THE ROLES OF CASK AND MINT1 IN Ca^{2+} CHANNEL CLUSTERING AND FUNCTION IN BOVINE CHROMAFFIN CELLS

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Abstract

The kinetics of exocytotic secretion depend not only on the spatial relationship between calcium channels and the exocytotic apparatus, but also on the total amount of Ca^{2+} influx through Ca^{2+} channels, the free Ca^{2+} around the release site and the filling state of the release-ready vesicles. These factors may differ between neurons and endocrine cells. Bovine chromaffin cells (BCCs) are neuroendocrine cells responsible for catecholamine release from the adrenal glands. Ca^{2+} imaging experiments have shown that localized zones of Ca^{2+} influx exist on BCC membranes, but how different Ca^{2+} channel subtypes are distributed, and the mechanisms by which they are targeted, remain to be elucidated. CASK (calcium, calmodulin associated serine kinase) and Mint1 (Munc-18-interacting protein 1), which are modular adaptor proteins involved in synaptic targeting, have recently been found to function in targeting of α_{1B} Ca^{2+} channels in hippocampal neurons. These data led to the proposal that Ca^{2+} channels are clustered in BCCs and that CASK and Mint1 play important roles in targeting and/or anchoring channels to their proper location.

Using RT-PCR and Western blotting, CASK is demonstrated present in isolated BCCs. Mint1 is shown to be present by Western blotting as well. Immunocytochemical experiments and experiments in which BCCs were transfected with plasmids expressing α_{1A}, α_{1B}, and α_{1C} subunits labeled with green fluorescent protein, have shown that α_{1A} and α_{1B} subunits are clustered on the plasma membranes of BCCs, while the α_{1C} subunit is distributed in diffuse patches. With immunoprecipitation, it was determined that CASK interacts biochemically with α_{1A} and α_{1B} Ca^{2+} channels. Transfection of BCCs with NC3-GFP, which codes for the sequence of the α_{1B} Ca^{2+} channel that interacts with CASK and Mint1, results in a punctate pattern of fluorescence, which is consistent with the binding

of GFP labeled peptide to complexes of CASK and Mint1 at sites of release. Furthermore, immunocytochemical analysis of cells transfected with NC3-GFP showed that α_{1B} Ca^{2+} channels have a dispersed distribution suggesting that they have been displaced from the binding sites. These data suggest that CASK and Mint1 are important in clustering and targeting Ca^{2+} channels in the BCC plasma membrane. This study is the first to show the existence and function of CASK and Mint1 in BCCs, and may contribute to our understanding of the exocytotic process in neuroendocrine cells.

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List of Abbreviations

ACCs: adrenal chromaffin cell

AP: action potential

APP: amyloid precusor protein

BAPTA: 1, 2-bis- (o-aminophenoxy) ethane-N, N, N′, N′-tetra-acetic

BCCs: bovine chromaffin cells

bp: base pairs

BSA: bovine serum albumin

Ca^{2+}: calcium

CAPSO: N-Cyclohexyl-2-hydroxy-3-aminopropanesulfonic acid

CASK: calcium/calmodulin-dependent serine protein kinase

CCs: chromaffin cells

CNS: central nervous system

DBH: dopamine-beta-hydroxylase

DHP: dihydropyridine

DMEM: Dulbecco's Modified Eagle's Medium

ECL: enhanced chemiluminescence

EGFP: enhanced green fluorescent protein

EGTA: ethylene glycol tetra acetic acid

EPSP: excitatory post-synaptic potential

ER: endoplasmic reticulum

FCS: fetal calf serum

GFP: green fluorescent protein

GUK: guanylate kinase

HVA: high voltage activated

kDa: kilo daltons

LDCV: large dense core vesicles

LVA: low voltage activated

MAGUKs: membrane-associated guanylate kinases

Mint1: Munc-18-interacting proteins

MPC: mouse pheochromocytoma

ms: milliseconds

nm: nanomter

NMDA: N-methyl-D-asparate

NSF: N-ethylmaleimide-sensitive fusion protein

PBS: phosphate-buffered saline

PBST: phosphate-buffered saline transfer buffer

PSD: post synaptic densities

PTB: a phosphotyrosine binding

RT-PCR: Reverse Transcription Polymerase Chain Reaction

SCG: superior cervical ganglion

SDS-PAGE: sodium dodecyl sulphate–polyacrylamide gel electrophoresis

SH3: Src homology3

SNAP-25: synaptosomal associated protein of 25kDa

SNARE: Soluble N-ethylmaleimide-sensitive factor attachment protein receptor

sRNAi: short RNA interference

T-box: transcriptional factor

TH: Tyrosine hydroxylase

TIRF: total internal reflection microscopy

t-SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor

μm: micrometer

μM: micromolar

VAMP: vesicle associated membrane protein

VDCCs: voltage dependent calcium channels

v-SNARE: vesicular soluble N-ethylmaleimide-sensitive factor attachment protein receptor

