti-Mouse was used. All secondary antibodies were used in dilution 1:1000 in blocking buffer.

2.11. SDS-PAGE:

SDS-PAGE was done as described by Maniatis *et al* (2005). One dimension gel electrophoresis under denaturing conditions separates the proteins according to their molecular size, in the presence of 0.1% SDS. The polyacrylamide gel is casted as a separating gel topped by a stacking gel. The sample proteins were solubilized by boiling in 4x SDS loading buffer. Coomassie brilliant blue R-250 binds non specifically to almost all proteins which allow detection of protein bands in polyacrylamide gels. Then the gels were stained with Coomassie staining solution with gentle shaking for 30 minutes at room temperature. The background was reduced by soaking the gel in acrylamide gel destaining solution. Then the gels were documented using either HP scanner or Bio RAD Gel Doc apparatus.

2.12. Western Blotting:

Proteins were separated by SDS-PAGE and transferred from the polyacrylamide gel to a nitrocellulose membrane with a constant voltage (3 hrs; 50Volts at 4ºC). After electroblotting, the membrane was stained with Ponceau S for 2 minutes. The Ponceau S stained membrane was documented using a scanner. Then, the membrane was destained using PBS (1x) and blocked with 5% skimmed milk powder (MMP) for 1 hour. For immunodetection of proteins, the primary antibodies were diluted in 5% skimmed milk and the membrane was incubated for over night in cold room with constant shaking of milk. Then the membrane was washed three times with PBS (1x). Afterwards, the secondary antibody was diluted in 5% MMP and the membrane was incubated at room temperature for 1 hour. Again, the membrane was washed with PBS (1x) for 3 times. Then, the membrane was incubated with ECL1 and ECL2 mixture (1:1 ratio; Chemiluminescence detection solution) and the signals were documented with BIO RAD Doc apparatus. Also the intensity was quantified using BIO RAD Gel Doc apparatus and Gel Doc software.

2.13. Bovine retina co-immunoprecipitation:

All steps were performed at 4ºC if not denoted otherwise. For each immunoprecipitation, 1 bovine retina was immediately homogenised with ultra turrax in the presence of 2ml of Lysis Buffer (150mM Tris HCl pH7.9, 200mM NaCl, 1.5mM EDTA) containing 1.5mM Triton-X 100 at 4ºC, at 960 rpm for 3 minutes on ice . The sample was transferred to 5 ml syringe and forcefully ejected through 27 gauge needle to mechanically disrupt the retinal tissue. Mechanical crushing through 27 gauge needle was repeated 40-50 times. After mechanical crushing, lysis was allowed to proceed for 30 minutes on ice. The sample was centrifuged at 13000 rpm for 30 minutes at 4ºC. The supernatant was incubated with 15µl of Munc119 V2T2 (120 IT) pre-immune serum and 20ml of washed protein A-sepharose beads for 1 hour at 4ºC on overhead rotator. Then, the samples were centrifuged at 13000 rpm, 4ºC for 15 minutes. Afterwards, the pre-cleared lysate was equally divided into two aliquots. Each aliquot, one with 15µl of Munc119 V2T2 (120 IT) immune serum and the second with Munc119 V2T2 (120 IT) pre-immune serum (IgG control), was incubated with 20µl of washed protein A-sepharose beads, overnight at 4 ºC on overhead rotator. After overnight incubation,

samples were centrifuged at 3500 rpm for 2 minutes at 4ºC to pellet the protein A-sepharose beads. The pellet was washed three times with 1ml of lysis buffer. The final pellet was boiled with SDS loading buffer and subjected to SDS-PAGE followed by Western Blotting.

2.14. Stripping of nitrocellulose membrane:

The nitrocellulose membrane was reprobed by stripping it off with boiled 1%SDS (in PBS) in the presence of 1ml ß-Mercaptoethanol. The incubation was carried out at room temperature for one hour with constant mild shaking. The excess of stripping solution was removed by washing the membrane with PBS (1x) for 3 times. Then the membrane was blocked as described earlier and reprobed with the desired antibody and the signals were detected as stated above.

2.15. Measurement of protein concentration:

The Bradford assay was used to estimate the protein concentration (Bradford, 1975). In the presence of acidic environment of the reagent, proteins bind to Coomassie dye. This results in a spectral shift from reddish brown form (absorbance at 465nm) of the dye to blue form (absorbance at 595nm) which gives a linear concentration for the soluble proteins in a distinct range of concentration. The standard calibration curve was obtained by using duplicates of a known concentration of BSA and unknown concentration was determined.

2.16. Preabsorption analyses for Western Blotting:

For preabsorption experiment, 50µl of Intersectin EH-HOM immune serum (final serum) was added to GST-EH-HOM (100µg) and GST(100µg) fusion protein bound to beads in a final volume of 70µl and incubated overnight at 4ºC in an overhead rotator. After incubation, samples were centrifuged at 13,000rpm for 3 minutes at 4ºC and the respective supernatants were taken for the subsequent experiments. For Western Blot analyses of bovine crude retinal extract, the two preabsorbed antisera described above were used at a dilution of 1:1000 in blocking buffer (5% skimmed milk powder in PBS).

2.17. Preabsorption analyses for immunolabelling experiments:

Preabsorbance with fusion protein for immunofluorescence microscopy carried out as described above for Western Blotting. The preabsorbed antisera (preabsorbed either with GST or Intersectin1/EH-HOM-GST) were subsequently tested at identical dilutions for immunolabeling on cryostat sections of bovine retina.

For Caskin1, Immunolabelling experiment was done using different dilutions of Caskin1to find out the dilution at which the immunosignals of Caskin1 were still visible. The single immunolabelling experiment was done with Caskin1 in various dilutions (1:500, 1:3500 and 1:5000).The dilution 1:3500 was used for blocking experiment because further dilutions resulted in complete disappearance of Caskin1 immune signals. For preabsorption experiment, 1µl of Caskin1 antibody was added to 5µg of Caskin1 peptide and 15µg of control (LPH) peptide in separate eppis, in a final volume of 3500µl and incubated overnight at 4ºC in an overhead rotator. After over night incubation, samples were centrifuged and the respective supernatants were used at identical dilutions for immunolabeling experiment on cryostat sections of bovine retina.

CHAPTER 3

RESULTS- 1

3.1. Immunoblot analysis of Caskin1 in bovine retina:

The immunoblotting of bovine retina with the antibody directed against Caskin1 uncovered a single major polypeptide band with a typical molecular weight 180 kDa. This result suggested that the band observed in the crude bovine retina corresponds to Caskin1 and it is highly expressed in the bovine retina.

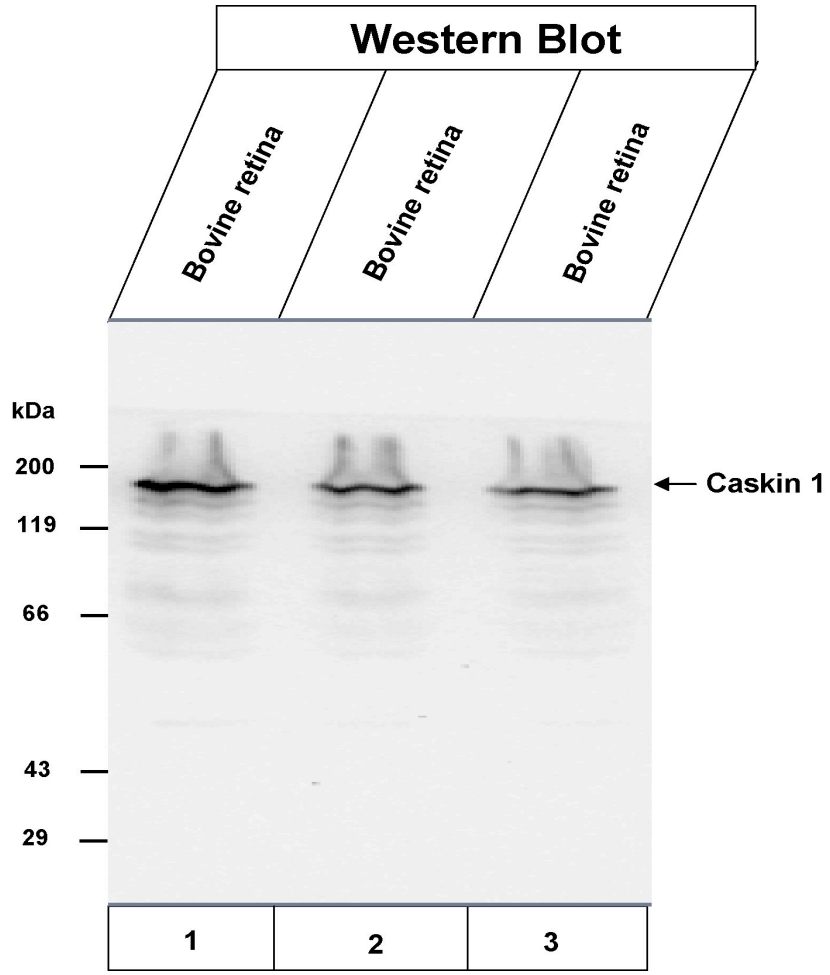

Figure 11. Immunoblot analysis of Caskin1 from bovine retina lysate. The retinal proteins separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to nitrocellulose membrane and probed for Caskin1 with polyclonal Caskin1 antibody. Signals were visualized with ECL. A polypeptide band at approx. 180 kDa, the typical molecular weight of Caskin1, was recognized. The numbers at the left indicate the positions of the molecular weight markers.

3.2. Distribution of Caskin1 in the bovine retina:

Immunocytochemistry experiment was used to determine the localization of Caskin1 in bovine retina cryosections. The single immunolabeling experiments with antibodies against Caskin1 revealed strong immune signals in the inner plexiform layer of bovine retina. In contrast, few dot like immune signals were present in the outer plexiform layer of the bovine retina. The negative control experiments were performed by omitting the primary antibody i.e. Caskin1, and incubation was carried out with secondary antibody GAR-Cy3, only a discrete dark background was observed, with the absence of significant signals in different layers of retina (result not shown). The immunocytochemistry analyses showed that Caskin1 is differently distributed in the synaptic layers of bovine retinal synapses.

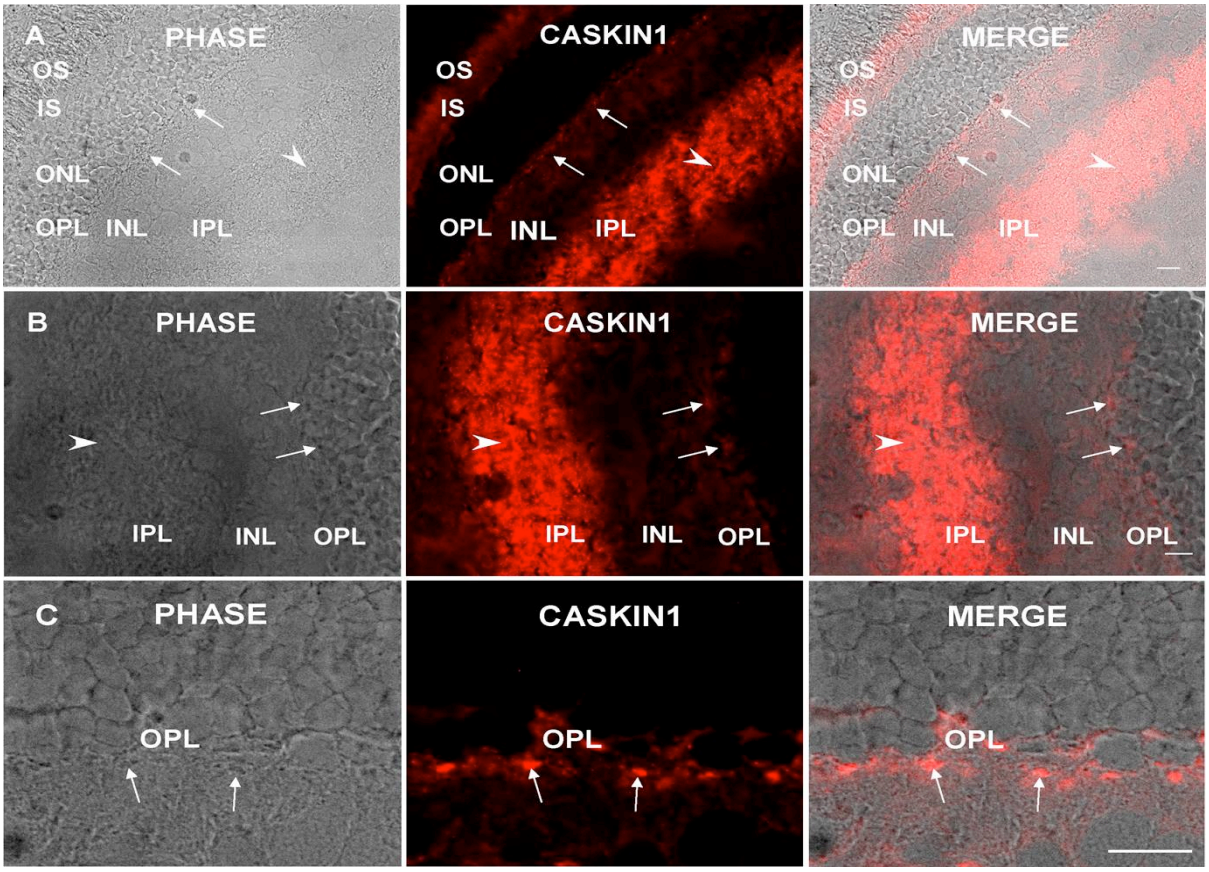

Figure 12: Distribution of Caskin1 in cryostat sections of bovine retina labeled with antibody against Caskin1. (A, B) show strong immunoreactivity in IPL (arrow heads) as well as dot like immunofluorescent structures in OPL (long arrows). (C) shows dot like immunoflorescent structures in OPL. The section analysed with conventional florescence microscope. Abbreviations: OS,outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer;IPL, inner plexiform layer. Scale bar: 10µm.

3.3. Caskin 1 is present in the synapses of inner plexiform layer:

Double immuno-florescence experiment was performed with mouse monoclonal antibody against Synaptophysin and rabbit polyclonal antibody against Caskin1. Synaptophysin is a membrane protein of synaptic vesicles (Wiedenmann and Franke, 1985). This experiment demonstrated that Caskin1 is co-localized with anti Synaptophysin in the inner plexiform layer of the mammalian retina (Fig.13. A, B).

For double immunofloresence experiment, mouse monoclonal CtBP2/RIBEYE(B) was used. CtBP2/RIBEYE(B) is identical to RIBEYE(B) domain. RIBEYE (B)/CtBP2 is distributed in the IPL as well as the OPL layers in the retina where it labels the synaptic ribbons. The double immunofloresence experiments demonstrated that the signals of Caskin1 co-localized with RIBEYE (B) in the inner plexiform layer of the mammalian retina plexiform layer of retina (Fig.14). The few dot like signals of Caskin1 in outer plexiform layer were co-localized with the immunosignals of RIBEYE (B) in bovine retina (Fig.14. A, B, C and D). This analysis demonstrated the presence of Caskin1 in the ribbon synapses and conventional synapses of bovine retina.

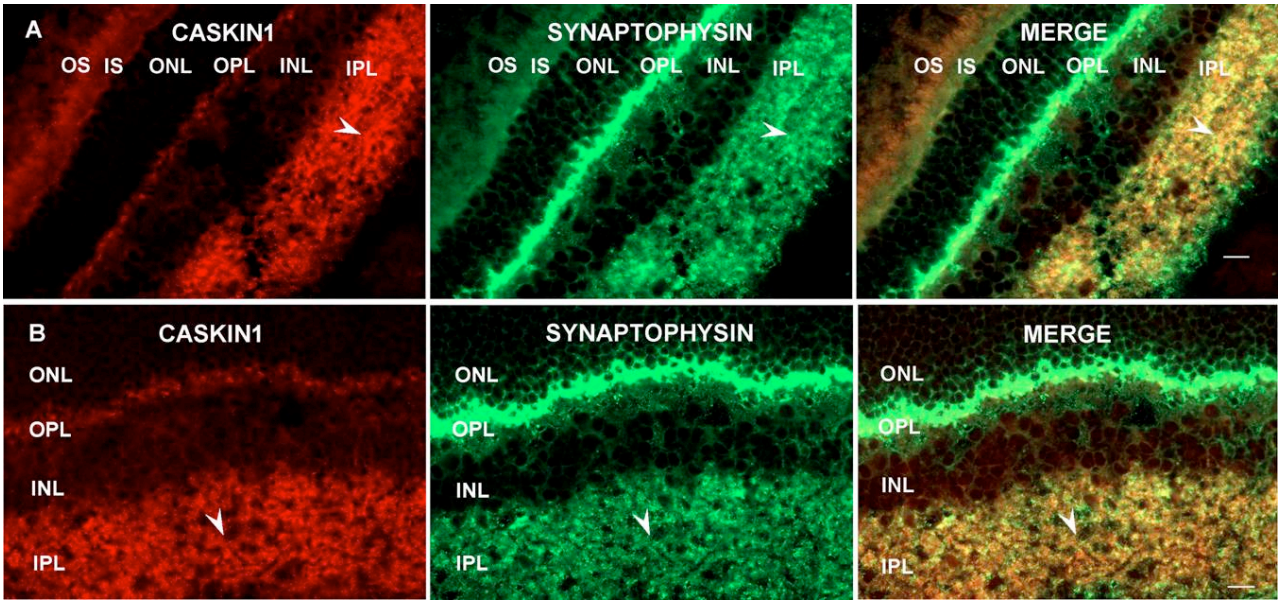

Figure 13: The immunosignals of Caskin1 co-localize with the immunosignals of Synaptophysin in IPL in bovine retina cryosections. The sections (arrow heads in A, B) analysed with the conventional florescence microscope (A, B). Abbreviations: OS,outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer;IPL, inner plexiform layer. Scale bar: 10µm.

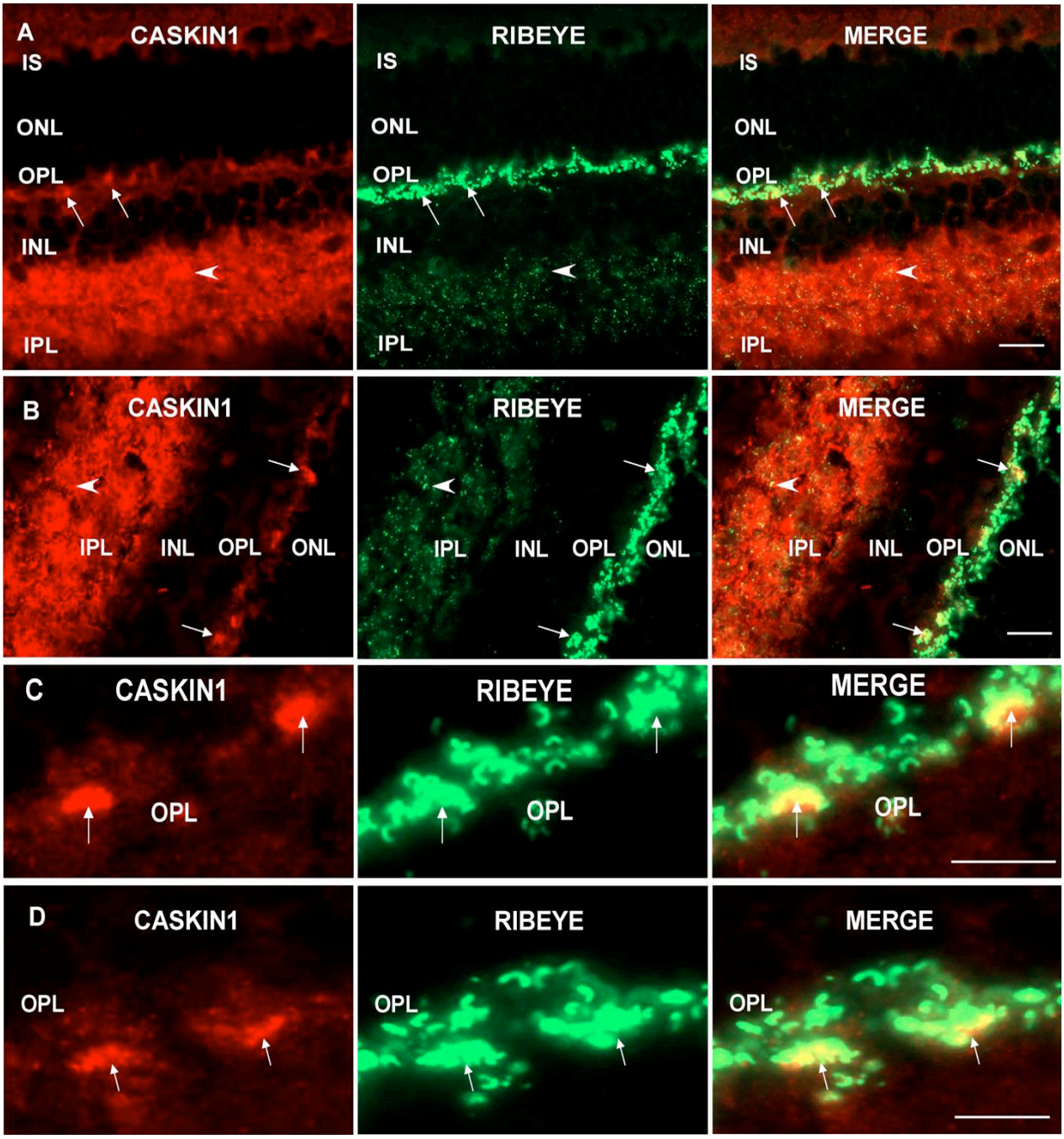

Figure 14: The immunosignals of Caskin1 co-localize with the immunosignals of RIBEYE (B) in IPL in bovine retina cryosections: A, B show respective images of inner plexiform layer (arrow heads) of the bovine retina. The merge shows co- localization of Caskin1 and RIBEYE. The cone photoreceptors (long arrows) in OPL labelled for Caskin1 (Red) and RIBEYE (green). The dot like signals of Caskin1 in OPL also colocalize with RIBEYE (B) (long arrows) in (A, B, C, D). The sections analysed with conventional florescence microscope (A, B, C, D) Abbreviations: OS,outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer;IPL, inner plexiform layer. Scale bar: 10µm.

3.4. Caskin1 is expressed in the cone photoreceptor ribbon synapses:

The dot like immuno signals of Caskin1 in the outer plexiform layer of the bovine retina suggested an association with the cone photoreceptors synapses. Therefore, PNA agglutinin was used as a specific marker for the cone synapses. In Fig.15, cone synapses

were identified with fluorescently labelled peanut agglutinin (PNA) (Wang *et al*., 2003; Morgans *et al*., 2009). In these incubations, Caskin1 immunosignals co-localized with PNA agglutinin (Fig.15, A, B).