1. Introduction

Exocytotic release of neurotransmitters and hormones is central to intercellular communication and synaptic transmission. Before a vesicle can fuse with the plasma membrane, it needs to be filled with neurotransmitter, translocated to and docked at the membrane, primed for release, and finally undergo exocytosis upon arrival of a Ca^{2+} trigger (Zimmermann, 1990; Parnas and Parnas, 1994; Morgan, 1995; Matthews, 1996; Cuchillo-Ibanez et al., 2002)The release is mediated by voltage dependent Ca^{2+} channels (VDCCs). The secretion kinetics depends on the distribution of VDCCs as well as the spatial relationship between VDCCs and the exocytotic apparatus. This relationship may differ between neurons and endocrine cells (Atlas et al., 2001; Cuchillo-Ibanez et al., 2002). Neurons and endocrine cells possess two distinct types of secretory vesicles: the small synaptic vesicles and the large dense core vesicles (LDCV) or granules that undergo Ca^{2+} dependent release. The density of small synaptic vesicles (≈50 nm) has been shown high in the active zones in neurons where the concentration of Ca^{2+} can exceed 100 micromolar (μM) when exocytosis happens. The release of LDCV or granules (≈200 nm) by neuroendocrine cells displays different Ca^{2+} dependence than found in neurons: the peak Ca^{2+} concentration at release sites during short depolarizations is lower than 10 μM (Barclay et al., 2005). The time constant for the exocytosis of small vesicles in synapses is reported to be 0.2-1.2 milliseconds (ms) while in neuroendocrine cells, adrenal chromaffin cells (ACCs), it is 150-1000 ms (Chow et al., 1994; Cremona et al., 2004). It is generally accepted that the fast release in synapses is dependent on a tight association between Ca^{2+} channels and the exocytotic machinery, while the slow release of hormone in endocrine cells is dependent on the global build-up of Ca^{2+} influx through

dispersed Ca^{2+} channels (Mansvelder and Kits, 2000). So it was first indicated secretion occurs with a significant delay after short step depolarization in BCCs and the neuron-like excitation-secretion coupling in neuroendocrine cells is neither possible nor necessary (Chow et al., 1992).

However, later on, by applying three kinds of stimulations (Ca^{2+} dialysis, rapid Ca^{2+} influx via VDCCs and the slow release of Ca^{2+} from intracellular stores), investigators used bovine chromaffin cells (BCCs) as a model to study time-resolved, stimulus-evoked Ca^{2+} dependent exocytosis release in neuroendocrine cells (Walker et al., 1996). They found that these neuroendocrine cells display two phases of secretion, a small amplitude fast phase followed by a gradually diminishing slow release phase (Heinemann et al., 1993). Capacitance recordings from single chromaffin cells in mouse adrenal slice have revealed a very fast exocytotic component (time constant 10 ms) in response to short depolarization (Moser and Neher, 1997). One possible explanation is that there is a tight molecular coupling of Ca^{2+} channels with the release sites. Another is that Ca^{2+} influx occurs through clusters of Ca^{2+} channels and that release is occurring preferentially near such sites (Atlas et al., 2001). These findings indicate that there should be a local large increase of Ca^{2+} after stimulation in neuroendocrine cells instead of the global build up of Ca^{2+}. But insight into the mechanisms by which local large increases in Ca^{2+} occur beneath the plasma membrane is still limited.

Different Ca^{2+} channels have been characterized in BCCs using pharmacological, molecular biological and electrophysiological approaches (Santana et al., 1999). Different channel types may display distinct efficacies in evoking release. This leads to several questions: What subtypes of Ca^{2+} channels are involved in the release process? How are they distributed? Are they clustered? How are Ca^{2+} channels targeted to exocytotic

locations in BCCs? What proteins are involved? This study attempts to answer these questions to contribute to a better understanding of the basic mechanism for exocytosis in neuroendocrine systems.

1.1. Ca^{2+} influx triggers exocytosis in BCCs

The idea that Ca^{2+} plays the crucial role in triggering the release of neurotransmitters and hormones by exocytosis was provided by Douglas (Douglas, 1981). This idea has been supported by recent advanced technologies, such as the use of Ca^{2+}-sensitive fluorescent indicator dyes in intact chromaffin cells to follow the stimulus-induced changes in internal Ca^{2+} (Cheek et al., 1989). By this technique, another study also provided evidence that after stimulation, internal Ca^{2+} increases to 300-1000 nM from the resting 10-100 nM (Kits and Mansvelder, 2000). The use of video-imaging techniques made it possible to visualize the stimulus-induced changes in the concentration of cytosolic Ca^{2+} in Fura-2- loaded chromaffin cells (Cheek et al., 1989). Moreover, the whole-cell patch-clamp coupled with flash photolysis technique, provided evidence that the entry of external Ca^{2+}, rather than the release of Ca^{2+} from intracellular stores, is the vital trigger for secretion from the BCCs (Neher and Zucker, 1993).

1.2. Ca^{2+} channel structure and function

1.2.1. Subunits, structure, biochemical characteristics and classification

VDCCs in neurons are heteromeric complexes consisting of different subunits: α_1,

β, α_2, δ and γ (Randall and Benham, 1999). VDCCs contain the voltage sensor in the α_1 subunit. The α_1 subunit folds up to form the ion selective pore of the channel and confers most of the pharmacological properties. The α_1 subunit consists of a single polypeptide chain with four homologous transmembrane domains (I-IV) linked by intracellular hydrophilic loops of various lengths. Domains I, III, and IV contribute strongly to the voltage dependence. Domain I is mainly responsible for channel activation kinetics (Li et al., 2004; Li et al., 2005). Each domain contains six α-helical transmembrane regions (S1-S6). The S4 region, the fourth membrane-spanning α-helical region, is positively charged and forms part of the voltage sensor with two additional domains between S5 & S6 forming the lining of the ion-conducting pore. The structure of Ca^{2+} channel subunits is depicted in figure 1.1.