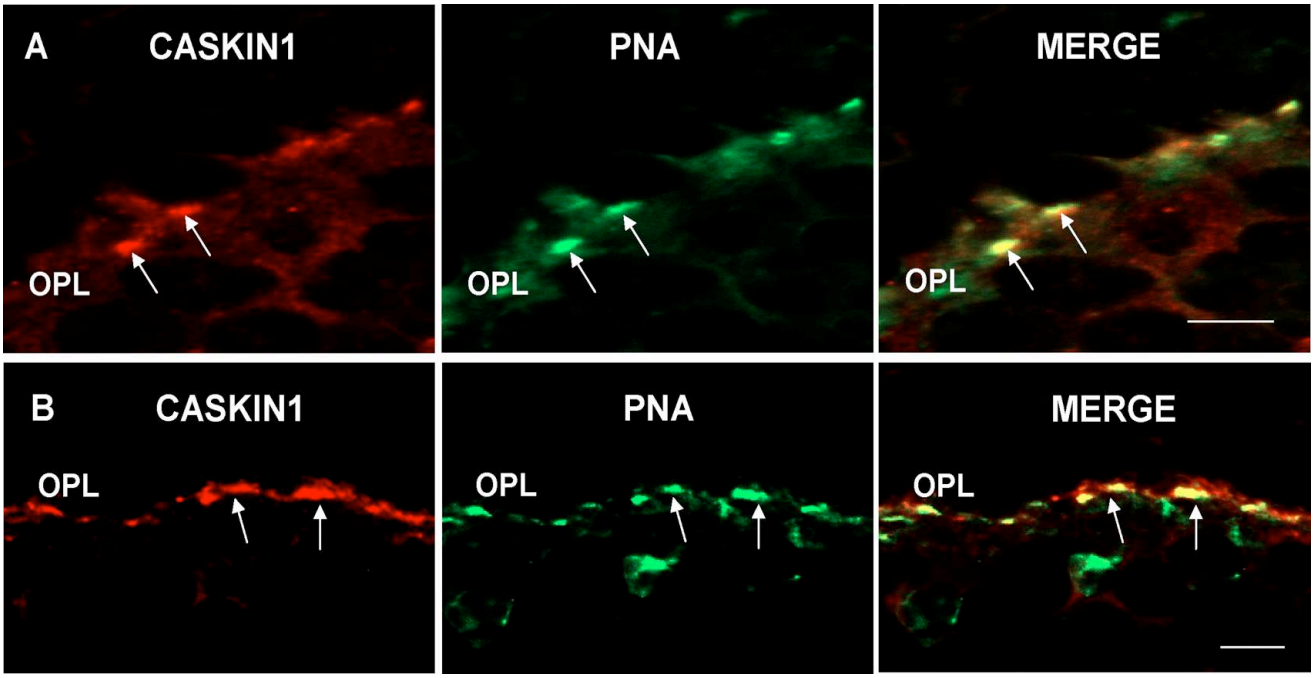

Figure 15: Co-localization of Caskin1 with PNA agglutinin: A, B show respective images of outer plexiform layer of the bovine retina. The cone photoreceptors (long arrows) double labelled for Caskin1 (Red) and PNA agglutinin (green). The merge shows co- localization of Caskin1 and PNA. Abbreviations: OS,outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer;IPL, inner plexiform layer. Scale bar: 10µm.

3.5. Labelling of Caskin1 immunosignals can be blocked by preabsorption with Caskin1 peptide and not by control peptide:

The minimum working concentration of Caskin1 at which immunosignals are still detectable is 1: 3500 (Fig.16 B). However, further dilution of Caskin1 did not reveal any immunosignals (Fig.16C) in the inner plexiform layer. Therefore, for blocking experiment, the dilution for Caskin1 used was 1:3500.

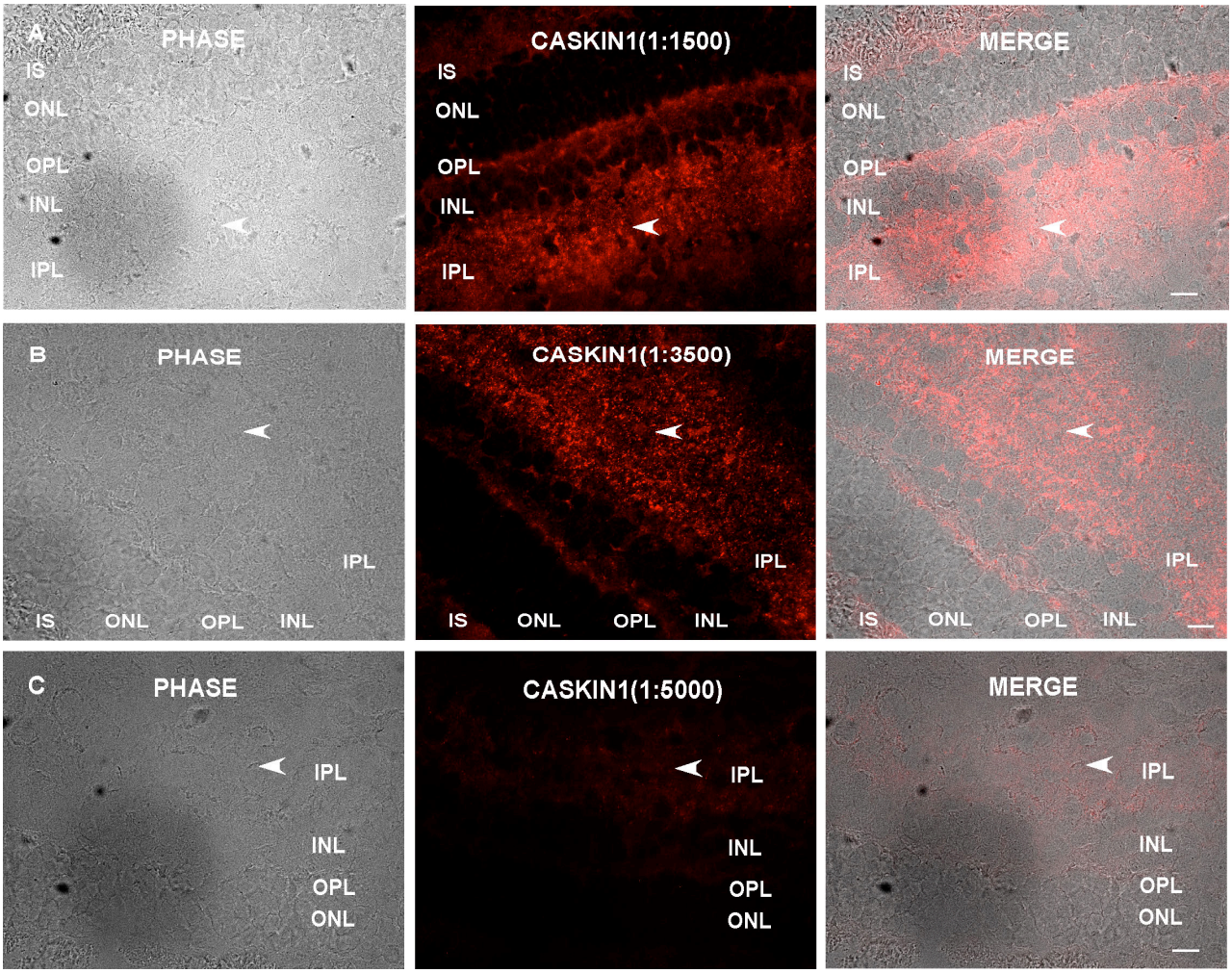

Figure 16: **Caskin1 immunosignals in different dilutions.** Cryostat sections immunolabeled with polyclonal Caskin1 antiserum showed immunosignals in inner plexiform layer (B) when used in dilution 1: 3500. The Caskin1 immunolabeling in the IPL did not demonstrate immunosignals in IPL (C) when dilution of Caskin1 was further increased. Abbreviations:OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bars: 10 µm.

The Caskin1 immune signals were specific because the signals could be completely blocked by preabsorbing the polyclonal antiserum with Caskin1 peptide (Fig. 17A) but not with control peptide (Fig.17B). After pre-absorption of the Caskin1 polyclonal antiserum with Caskin1 peptide, immune signals disappeared from inner plexiform layer of bovine retina (Fig.17A). On the contrary, for preabsorption with LPH control peptide, the immune signals remained unchanged (Fig. 17B).

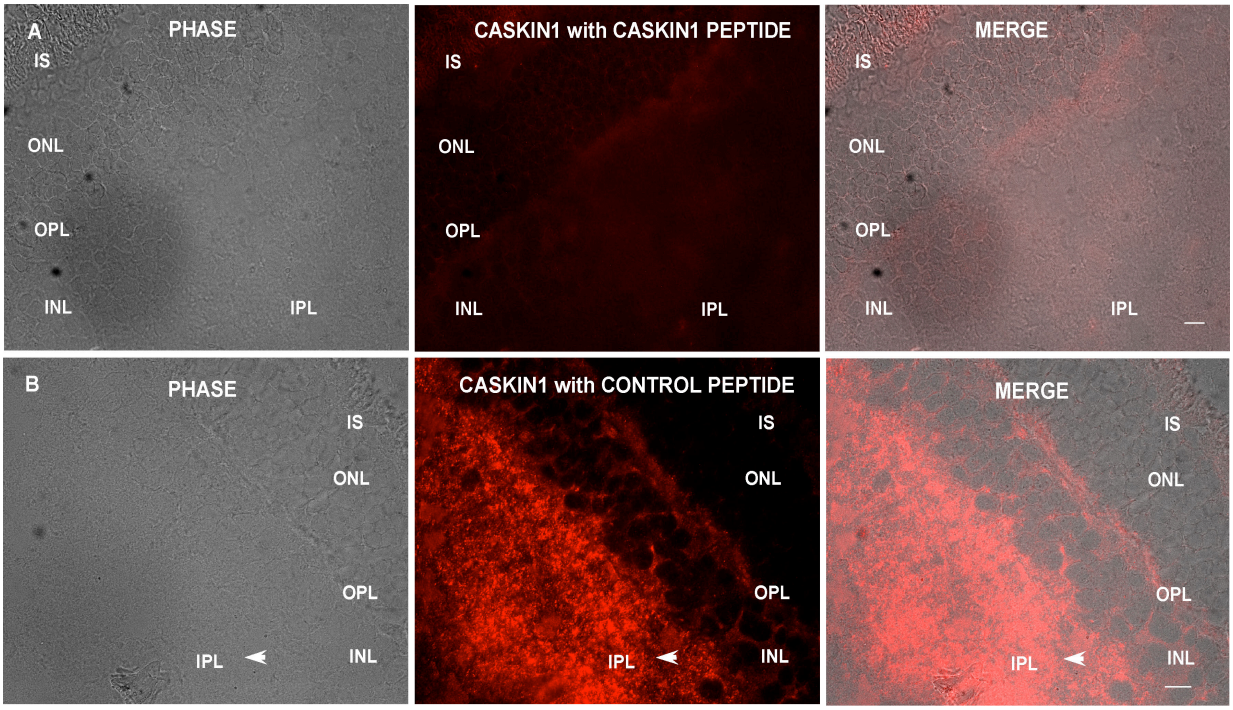

Figure 17: **Labeling of Caskin1 immunosignals can be blocked by preabsorption with Caskin1 peptide but not by LPH control peptide pre-absorption**. Cryostat sections immunolabeled with polyclonal anti-Caskin1 antiserum pre-absorbed with Caskin1 peptide (A) and LPH control peptide (B). The Caskin1 immunolabeling in the IPL cannot be blocked by pre-absorption with LPH control peptide (B) but by pre-absorption with Caskin1 peptide (A). Abbreviations:OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bars: 10 µm.

3.6. Detection of Intersectin1 in bovine retina by Western Blot analysis:

The immunoblotting of bovine retinal lysate with antibodies directed against GST fusion proteins of EHSH1 (Intersectin 750, 227, 229 and EH-HOM) detected a strong band of~200kDa. In addition, another band typical for the short isoform of EHSH1/Intersectin 145kDa was also recognised by these antibodies (Fig.18A, B; Fig.19C, D). In contrast, Western blot for EHSH1 229 detected an additional lower molecular weight band of almost 43kDa (Fig.19D) which is not present in Western blots for Intersectin-227,750 and EH-HOM.

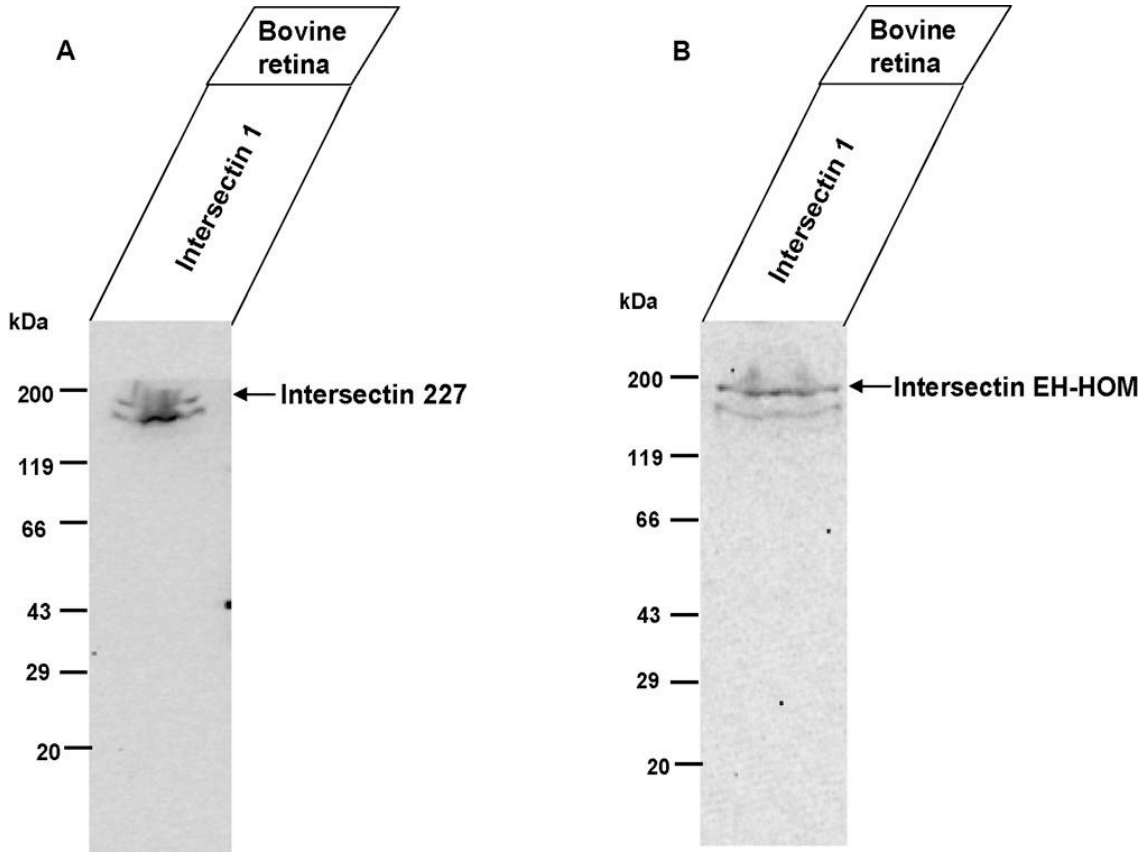

Figure 18. Immunoblot analysis of Intersectin1 from bovine retina. The retinal proteins separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was transferred to nitrocellulose membrane and probed for Intersectin1 with polyclonal Intersectin 227, Intersectin EH-HOM antibody. Signals were visualized with ECL. A polypeptide band at approx. 200 kDa, the typical molecular weight of Intersectin1, was recognized. The numbers at the left indicate the positions of the molecular weight markers.

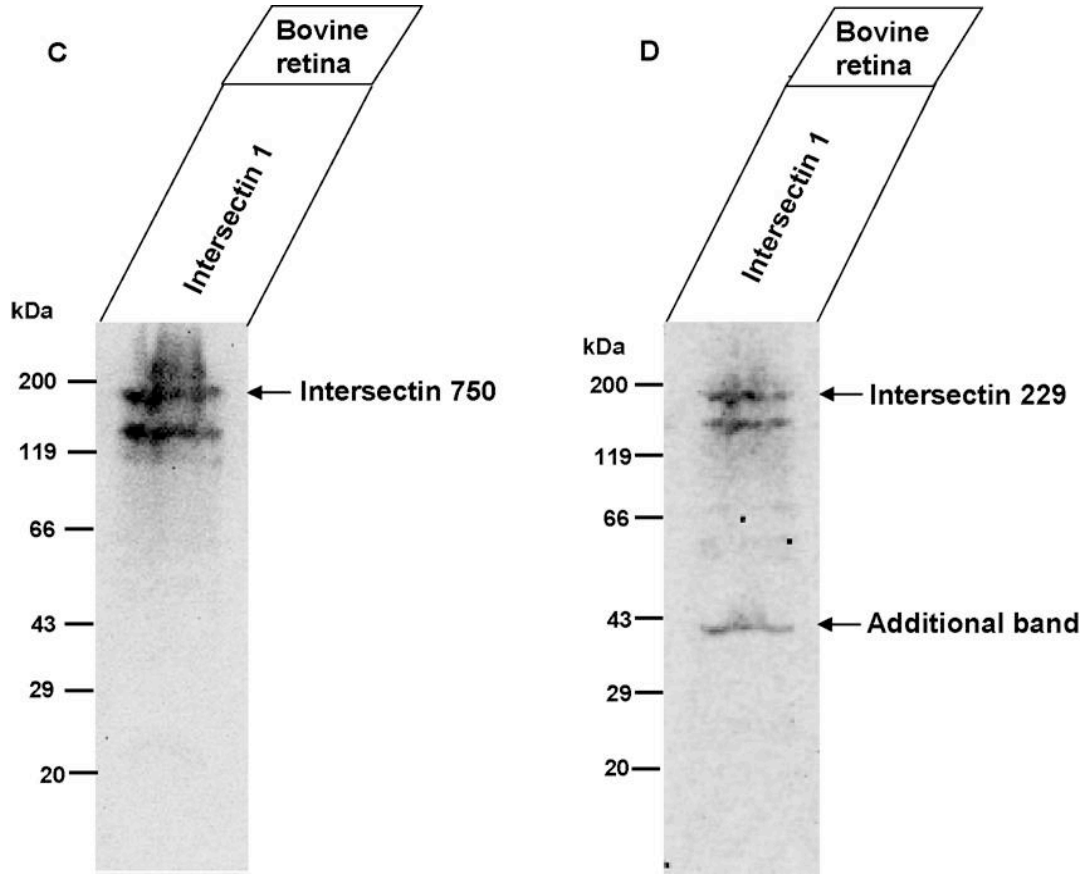

Figure 19. Immunoblot analysis of Intersectin1 from bovine retina . The retinal proteins separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was transferred to nitrocellulose membrane and probed for Intersectin1 with polyclonal Intersectin 750 and Intersectin 229 antibody. Signals were visualized with ECL. A polypeptide band at approx. 200 kDa, the typical molecular weight of Intersectin1, was recognized. The numbers at the left indicate the positions of the molecular weight markers. For Intersectin 229, an additional low molecular weight band (Fig.19D) was also recognized.

3.7. Detection of Intersectin1 in bovine retina can be blocked by Intersectin1-GST preabsorption but not by GST preabsorption:

Intersectin1 is highly specific because the detection of Intersectin1 in the bovine retina was completely blocked when the nitrocellulose membrane blot was incubated with polyclonal Intersectin EH-HOM antiserum preabsorbed with EH-HOM-GST fusion protein (Fig. 20A, lane 2). In contrast to this, Intersectin1 was still detectable in the bovine retinal lysate when the blot was incubated with GST fusion protein alone and in the crude bovine retina (Fig.20A, lane 3, 4). In Fig. 20B, the same blot as in Fig. 20A was stripped off and reprobed with mouse monoclonal antibody against Synaptophysin.

This blot (Fig. 20B) demonstrated the presence of Synaptophysin (Fig. 20B, lane1, 2, 3and 4) thus indicating the specificity of Intersectin1 (EH-HOM) preabsorption.

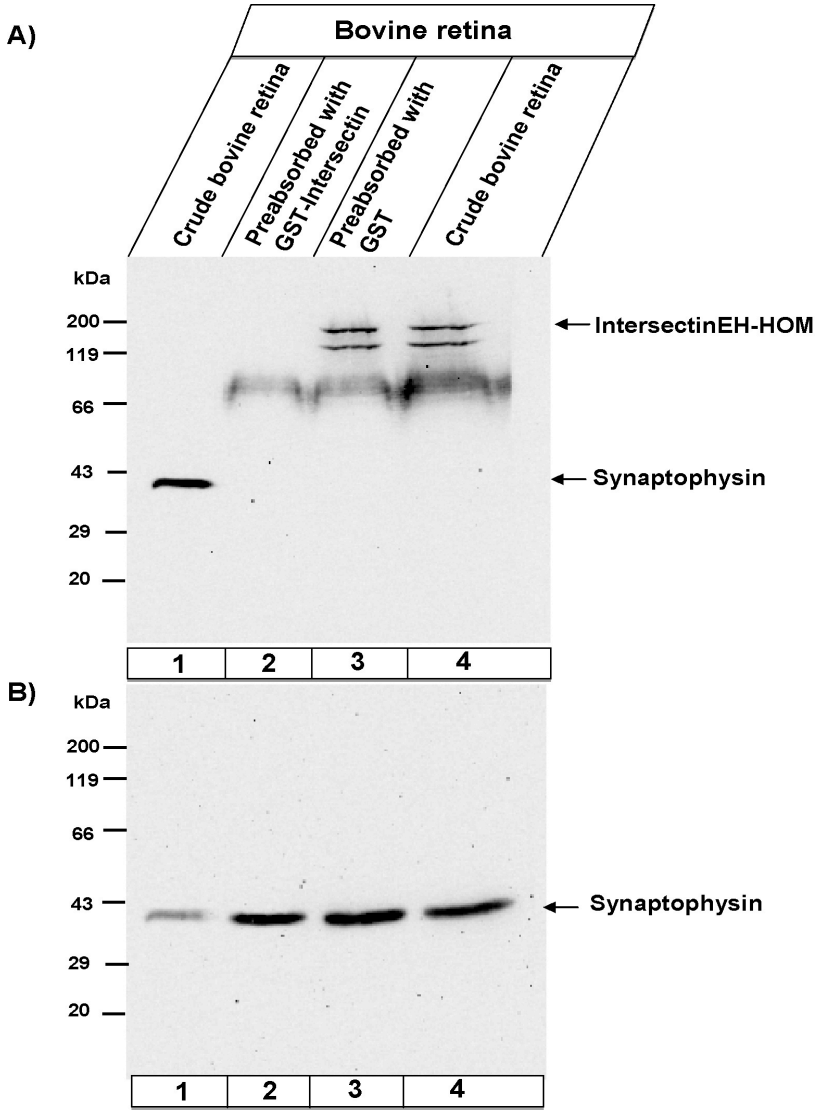

Figure 20. Detection of Intersectin1 (EH-HOM) in bovine retina can be blocked by Intersectin1(EH-HOM)-GST but not by GST preabsorption. The polyclonal Intersectin EH-HOM antibody specifically detected Intersectin1 in a crude retinal extract as a band of the expected molecular weight of approx. 200kDa (lane 4). This band is specific because it is completely blocked if our EH-HOM antiserum was pre-absorbed with its antigen (EH-HOM-GST, lane 2) but not by GST alone (lane 3). Intersectin1 can be detected in crude bovine retina (lane4) as a positive control. Lane 1 shows loading control by anti Synaptophysin in bovine retina. In Fig.20 B., the same blot was stripped off and re-incubated with anti Synaptophysin antibody. It indicated the detection of Synaptophysin in all lanes (1, 2, 3, and 4).

3.8. Intersectin1 and Munc 119 can be co-immunoprecipitated from bovine retina:

Co-immunoprecipitation experiment was done using extracts from bovine retina. Munc119 (V2T2, 120IT) immune serum co-immunoprecipitated Intersectin1 (EHSH1-227) from bovine retinal lysate (Fig. 21b, lane 2) while Munc119 (V2T2, 120 IT) pre-immune serum did not co-immunoprecipitate Intersectin1 (EHSH1 227) (Fig. 21b, lane

1). Munc119 immune serum co-immunoprecipitated Munc119 from the bovine retina lysate (Fig.21a, lane 2). However, Munc119 preimmune serum did not co-immunoprecipitate Munc119 (Fig.21a, lane 1) and Intersectin1 (EHSH1 227) (Fig. 21b, lane 1). Hence, this experiment demonstrates a certain extent interaction between Munc119 and Intersectin1 (EHSH1 227) in the bovine retina.