The amino acid sequences of all cloned subunits share overall structural features. The homologous domains exhibit a high degree of sequence conservation among subtypes. However, the cytoplasmic loops linking the domains are highly divergent. The α_1 subunit therefore carries the basic fingerprint of the channel.

Based on electrophysiological and biophysical properties, VDCCs were first categorized into five major groups: L-, N-, P/Q-, T-, and R-types (Randall and Benham, 1999). They can be further divided into two classes depending on their voltage dependence. The family of high-voltage-activated (HVA) Ca^{2+} channels activate at more positive membrane potentials and include L-, N- P/Q-, and R-types (Catterall, 2000). The low-voltage-activated (LVA) Ca^{2+} channels, which typically activate at fairly negative potentials (−70 to −50 mV) are termed T-type Ca^{2+} channels (Lacinova et al., 2000).

The Ca^{2+} channels were also characterized by their pharmacological properties (Catterall, 2000). The L-type Ca^{2+} channels are sensitive to 1, 4-dihydropyridine (DHP);

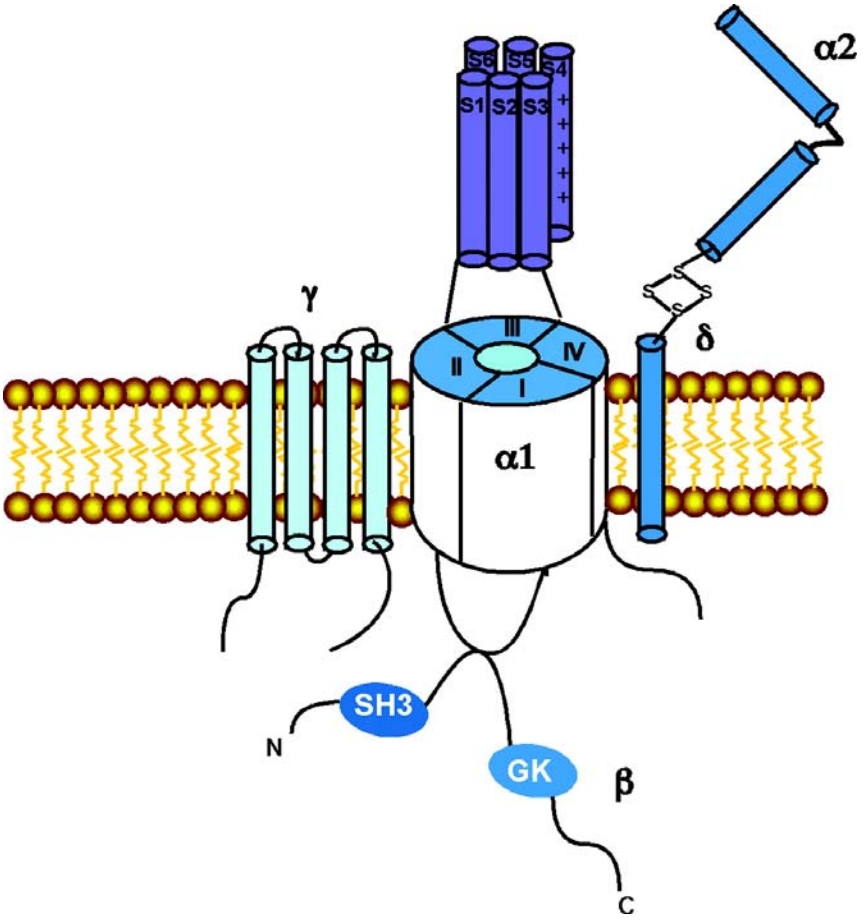

Figure 1.1. Subunit composition of VDCCs

Schematic representation of the subunits of voltage-dependent calcium channels. Inset within the α_1-subunit represents the six-transmembrane domains found in each of the four repeats of this 24 transmembrane pore-forming subunit. The Src homology domain 3 (SH3) and guanylate kinase domain (GK) are shown in the β-subunit (Strock and Diverse-Pierluissi, 2004).

N-type Ca^{2+} channels are sensitive to ω-conotoxin GVIA and P/Q- type Ca^{2+} channels are sensitive to ω-agatoxin IVA. R-type Ca^{2+} channels were so named because of their resistance to all the blockers of L-, P/Q- and N-type channels (Zhang et al., 1993; Yu and Shinnick-Gallagher, 1997). Later on, the genes coding for these channels were cloned. Until now, 10 distinct genes coding for α_1 subunits have been cloned and expressed (Fisher and Bourque, 2001). They were classified into three different subfamilies according to their genetic homology. The Ca_v1 subfamily is formed by four members, $Ca_v1.1$-$Ca_v1.4$, originally called α_{1S}, α_{1C}, α_{1D} and α_{1F}, which mediate L-type Ca^{2+} currents. The Ca_v2 subfamily ($Ca_v2.1$-$Ca_v2.3$) includes $\alpha_{1A,}$ α_{1B} and α_{1E} subunits, which mediate P/Q-, N-, and R-type Ca^{2+} currents. The non L-type HVA N- and P/Q Ca^{2+} channels are encoded by α_{1A} ($Ca_v2.1$) and α_{1B} ($Ca_v2.2$). The physiological function of α_{1E} ($Ca_v2.3$), which coded for R-type Ca^{2+} channels is still uncertain, but multiple isoforms of R-type Ca^{2+} channels with distinct pharmacological properties have been reported (Tottene et al., 1996). The $Ca_v3.3$ subfamily ($Ca_v3.1$-$Ca_v3.3$) include channels containing α_{1G}, α_{1H} and α_{1I} that code for LVA T-type Ca^{2+} channels. T-type Ca^{2+} channels have been cloned recently (Catterall et al., 2003). In this thesis, the original nomenclature for different subtypes of Ca^{2+} channels is used (i.e. $\alpha_{1A,}$ α_{1B} and α_{1C} channels). Table 1 shows the classification of Ca^{2+} channels.