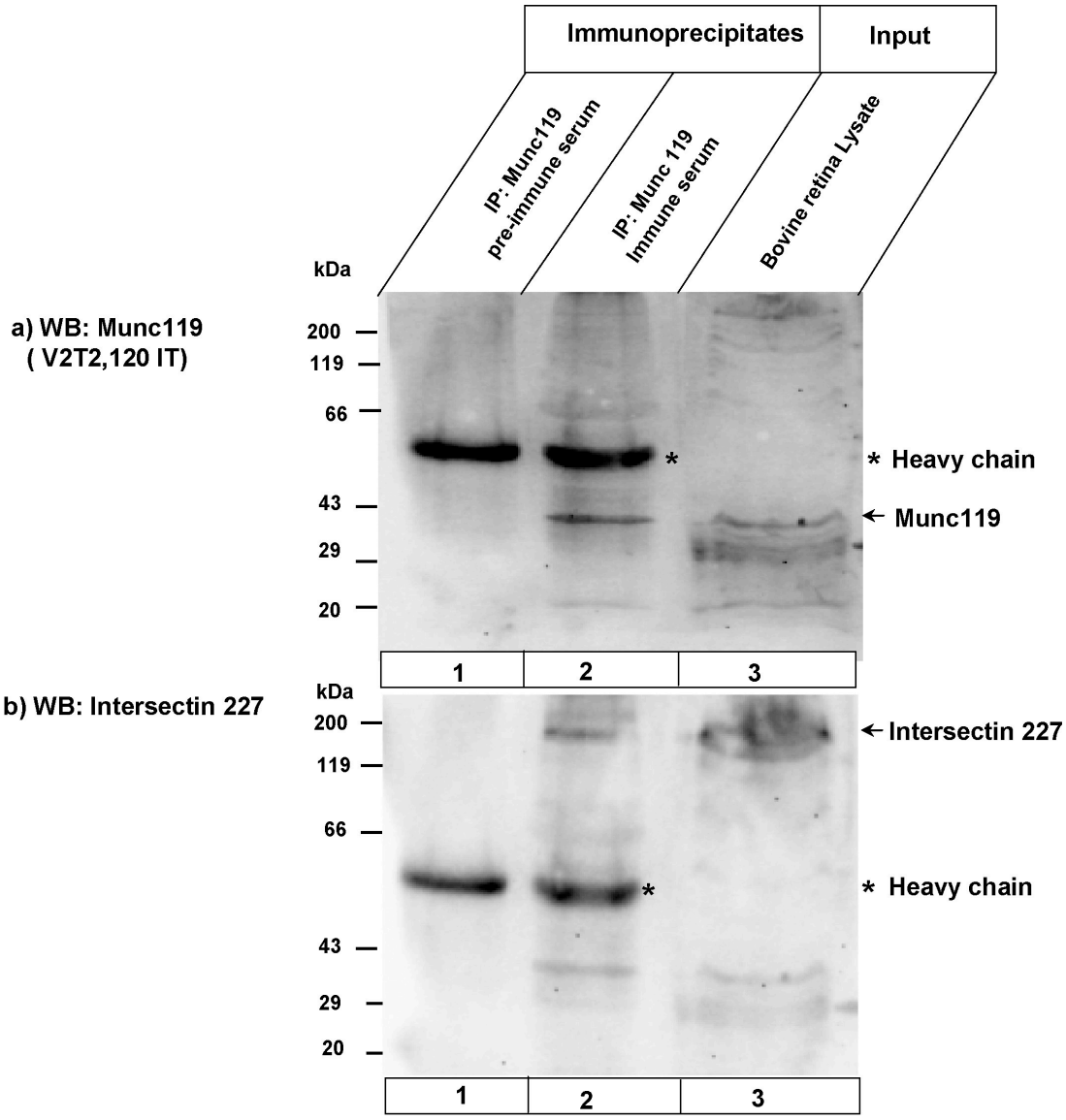

Figure 21. Co-immunprecipitation of Munc119 and Intersectin1 from the bovine retina. Munc119 immune serum and Munc119 pre-immune serum were tested for their capability to co-immunoprecipitate Intersectin1. Fig. 21 a) Munc119 is immunoprecipitated by Munc119 immune serum (lane 2, Fig. 21a) but not by Munc119 pre-immune serum (lane 1, Fig.21a). Asterisks indicate the immunoglobulin heavy chains (lanes2). Fig. 21b shows the same blot (as in Fig. 21 a) but reprobed with rabbit polyclonal Anti Intersectin 227 antibody. This blot shows the presence of Intersectin 227 precipitated by the Munc119 immune serum (lane 2) but not by the pre-immune serum (lane 1). Asterisks indicate the immunoglobulin heavy chains (lanes2). In the input lanes, 3% of total input was loaded.

3.9. Intersectin1 is present in the OPL of bovine retina:

The single immunolabelling experiments with rabbit polyclonal Intersectin1 (EH-HOM, EHSH1-227,750 and 229) revealed very strong immune signals in the outer plexiform layer of the bovine retina (Fig.22, 23). Interestingly, EHSH1 229 demonstrated puncted immune signals in the OPL under lower magnification and with higher magnification these immunosignals from EHSH1 229 antibody, labeled the synaptic ribbons (Fig. 23 C, D, E).

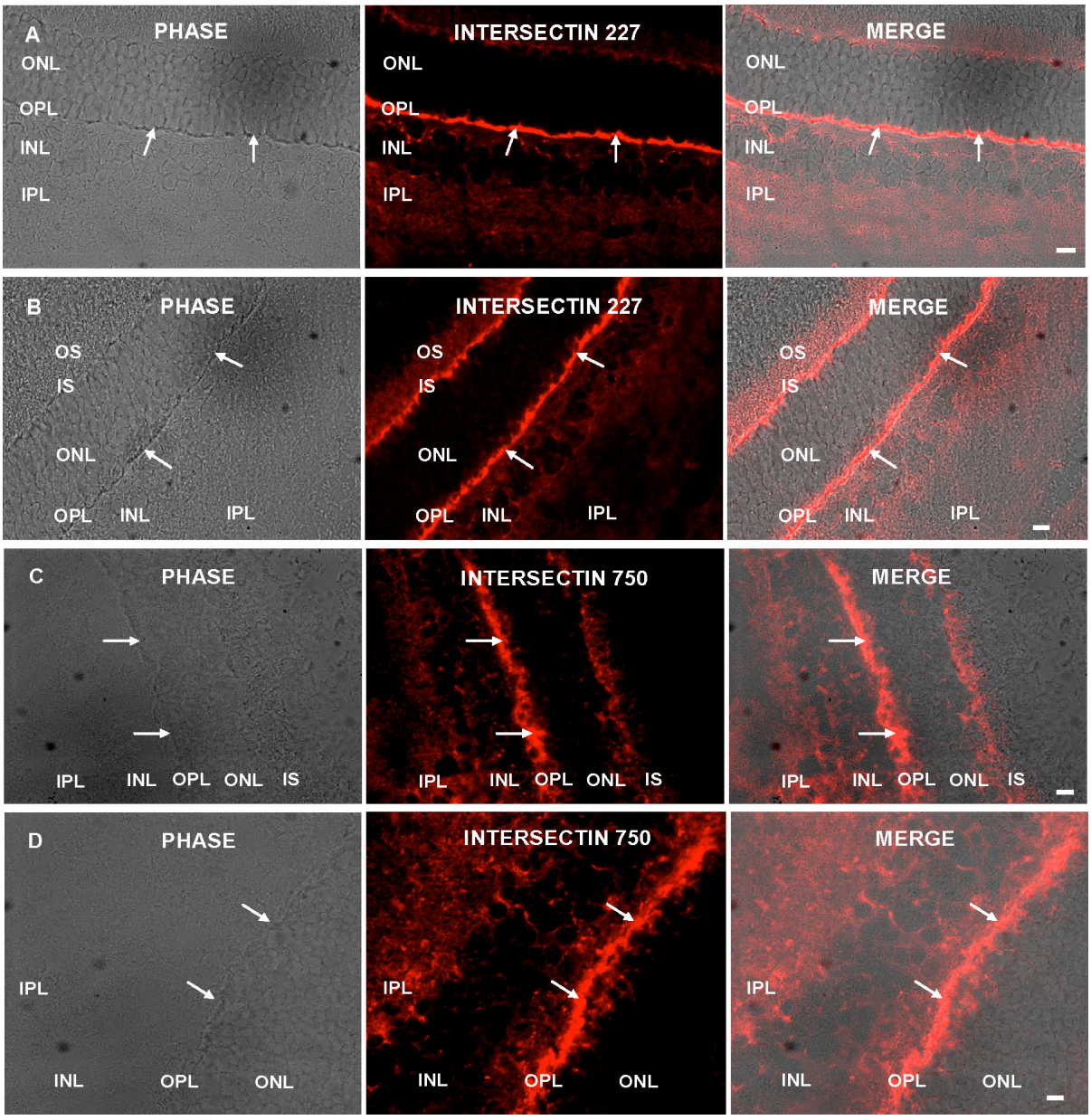

Figure 22: Distribution of Intersectin1 in cryostat sections of bovine retina labelled with antibody against Intersectin 227 and Intersectin 750. (A, B, C, D) show strong immunoreactivity in OPL (long arrows).The sections analysed with conventional florescence microscope. Abbreviations: OS,outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer;IPL, inner plexiform layer. Scale bar: 10µm.

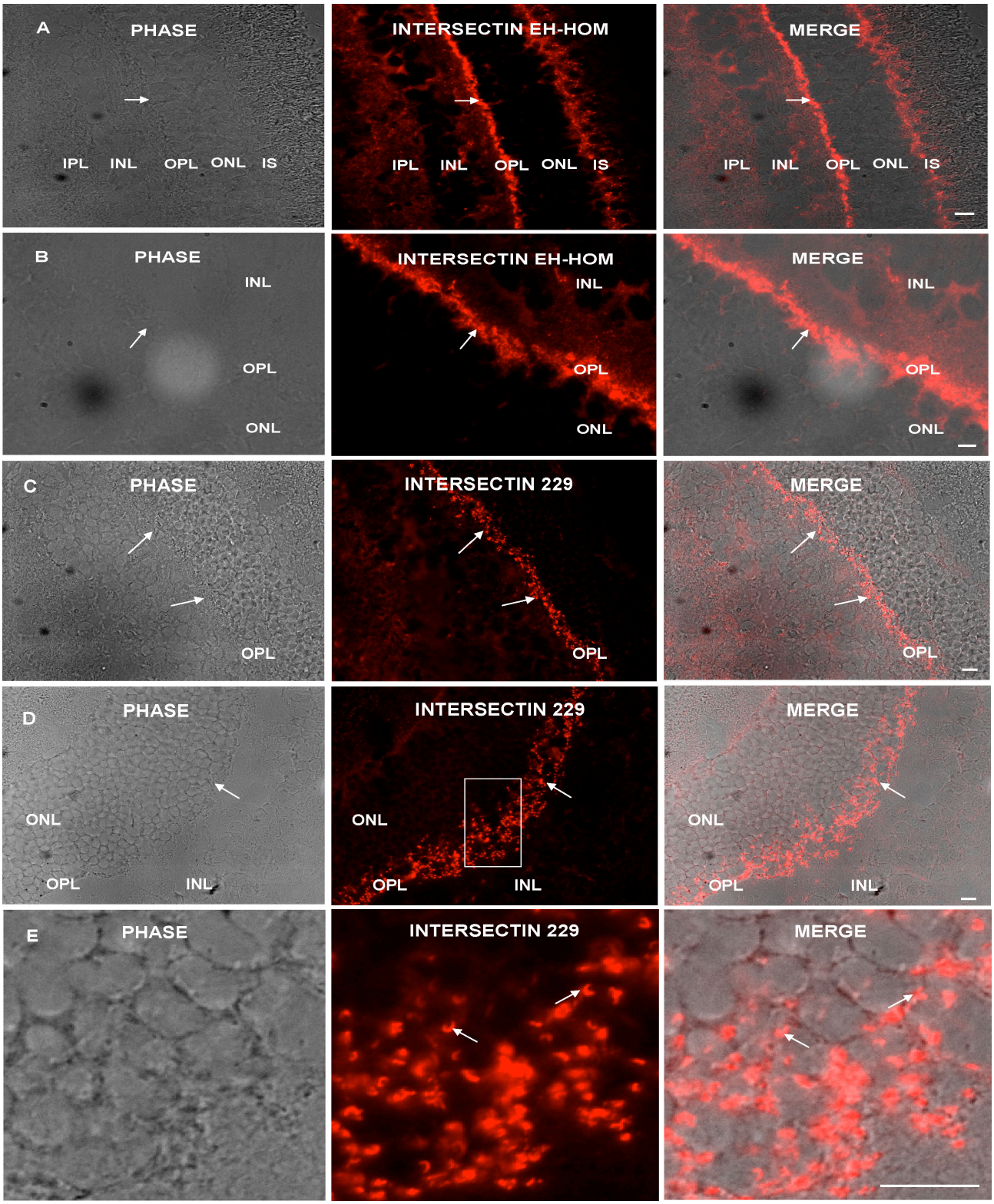

Figure 23: Distribution of Intersectin1 in cryostat sections of bovine retina labelled with antibody against Intersectin EH-HOM and Intersectin 229. Intersectin EH-HOM (A, B) show strong immunoreactivity in OPL (long arrows). Intersectin 229 (C, D, and E) show the puncted signals labelling the synaptic ribbons. The section analysed with conventional florescence microscope. Abbreviations: OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar: 10µm.