VDCCs are formed by complexes of α_1 subunit and other auxiliary subunits, of which β subunits is most important. Four β subunit genes (termed $\beta_{1,}$ $\beta_{2,}$ $\beta_{3,}$ and $\beta_{4,}$ with multiple splice variants) have been identified and their functions have been extensively characterized in heterogenous expression system (Ruth et al., 1989; Dolphin, 2003). The β subunits bind to the α_1 subunit through the I-II loop of the α_1 subunit and modulate the biophysical properties of the channels. Also, the β subunits have been shown to be

Channel gene (new name)		Subunit channel gene	Voltage dependence	Biophysical subtype	Inactivation profile	Antagonists
Ca_v1	$Ca_v1.1$	α_{1S}	high voltage activated (HVA)	L	slow	dihydropyridines
	$Ca_v1.2$	α_{1C}				
	$Ca_v1.3$	α_{1D}				
	$Ca_v1.4$	α_{1F}				
Ca_v2	$Ca_v2.1$	α_{1A}		P	very slow	ω-agatoxin IVA
		α_{1A}		Q	moderate	ω-agatoxin IVA
	$Ca_v2.2$	α_{1B}		N	fast	ω-conotoxin GVIA
	$Ca_v2.3$	α_{1E}		R	fast	SNX-482
Ca_v3	$Ca_v3.1$	α_{1G}	low voltage activated (LVA)	T	very fast	kurtoxin
	$Ca_v3.2$	α_{1H}				
	$Ca_v3.3$	α_{1I}				

Table 1.1. Characteristics and Classification of Voltage-Dependent Calcium Channels

necessary for the functional expression of VDCCs and trafficking channels to the plasma membrane (Pragnell et al., 1994; Dolphin, 2003). The I-II loop of α_1 subunit contains a reticular retention signal that restricts its incorporation to the plasma membrane. In addition, this sequence is antagonized by the β subunit, releasing the α_1 subunit from the endoplasmic reticulum (ER) and facilitating the incorporation of this subunit to the plasma membrane (Hanlon and Wallace, 2002). This property suggests that an overexpression of a construct containing the loop I-II from an α_1 subunit would act as a lure sequestering the endogenous β subunit and altering its intracellular function. Besides the role in channel complex targeting, the Ca^{2+} channel β_4 subunit by itself contains structural information in its N- and C-termini that results in transporting and clustering of the β_4 subunit to and at presynaptic sites (Wittemann et al., 2000).

The α_2 and δ are products of the same gene, and arise by post-translational proteolytic cleavage. The $\alpha_2\delta$ subunits, which are disulfide-linked subunit dimers, play a function in regulating the properties of Ca^{2+} channels, including inactivation and Ca^{2+} targeting (Brickley et al., 1995; Gurnett et al., 1996; Gao et al., 2000). However, less is known about the function of $\alpha_2\delta$ subunits than about the role of the β subunits due to the difficulty in defining the large extracellular interaction sites of $\alpha_2\delta$ subunits (Pragnell et al., 1994).

The γ subunit is a transmembrane protein (Chu et al., 2001). The function of this subunit is still under study.

1.2.3. Ca^{2+} channels localization and function

VDCCs are main sources of Ca^{2+} entry into the cytosol of cells and are central to the function of excitable cells as well as nonexcitable clells (Penner and Neher, 1988).

They contribute much to the generation of electrical signaling and the highly regulated mechanisms that deliver Ca^{2+} into specific intracellular locations for a wide variety of Ca^{2+} dependent physiological functions, including excitation-contraction coupling in muscles, hormone release in glands, acrsome reaction in sperms, neurotransmitter release, neuronal excitability (by activation of Ca^{2+} dependent currents), neurite outgrowth, and other Ca^{2+}-dependent processes, such as activation of Ca^{2+}-dependent enzymes and second messenger cascades (Morgan and Curran, 1988; Bezprozvanny et al., 1995; Dunlap et al., 1995; Bito et al., 1997; Bergsman and Tsien, 2000).

The different physiological functions of the VDCCs indicated that various Ca^{2+} channel types with specific biophysical properties and potential for modulation have to be located in specialized subcellular locations (Fisher and Bourque, 2001). VDCCs show a highly non-homogeneous distribution pattern in many cells types, including neurons and other secretory cells. In each secretion system, one or more subtypes of Ca^{2+} channels are responsible for mediating Ca^{2+} influx to trigger release. In neurons, the VDCCs subtypes are differentially distributed between cell bodies, dendrites and presynaptic terminals (Westenbroek et al., 1992; Day et al., 1996). The initial proposal that Ca^{2+} channels exist in the nerve terminals came from electrophysiological experiments on the squid giant synapse (Augustine et al., 1985; Llano and Bookman, 1986).