3.10. Intersectin1 partly co-localizes with the synaptic ribbons in the photoreceptor synapses:

The double immunofloresence experiments with rabbit polyclonal antibodies EHSH1-227, 229, 750 and EH-HOM generated very strong immune signals in the OPL (outer plexiform layer) of the bovine retina and these signals were co-localised with the synaptic ribbons which were labelled with RIBEYE (B)/CtBP2 antibodies (Fig. 24, 25 , 26 and 27).

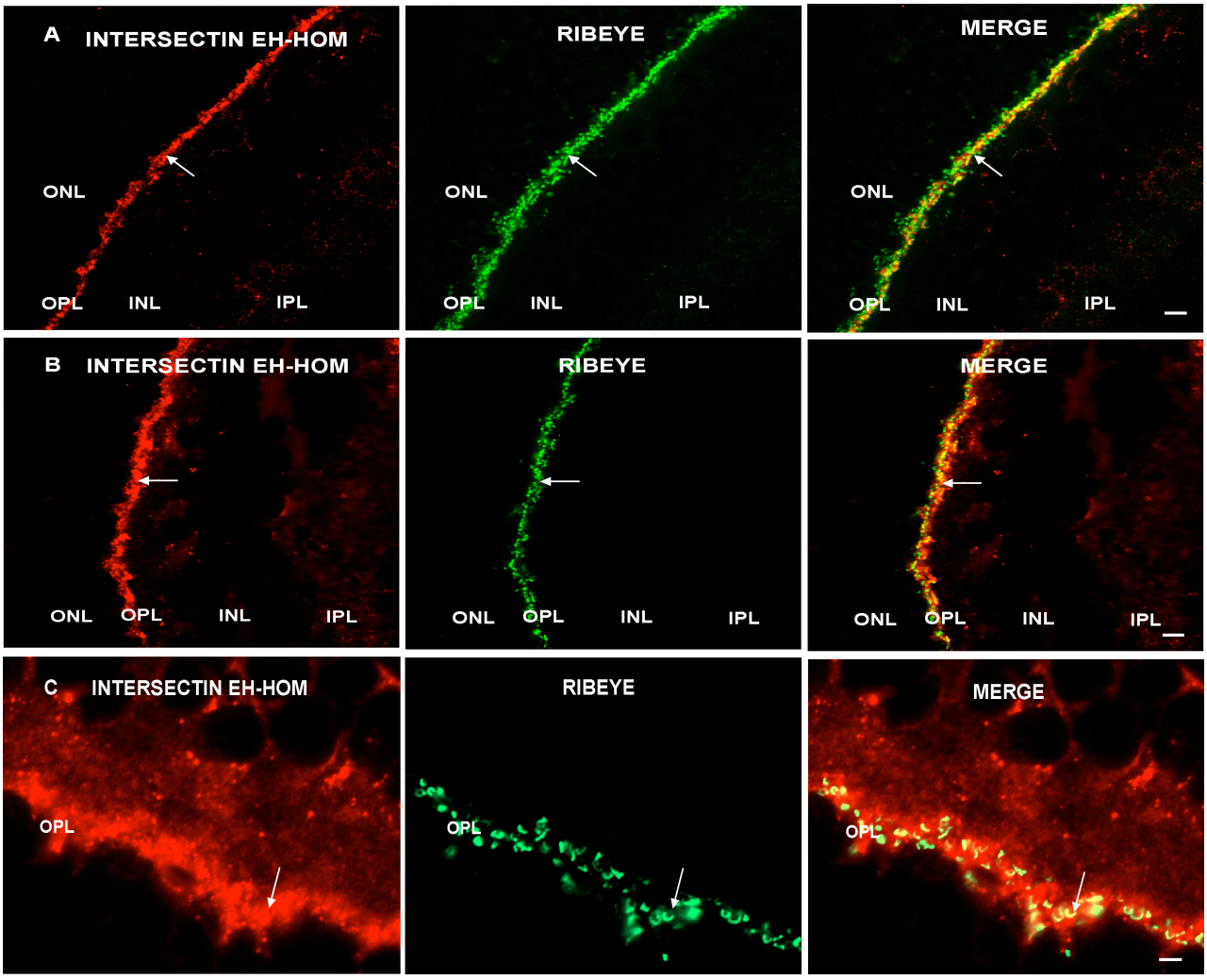

Figure 24: The immunosignals of Intersectin (EH-HOM) co-localize with the immunosignal of RIBEYE (B)/CtBP2 in bovine retina cryosection (arrows in A, B, C). The sections analysed with conventional florescence microscope (A,B,C):Abbreviations: OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar: 10µm.

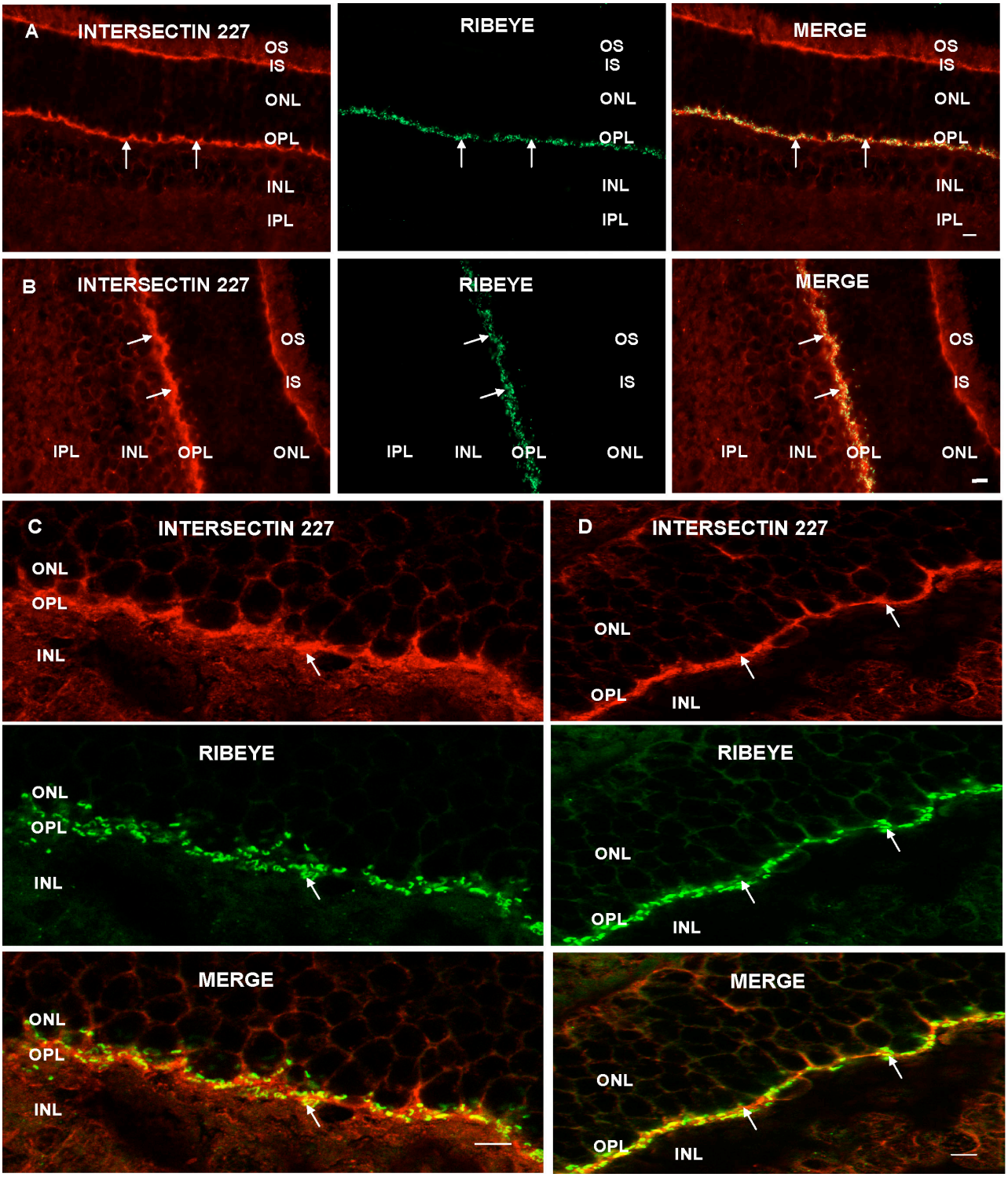

Figure 25: The immunosignals of Intersectin 227 co-localize with the immunosignals of RIBEYE (B)/CtBP2 in bovine retina cryosections (arrows in A, B, C, D).The sections analysed with conventional florescence microscope (A, B) and with confocal microscope (C, D). Abbreviations: OS,outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer;IPL, inner plexiform layer. Scale bar: 10µm.

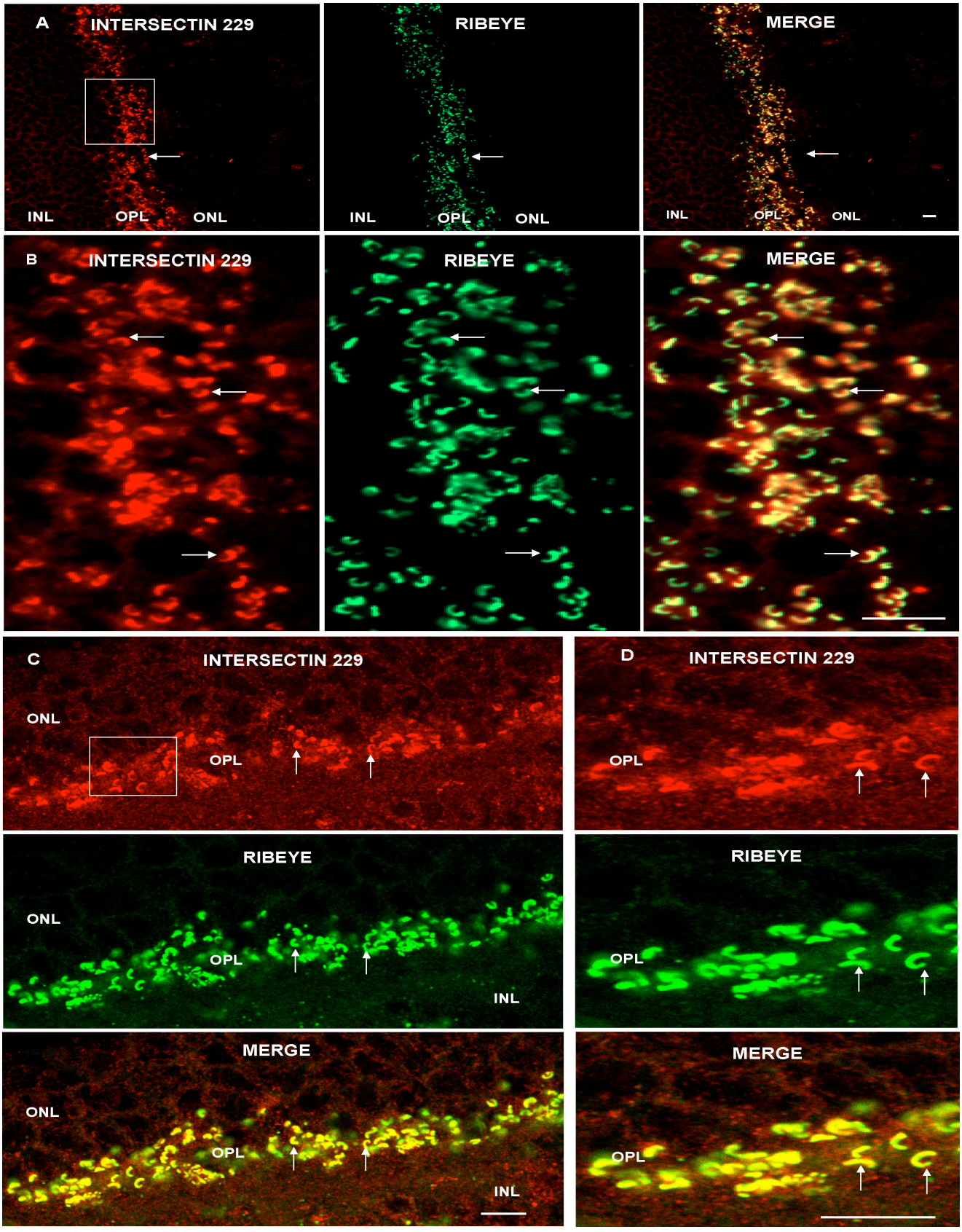

Figure 26: The immunosignals of Intersectin 229 co-localize with the immunosignals of RIBEYE (B)/CtBP2 in bovine retina cryosections (arrows in A, B, C, D). The sections analysed with conventional florescence microscope (A, B) and with confocal microscope (C, D). Abbreviations: OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar: 10µm.