The α_{1A}, α_{1B} and α_{1E} types are found predominantly in the central and peripheral nervous systems and have major roles in controlling neurotransmitter release. Studies have shown that the α_{1A} and α_{1B} type channels are essential to provide Ca^{2+} for neurotransmitter release, either individually or in combination with each other (Turner et al., 1993; Wheeler et al., 1994), indicating a presynaptic localization for these channels. α_{1B} channels have been suggested to play a dominant role in synaptic transmission in

some neurons and have been therefore detected in various presynaptic terminals. In goldfish retinal bipolar synaptic terminals, the only Ca^{2+} current is the α_{1B} type Ca^{2+} channel (Heidelberger and Matthews, 1992). Compared to α_{1B} type Ca^{2+} channels, α_{1A} Ca^{2+} channels are concentrated in a larger number of presynaptic nerve terminals implying a more prominent role in neurotransmitter release at many central synapses (Lin and Lin-Shiau, 1997). Evidence thus points to the α_{1A} type channel as the most prominent in many different nerve terminals, with α_{1B} type also playing a very significant role. α_{1E} type Ca^{2+} channels are distributed on proximal dendrites and may participate in neurotransmitter release in some neurons (Hanson and Smith, 2002).

In contrast, there is no evidence that the α_{1C} and α_{1D} Ca^{2+} channels are involved in transmitter release. Instead, they have been implicated in regulating gene expression and Ca^{2+} dependent enzymes (Bading et al., 1993; Hell et al., 1993; Deisseroth et al., 1998). They are typically found in cell bodies and proximal dendrites with significant staining of distal dendrites (Bito et al., 1996). They have been shown to be localized electrophysiologically and immunocytochemically in these regions (Hell et al., 1993; Pearson et al., 1995; Melliti et al., 1996). α_{1C} type Ca^{2+} channels are central to excitation-contraction coupling and found in neuronal, endocrine, cardiac, smooth, and skeletal muscles as well as fibroblasts and kidney cells. α_{1C} channels have also been shown to play a role in enzyme activity in cortical and hippocampal neurons and long term potentiation and in the release of dynorphin from hippocampal granule cell bodies (Song et al., 1996; Borroni et al., 2000). In comparison, the α_{1D} subunit was observed to be generally distributed on the surface of cell bodies and proximal dendrites with little or no staining of the distal dendrites (Hell et al., 1993).

$\alpha_{1G,}$ α_{1H} and α_{1I} type Ca^{2+} channels have not been detected in presynaptical

terminals and thus may not play a role in synaptic transmission (Momiyama and Takahashi, 1994). They are localized to the soma and dendrites where they are involved in pacemaker activity, such as sleep/wakefulness regulation, membrane voltage oscillation, motor coordination and neuronal circuit specification during ontogenesis (Llinas, 1988).

In neurons and other polarized cells, VDCCs must be sorted to the proper location on membrane domains for the proper functioning of the cell. The correct trafficking and localization of VDCCs is very important for the normal functioning of cells. So specific sorting and transport mechanisms exist to transport the channels to their site of action and then precisely anchor the channel complex at the somata, dendritic spines or presynaptic terminals (Dolphin, 1998). Studies showed that there is a polarized distribution of VDCCs when expressed in a polarized epithelial cell line, and the accessory β subunits contributed to the polarized distribution of α_1 subunits (Brice and Dolphin, 1999). Endocrine cells (such as pancreatic β cells) and neuroendocrine cells (such as BCCs) are not polarized. Channel localization may, however, still be important. Functions of Ca^{2+} channel subtypes are varied between synaptic and non-synaptic release. In many endocrine cells, like pancreatic β cells, there is a long delay between stimulation and hormone release. α_{1C} Ca^{2+} channels are predominant and the release is mainly mediated by α_{1C} type Ca^{2+} channels (Bokvist et al., 1995). Secretion kinetics from neuroendocrine cells is intermediate between neurons and endocrine cells.

At intermediate distances, molecules with fast Ca^{2+} binding kinetics, like 1, 2-bis-(o-aminophenoxy) ethane-N, N, N′, N′-tetra-acetic (BAPTA), are more efficient in reducing [Ca^{2+}] than those with slow kinetics, like ethylene glycol tetra acetic acid (EGTA). So such buffers are often added to the intracellular milieu to make inferences

about the spatial relationship between Ca^{2+} channels and the targets of a Ca^{2+} signal. The Neher group showed that BAPTA hinders release much faster than EGTA does in BCCs and there is a fast release phase in the chromaffin cells by capacitance measurement (Moser and Neher, 1997). This suggested that the vesicles are located in close proximity to Ca^{2+} channels. But how the Ca^{2+} channels are distributed in BCCs and the mechanism underlying their distribution are questions remaining to be elucidated.