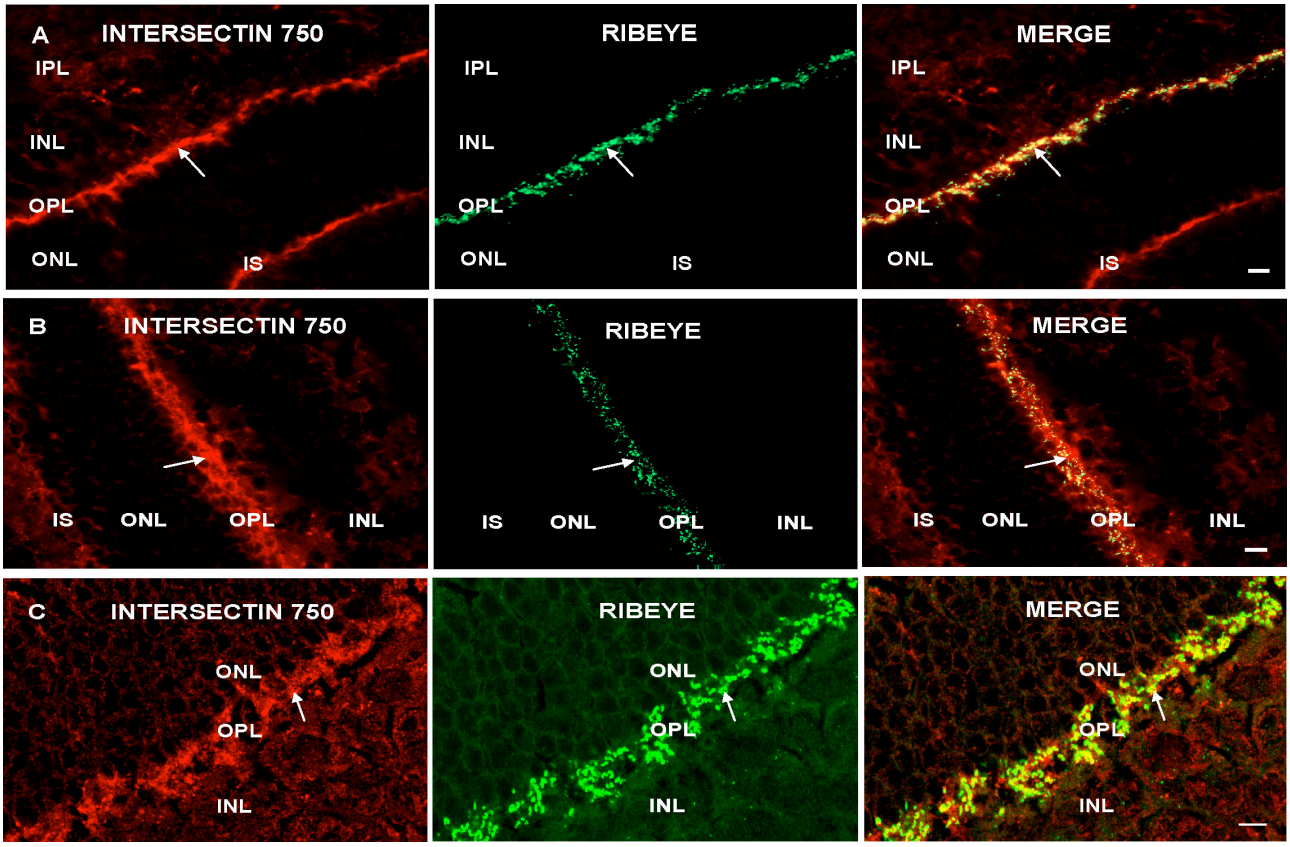

Figure 27: **The immunosignals of Intersectin 750 co-localize with the immunosignals of RIBEYE (B)/CtBP2 in bovine retina cryosections** (arrows in A, B, C).The sections analysed with conventional florescence microscope (A, B) and with confocal microscope (C). Abbreviations: OS,outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer;IPL, inner plexiform layer. Scale bar: 10µm.

3.11. Labelling of Intersectin1 immunosignals can be blocked by preabsorption with Intersectin-GST and not by GST alone:

The Intersectin/EH-HOM immune signals were specific because the signals could be completely blocked by preabsorbing the polyclonal antiserum with EH-HOM-GST fusion protein but not with GST fusion protein alone (Fig.28). After pre-absorption of the EH-HOM polyclonal antiserum with EH-HOM-GST protein, immune signals were disappeared from outer plexiform layer of bovine retina (Fig.28B). On the contrary, for preabsorption with GST fusion protein alone, the immune signals remained unchanged (Fig. 28A). Preabsorption of EH-HOM antiserum with GST (Fig. 28D) does not influence the Intersectin EH-HOM as well as the RIBEYE (B)/ CtBP2 immune signals in the ribbon synapses of OPL (Fig.28D). Conversely, when preabsorption was carried out with EH-HOM-GST protein it abolished the EH-HOM immune signals without

affecting the RIBEYE (B)/CtBP2 signals in outer plexiform layers of the bovine retina (Fig:28C).

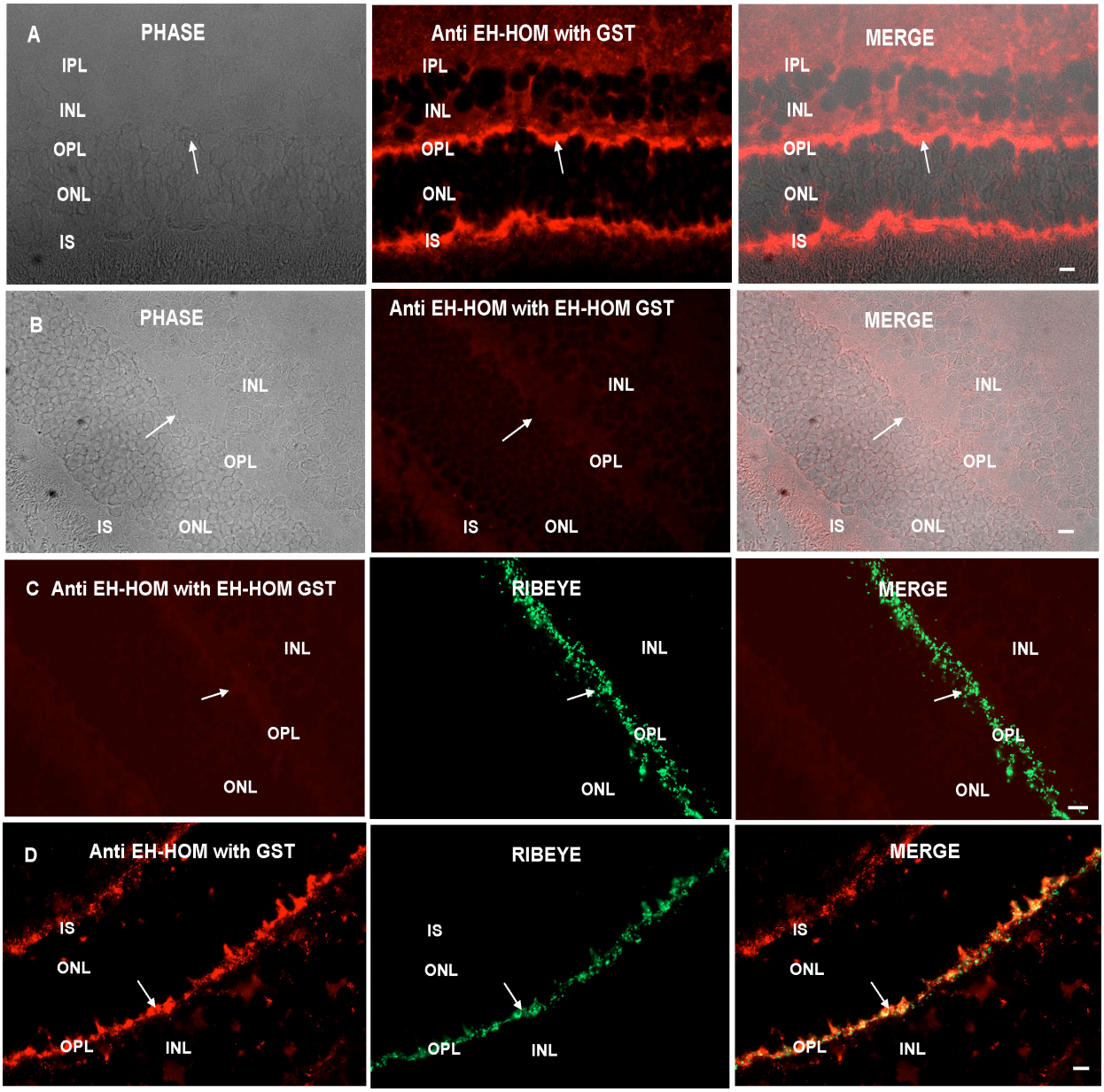

Figure 28. **Labeling of presynaptic Intersectin1(EH-HOM) immunosignals can be blocked by EH-HOM-GST but not by GSTpre-absorption.** Cryostat sections immunolabeled with polyclonal anti-EH-HOM antiserum pre-absorbed with GST (**A, D**) and EH-HOM-GST (**B, C**). The Intersectin EH-HOM immunolabeling in the OPL cannot be blocked by pre-absorption with GST (**A**) but EH-HOM immunosignals are blocked by pre-absorption with EH-HOM-GST (**B**). After preabsorption of the Intersectin EH-HOM polyclonal antiserum with EH-HOM-GST, the Intersectin EH-HOM immunosignals is gone (**C**) whereas after pre-absorption with GST the EH-HOM immunosignals remained unchanged (**D**). Pre-absorption of EH-HOM antiserum with GST does not influence the EH-HOM as well as the RIBEYE immunosignal in ribbon synapses of the OPL (**D**). However, pre-absorption of the EH-HOM antiserum with EH-HOM-GST (**C**) abolishes EH-HOM immunosignals but not the RIBEYE immunosignals. Abbreviations:OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bars: 10 µm.

4. DISCUSSION

Proper function of the mammalian brain relies on the establishment of highly specific synaptic connections among billions of neurons. In order to understand the structural basis of the synaptic functions, it is crucial to precisely describe the morphology and molecular composition of the synapses. The ribbon synapses of retina are highly specialized tonically active synapses. The typical landmark of ribbon synapses is the synaptic ribbon, a highly dynamic organelle suggested to be involved in exocytosis, membrane trafficking and endocytosis (Schmitz, 2009; Sterling and Matthews, 2005). RIBEYE, a major component of synaptic ribbons, appears to be involved in the synaptic ribbon formation (Magupalli *et al.,* 2008; Schmitz, 2009). Although a lot is known about the physiology of ribbon synapses, the functional role of synaptic ribbons in the ribbon synapses is unclear. Hence, analyses of other synaptic proteins in the retinal synapses would provide more information about the protein machinery and functional aspects which could further highlight the differences between the conventional and ribbon synapses. In my experimental work, I have demonstrated the presence of Caskin1 and Intersectin1 in the synaptic layers of mammalian retina.