1.3. The process of Ca^{2+} dependent exocytosis involves multiple proteins

1.3.1. Ca^{2+} dependence of exocytosis

In neurons, Ca^{2+} dependent processes such as transmitter release, require very high concentrations of influx Ca^{2+} for triggering (Zucker, 1993). When the action potential (AP) invades, it opens VDCCs that are enriched near the active zone. Large and localized Ca^{2+} elevations are thought to occur only in the immediate vicinity of Ca^{2+} channels (Neher and Almers, 1986b, 1986a; Augustine et al., 1992).

The immediate proximity of Ca^{2+} channels with the exocytotic machinery has been shown to be crucial in fast synaptic transmission (Benfenati et al., 1999). The high speed and precise targeting of release are achieved by the organization of the presynaptic active zones, which brings together vesicles, docking and release machinery and Ca^{2+} channels in molecular proximity (Sudhof, 1995). Dramatic progress has been made in the identification of the proteins involved and this has facilitated the understanding of the process of Ca^{2+} dependent exocytotic secretion. Most of the molecular players that

orchestrate the formation, targeting, docking, and fusion of secretory granules have been identified in chromaffin cells. In particular, a number of proteins have been shown to associate with certain Ca^{2+} channels and have roles in the localization or clustering of Ca^{2+} channels.

1.3.2. The SNARE hypothesis

The soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins (SNARE) proteins are probably the most studied proteins that interact with Ca^{2+} channels (Hay, 2001). The SNARE hypothesis proposed that neurotransmitter-loaded secretory vesicles fuse with presynaptic membranes in ms by zippering v-SNAREs on vesicle membranes and t-SNAREs on target membranes into a 4-helix coiled structure. SNARE proteins forming the core of the machinery for intracellular membrane fusion are of crucial importance in exocytosis (Poirier et al., 1998; Gerst, 1999).

1.3.2.1. The SNARE family and function in exocytosis

The importance of the SNARE proteins in synaptic transmission has been studied widely (Pevsner et al., 1994; Sheng et al., 1994; Mochida et al.; Bergsman and Tsien, 2000; Voets et al., 2001; Mochida et al., 2003; Spafford et al., 2003). SNAREs are composed of syntaxin, synaptosomal associated protein of 25kDa (SNAP-25), and vesicle associated membrane protein (synaptobrevin/VAMP). Syntaxin and SNAP-25 reside in the plasma membrane, whereas synaptobrevin is present on the synaptic vesicle membrane. These proteins mediate intracellular membrane fusion through the formation of rod-shaped helical bundles that span opposing membranes. The formation of the complex, composed of the cytoplasmic parts of all three SNAREs, is followed by the interaction of the complementary transmembrane domains of syntaxin and synaptobrevin,

finally mixing membranes and cause them to fuse. The SNARE proteins are implicated in the final steps of vesicular exocytosis, including vesicle docking and fusion.

1.3.2.2. SNAREs interaction with Ca^{2+} channels

1.3.2.2.1. Ca^{2+} channel modulation by SNARE proteins

Retrograde signaling from the SNARE protein complex also modulates Ca^{2+} channel function. The presence of either syntaxin1A, syntaxin1B or SNAP25 resulted in decreased channel availability because of a hyperpolarizing shift in the voltage-dependence of inactivation of transiently expressed and native α_{1A} and α_{1B} channels (Bergsman and Tsien, 2000; Jarvis et al., 2000; Jarvis and Zamponi, 2001; Berlanga et al., 2004). It was found that coexpression of syntaxin with α_{1A} or α_{1B} channels in *Xenopus* oocytes caused a change in the voltage dependence of inactivation of the channels (Bezprozvanny et al., 1995). Wiser Adam and coworkers found by overexpression of synaptotagmin, that there is an inhibition of Ca^{2+} channel expression and modulation of voltage dependence of the inactivation of channels (Trus et al., 2001). In addition, deletion of the II-III loop (synaptic protein interaction site) results in a decrease in the level of α_{1A} in presynaptic terminals (Mochida et al., 2003).

1.3.2.2.2. The association between Ca^{2+} channels and synaptic proteins is of critical importance in neurotransmitter release

The idea that the interaction between Ca^{2+} channels and SNARE proteins is the primary mechanism in Ca^{2+} channels targeting and neurotransmitter release has been supported by a series of studies.

Association of α_{1B} Ca^{2+} channels with synaptotagmin was first demonstrated in

immunoprecipitation experiments (Li et al., 1995; Wiser et al., 1996). Synaptotagmin, functions as a Ca^{2+} sensor for fast, Ca^{2+} dependent neurotransmitter release. The role for synaptotagmin in synaptic vesicle docking/fusion has been suggested based on its interaction with α_{1B} type Ca^{2+} channels (Koh and Bellen, 2003). Synaptotagmin is present on synaptic vesicles and also on chromaffin and other endocrine granules. This suggests that it may regulate exocytosis of both vesicle classes (Littleton and Bellen, 1995).