4.1. Characterization Caskin1 in mammalian retina:

Caskin1, a CASK [calcium/calmodulin-(CaM)-activated serine-threonine] interacting protein, belongs to scaffolding protein group and it consists of multiple domains which interact with different proteins (Balázs *et al*., 2009). In brain, Caskin1 is present in presynaptic active zones of conventional synapses (Tabuchi *et al*., 2002). Caskin1 has been demonstrated to interact with Synaptotagmin and neurexins2 and it is a strong interacting partner of CASK protein (Balázs *et al*., 2009, Weng *et al*., 2011, Tabuchi *et al*., 2002). CASK is identified as an important protein involved in synaptic protein targeting, synapse formation, brain development and regulates gene expression (for review, see Hsueh *et al*., 2006). It is suggested that in brain, CASK and Caskin1 constitute a part of the fibrous mesh of proteins and help in organizing the active zone of neuronal synapses (Stafford *et al*., 2011). The interaction between CASK and

Caskin1 takes place when CASK binds to a region known as CASK interacting domain (CID) of caskin1 (Stafford *et al*., 2011). Caskin1 consists of a short linear EEIWVLRK peptide motif and this peptide motif is very essential for the binding of Caskin1 with Cask protein (Stafford *et al*., 2011). Presence of a binding pocket on the CASK CaMK domain has been identified and autophsphorylation of CASK takes place adjacent to the binding pocket which regulates binding of Caskin1 to CASK (Stafford *et al*., 2011). Caskin1 is specifically present in brain (Tabuchi *et al*., 2002). In the present study, it is demonstrated for the first time that Caskin1 is highly enriched in the mammalian retina. This finding is further supported by the immunocytochemistry experiments that Caskin1 is predominantly expressed in the inner plexiform layer of the retina and it is present in the outer plexiform layer but it is not labeling all terminals of the photoreceptors. The bovine retina is a mixed retina that contains both rods and cones photoreceptors. The rod synapses contain a single synaptic ribbon, and the cone synapses can be discriminated from the rod synapses based on their larger size and the presence of numerous synaptic ribbons (Schmitz et al., 1996; 2000). By light microscopy, the ribbons in a cone terminal appear like a chain of dots (tom Dieck *et al*., 2006). On the basis of co-localization of Caskin1 with RIBEYE(B) in the OPL, we assumed that Caskin1 might be present in cone photoreceptor synapses. To verify Caskin1 cone specific labeling, PNA (Peanut agglutinin) was used as a specific marker for cone synapses (Wang *et al*., 2003; Morgans *et al*., 2009). Co-localization of Caskin1 with PNA confirmed that Caskin1 is present only in the cone photoreceptor synapses of the bovine retina. However, the rod photoreceptor synapses do not contain Caskin1. This further explains diversity of protein components among different types of ribbon synapses of bipolar cells, rod photoreceptor cells and cone photoreceptor cells ((Matthews *et al*., 2005). The precise function of Caskin1 in cone photoreceptor synapses is not known at present. This experimental work has demonstrated that Caskin1 is absent in rod synapses , therefore, it could be possible that other proteins might be responsible to perform the same function in the rod photoreceptor synapses that is carried out by Caskin1 in non ribbon synapses and in the ribbon synapses of the cone photoreceptors. Caskin1 and Mint1 are expressed predominantly in brain (Tabuchi *et al*., 2002). Moreover, both of these proteins compete with each other to bind with the same CaM kinase domain of CASK protein. This could be explained because of the

presence of common peptide motifs in both Caskin1 and Mint1 proteins (Stafford *et al*., 2011). In brain, CASK forms alternate stable complexes with Mint1/CASK/neurexin1 and also with Caskin1/CASK/neurexin1 (Tabuchi et al., 2002). From these findings we can suppose that a similar function might take place in retinal synapses but it needs more evaluation. In short, this data suggested that Caskin1 appeared to be a component of synaptic cytoskeleton in conventional synapses and the cone ribbon synapses but not the rod ribbon synapses. Further experiments are needed to elucidate the precise role of Caskin1 in mammalian retina

Caskin1 strongly interacts with CASK (Tabuchi *et al*., 2002). And, in humans association of CASK with X-linked mental retardation and microcephaly has been demonstrated (Hackett *et al*., 2010; Tarpey *et al*., 2009; Hsueh, 2009; Najm *et al*., 2008). Moreover, other CASK interacting proteins neurexin1 and Mint1 are associated with autism and Alzheimer's disease (Lisé and El-Husseini, 2006; Miller *et al*., 2006). A decline in the function of GABAergic synapses was noticed in the CASK knockout mice (Atasoy *et al*., 2007). Hence, it could be speculated that Caskin1 could also be associated in these pathological conditions but it is still not proven. However, future studies on Caskin1 knockout mouse might enlighten the role of Caskin1 in these pathologies.

4.2. Characterization of Intersectin1 in mammalian retina:

In the present study, I have identified that Intersectin1 is highly enriched in retina. Intersectin1 is an evolutionary conserved scaffold protein (O'Bryan *et al*., 2001). Intersectin1 consists of two Eps15 homology and five Src homology 3 domains (Yamabhai *et al*., 1998). It is a multidomain protein and these domains are involved in different phases of membrane trafficking (Okamoto *et al*., 1999). At the presynaptic plasma membrane, vesicles are inserted by exocytosis while, endocytosis results in the regeneration and recycling of the vesicles (for review see, Bennet *et al*., 1994). In neurons, clathrin coat mediated endocytosis is very important for the recycling of the synaptic vesicles (Pucharcos *et al*., 2001). There are more than 20 different proteins which are involved in clathrin mediated endocytosis (Okamoto *et al*., 1999). Intersectin1 has been found to play an important role in clathrin mediated endocytosis

in humans (Guipponi *et al*., 1998; Pucharcos *et al*., 1999). Intersectin1 interacts with various endocytic proteins as well as with the proteins associated with exocytosis (Okamoto *et al*., 1999; Sengar *et al*., 1999; Yamabhai *et al*., 1998; Fernández-Chacón *et al*., 2000). Functional mutations in Intersectin1 is associated with defects in endocytosis (Marie *et al*, 2004; Koh *et al*., 2004; Yu *et al*., 2008) and impaired recruitment of endocytic proteins (Henne *et al*., 2010). The short isoform of Intersectin1 is present in glial cells and the long isoform is neuron specific (Okamoto et al., 1999). In my study, it is shown that Intersectin1 is diffusely distributed in the presynaptic terminals of photoreceptors and it has shown co-localization with the RIBEYE(B) immunosignals. Remarkably, one Intersectin1 antibody (Intersectin 229) showed labeling of synaptic ribbons. The labeling of Intersectin1 (Intersectin EH-HOM) was specific as it could be blocked by GST-Intersectin. An interaction between RIBEYE and Munc119 has already been demonstrated (Alpadi *et al*., 2008). Munc119 directly interacts with RIBEYE (B) as the NADH-binding domain of RIBEYE interacts with PrBP/δ homology domain of Munc119. Presence of Munc119 in the presynaptic terminals of OPL of retina has been documented (Alpadi *et al*., 2008). In my study, an interaction between Munc119 and a small part of Intersectin1 (Intersectin 227) has been observed by co-immunoprecipitation. Munc119 appears to be a peripheral protein component of synaptic ribbon (Alpadi *et al*., 2008) and is essential for the synaptic vesicle trafficking at the photoreceptor ribbon synapses (Higashide *et al*., 1998). Although, the physiologic importance of Munc119 has been well documented, it is not yet clear how Munc119 works at the molecular level in the ribbon synapse. Therefore, more experiments are needed to address the possible functional significance of the presence of Intersectin1 in retina. Intersectin1, a membrane associated protein (Okamoto *et al*., 1998), strongly binds with SNAP25 (important member of Q-SNARE family) and Dynamin1 protein in brain. These proteins are involved in exocytosis and endocytosis respectively (Okamoto *et al*., 1998). It has been analyzed that SNAP25 and Dynamin1 are highly enriched in ribbon synapses in retina (Ullrich *et al*., 1994). A recent research has implicated that the multidomain scaffolding and adaptor protein Intersectin1 acts as a central regulator of synaptic vesicle cycling (Pechstein *et al*., 2010). However, the question how does Intersectin1 interact with Dynamin1 and SNAP25 in retinal synapses is unknown at present. The tonically active ribbon synapse requires a fast membrane trafficking

machinery to keep the pace constant for a prolonged period. Future advances regarding the functional role of Intersectin1 in retina will help to produce a more detailed picture of synaptic transmission in the retinal synapses.

Association of Intersectin1 with Down's syndrome and neurodegenerative diseases such as Huntington's disease, Alzheimer's disease has been documented (Guipponi *et al*., 1998; Scappini *et al*., 2007; Wong *et al*., 2012). Intersectin 1 gene is localized on human chromosome 21, which is considered to be the critical region for Down syndrome (Guipponi *et al*., 1998). It has been studied that there is an increased expression of ITSN1 gene in the individuals with Down syndrome as compared to the normal individuals (Pucharcos *et al*., 1999; Skrypkina *et al*., 2005). A clear relationship has been observed between the pathology of Down syndrome and abnormal endocytosis process (Keating *et al*., 2006). The neurons in Down syndrome patients are characterized by the presence of large sized early endosomes and also there is an increase in the total number of endosomes of various sizes (Cataldo *et al*., 2008). To explain the relationship between Intersectin1 and endocytic defects of Down syndrome, Yu *et al*., (2008) generated Intersectin1 null mice. It was found that in this mice model, not only the process of endocytosis was slowed down but there was an increase in the size of endosomes also. Moreover, the spatial regions of the brain contained decreased levels of nerve growth factor (Yu *et al*., 2008). This study suggested that Intersectin1 participates in endocytosis and a disturbance in its expression could disrupt vesicle trafficking and endocytosis process in the brain (Yu *et al*., 2008). Considerably less is known about the precise role of Intersectin1 in retina. Further research in future would provide a better understanding regarding the importance of Intersectin1 in the pathophysiology of retinal diseases.

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LIST OF FIGURES

LIST OF ABBREVIATIONS

µg	microgram
µl	microlitre
A260 nm	Absorbance at 260 nm
A280 nm	Absorbance at 280 nm
aa	Amino acids
BARS	Brefeldin A-ADP ribosylated substrate
BD	Binding Domain
BSA	Bovine serum albumin
C	Celsius
CME	Clathrin mediated endocytosis
CCPs	Clathrin coated pits
CCR	Central coiled reoion
CtBP1	C-terminal Binding Protein 1
CtBP2	C-terminal Binding Protein 2
Cy2	Carbocyanin
Cy3	Indocarbocyanin
ddH2O	double distilled water
DNA	Deoxyribonucleicacid
ECL	Enhanced chemiluminiscence
EDTA	Ethylenediaminetetrachloroacteic acid
EM	Electron microscopy
GCL	Ganglion cell layer
GST	Glutathione S-transferase
GAM	Goat anti Mouse
GAR	Goat anti Rabbit
Hc	Horizontal cell

hrs	hours
g	grams
INL	Inner nuclear layer
IPL	Inner plexiform layer
IT	Immunization tag (day)
IS	Inner segments
Kb	Kilobases
kDa	kilo Dalton
KO	Knock-out
LPH	Limulus polyphemus hemolymph
Munc119	Mammalian UNC119
M	Molar
min(s)	minute(s)
ml	milliliter
mM	milli Molar
MW	Molecular weight
NAD	oxidised Nicotinamide adenine dinucleotide
NADH	reduced Nicotinamide adenine dinucleotide
NBD	NADH binding sub-domain
ng	nanogram
nm	nanometer
NPG	N-propyl gallate
OD	Optical density
OLM	Outer Limiting Membrane
ONL	Outer nuclear layer
ONPG	o-Nitrophenyl-β-D-galactoside
OPL	Outer plexiform layer
OS	Outer segments

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMSF	Phenylmethanesulphonylfluoride
PNA	Peanut agglutinin
RE(A)	RIBEYE(A)-Domain
RE(B)	RIBEYE(B)-Domain
rpm	revolutions per minute
RT	room temperature
SAM	Sterile α motif
SBD	Substrate binding sub-domain
SD	Synthetic Drop out medium
SDS	Sodiumdodecylsulfate
SDS-PAGE	SDS Polyacrylamide gel electrophoresis
Tris	Trishydroxymethylaminomethane
U	Unit
V	Volts
V/V	volume/volume
w/v	weight/volume
-W	Yeast selection medium lacking Tryptophan
WT	Wild type
YTH	Yeast two-hybrid

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