It was first discovered that the interaction of α_{1B} Ca^{2+} channels with SNARE proteins through the synprint site (the synaptic protein interaction site) also may have direct functional effects on the release process (Mochida et al., 1996). Injection of rat synprint peptide, which mimics the synprint site, into presynaptic superior cervical ganglion (SCG) neurons to compete with the endogenous channel for the synaptic proteins, resulted in disruption of the coupling with the secretory apparatus and block all of release. The injection of synprint peptide resulted in a 50% decrease in the excitatory post-synaptic potential (EPSP) (Mochida et al., 1996). Studies identified that α_{1A} and α_{1B} type Ca^{2+} channels may interact specifically with syntaxin through a synprint site, a binding site in the intracellular loop connecting domain II and domain III (Sheng et al., 1998). This site is unique to vertebrate α_{1A} and α_{1B} channels, and the corresponding regions of other types, such as α_{1C} and α_{1E}, do not associate with SNAREs *in vitro* (Sheng et al., 1994). Furthermore, an α_{1B} Ca^{2+} channel mutant, in which the synprint site was deleted, were transfected into the mouse pheochromocytoma (MPC) cell line 9/3L, and it was found that the release was much decreased. MPC is a cell line that lacks endogenous VDCCs, but it has the machinery required for rapid exocytosis. These studies showed that the synprint site is necessary for efficient coupling of Ca^{2+} influx through α_{1B} containing Ca^{2+} channels to exocytosis (Harkins et al., 2004). More direct evidence for

functionally significant interaction with presynaptic Ca^{2+} by syntaxin comes from studies in which cleavage of syntaxin and SNAP-25 via botulinum toxins disrupts the coupling of the α_{1B} and α_{1C} type channels with syntaxin 1A and abolishes exocytosis in a *Xenopus* oocyte expression system (Trus et al., 2001). These studies suggest that the association between Ca^{2+} channels and the SNAREs is crucial in Ca^{2+} channel targeting and neurotransmitter release.

However, a recent study by Spafford and coworkers challenged the idea that SNARE proteins are necessary for Ca^{2+} channel targeting. Cloning of the α_{1A} and α_{1B} Ca^{2+} channels in invertebrate *Lymnaea* neurons which lack the synprint site and are unable to bind to synaptic proteins. However, they still exhibit normal rapid transmitter release. Therefore, synaptic transmission in *Lymnaea* neurons occurs independently of a physical interaction between presynaptic Ca^{2+} channels and SNARE proteins (Spafford et al., 2003). This suggests that there might be other proteins important in neurotransmitter release. Two other proteins, CASK and Mint1, were shown to have a role in Ca^{2+} channels clustering in *Lymnaea* neurons (see below).

1.3.3. The interaction of CASK and Mint1 with Ca^{2+} channels

1.3.3.1. Modular adaptor proteins CASK and Mint1

Membrane-associated guanylate kinases (MAGUKs) are cytoplasmic multi-domain proteins that serve as scaffold proteins at cell junctions and synapses. These proteins are defined by a Src homology3 (SH3) domain, a domain with homology to the enzyme guanylate kinase (GUK), and a post synaptic densities (PDZ) domain (Baines, 1996; Dimitratos et al.; Butz et al., 1998; Dimitratos et al., 1998; Hsueh et al., 2000; Nix et al., 2000; Zhang et al.; Li et al., 2002; Olsen and Bredt, 2003; Sanford et al., 2004;

Zhang et al., 2005; Gardner et al., 2006). These proteins all have one to three PDZ domains at the N-terminal followed by an SH3 and a GUK domain and these domains have been found to be involved in clustering of certain ion channels and receptors (Blackstone and Sheng, 1999). The protein-binding PDZ domains of MAGUKs are thought to bind the extreme carboxy-terminal cytoplasmic tail of transmembrane proteins in a sequence-specific fashion. They are responsible for the assembly of multiprotein complexes in distinct cellular compartments responsible for signaling pathways (Fanning and Anderson, 1996). Crystallographic analysis revealed that the Ca^{2+} channel β subunit represents a novel member of the MAGUK protein family (Opatowsky et al., 2004; Rousset et al., 2005). Calcium, calmodulin-associated serine/threonine kinase (CASK) is a member of the MAGUK family of proteins. It contains an N-terminal Ca^{2+}, calmodulin-dependent protein kinase-like domain, a C-terminal region that is similar to the intercellular junction proteins, a PDZ, a SH3, and a GUK-like domain. CASK is enriched in brain and interacts with various proteins, e.g. β-neurexin and Veli/LIN7 (Hata et al., 1996). The CASK PDZ domain binds to β-neurexin, a transmembrane protein localized in the presynaptic membrane and mediates the assembly of the presynaptic terminals through the C-terminal tail, thus linking VDCCs into the macromolecular structure (Butz et al., 1998). The many modules that CASK contains mediate protein-protein interactions and form scaffolds for protein networks at cell membranes (Hata et al., 1996). It also stabilizes the integrity of synapses in the brain. Further support for CASK's role in targeting comes from evidence that the network including PDZ domains ensures that membrane proteins are efficiently retained at the cell surface (Hsueh et al., 2000). A recent study shows that CASK is a central protein in a macromolecular complex that participates in trafficking and plasma membrane localization of Kir2 channels

(Leonoudakis et al., 2004). The close spatial positioning of functionally related proteins into a signal transduction complex (figure 1.2, 1.3) allows fast and efficient control of membrane transport processes (Spafford et al., 2003). Additionally, CASK is capable of acting as a transcriptional co-activator by interaction between its GUK domain and T-box transcriptional factor (T-br1) and localizes to neuronal nuclei in the developing brain (Bredt, 2000).

Munc-18 is a syntaxin-interacting protein that is required for synaptic vesicle exocytosis. These proteins are adaptors involved in the regulation of synaptic vesicle release (Hata et al., 1993 J. Pevsner et al, 1994, T. Voets, et al, 2001; Pevsner et al., 1994; Voets et al., 2001). Mint proteins are Munc-18-interacting proteins, first identified in 1997 (Okamoto and Sudhof, 1997). Three mammalian Mint isoforms have so far been characterized (Okamoto et al., 2000). Mint isoforms are either neuron-specific (Mint1 and Mint2) or ubiquitous (Mint3) (Okamoto and Sudhof, 1997, 1998; Biederer and Sudhof, 2000; Okamoto et al., 2000). Mint1 protein is a modular adaptor protein containing a divergent N-terminal sequence that bind to synaptic proteins, such as CASK and Munc18-1, a phosphotyrosine binding (PTB) domain, and two "orphan PDZ domains" that bind to widely distributed proteins such as amyloid precusor protein (APP), presenilins, and Ca^{2+} channels (Biederer and Sudhof, 2000; Ho et al., 2003). Mint1 is most abundant at the active zone and associated with synaptic vesicles in presynaptic terminals (Okamoto and Sudhof, 1997). CASK and Mint-1 also form a tripartite complex with Veli in the presynaptic terminus (Biederer and Sudhof, 2000).

1.3.3.2. The interaction of CASK and Mint1 with Ca^{2+} channels

Maximov et al (1999) were the first to report the interaction of Ca^{2+} channels with CASK and Mint1. They demonstrated that the cytosolic carboxyl terminus of the α_{1B} Ca^{2+} channel binds to the first PDZ domain of Mint1 and the proline-rich region present in the carboxyl termini of α_{1B} binds to the SH3 domain of CASK. They also showed that the long C-terminal isoforms of α_{1A} and α_{1B} Ca^{2+} channels specifically associate with the PDZ domain of Mint1. Figure 1.2. shows the interaction sites of Ca^{2+} channels with other cellular proteins. Furthermore, they proposed a macromolecular signaling complex (illustrated in figure 1.3.) in synapses, which creates the potential for the formation of CASK-Mint1-Ca^{2+} channel ternary complex in presynaptic neurons.

To investigate the role of CASK and Mint1, a peptide, NC3-GFP, coding for amino acids 2021-2039 of the human α_{1B} Ca^{2+} channel was designed (Maximov and Bezprozvanny, 2002). It includes the interaction site with CASK and Mint1. By expressing different constructs of α_{1B} channels in cultured rat hippocampal neurons, they found that the wild type α_{1B} channels were clustered. In contrast, expression of a mutation in the CASK and Mint binding motif of α_{1B} Ca^{2+} channels, resulted in a diffuse distribution. Moreover, overexpression of NC3-GFP disrupted the synaptic clustering of α_{1B} Ca^{2+} channels. They therefore showed for the first time that the targeting of α_{1B} type Ca^{2+} channel to the presynaptic nerve terminals is dependent on the ability of Ca^{2+} channels to bind to CASK and Mint1 (Maximov and Bezprozvanny, 2002). The investigation of the clustering of recombinant α_{1B} channels in hippocampal neurons inspired the present study to determine if CASK and Mint1 play roles in BCCs.

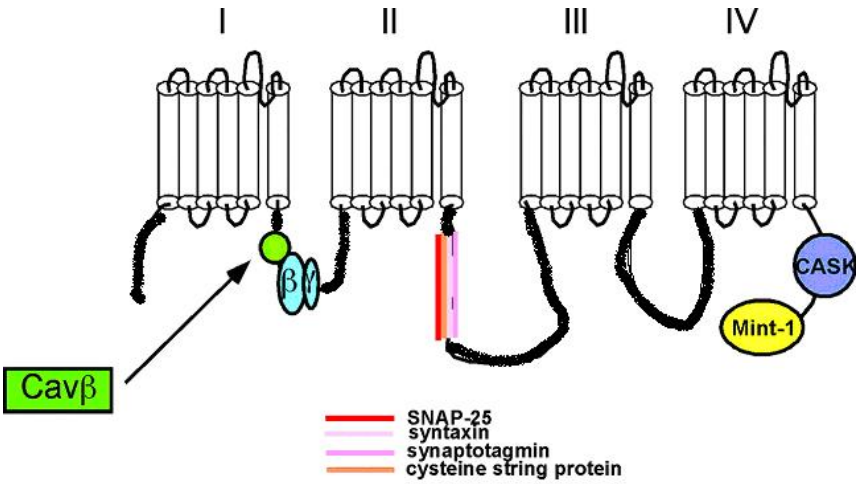

Figure 1.2. Interaction of the α_1-subunit with cellular proteins

Schematic representation of the 24-transmembrane spanning regions of α_{1B} subunit. The Ca^{2+} channel β-subunit and G protein bind to loop I-II. SNAP-25, syntaxin, synaptotagmin, and cysteine string protein bind to loop II–III amino acids 726–984; Mint1 and CASK bind to the C terminus of α_{1B} subunit (Strock and Diverse-Pierluissi, 2004)

Evolutionary analysis of presynaptic Ca^{2+} channels and their ancestors indicated that the Mint1 and CASK binding interaction sites evolved at a much earlier stage than the synprint site (Spafford et al., 2003). In addition, detailed sequence analysis of these channels in cultured molluscan neurons revealed that they all lack the synprint site, while they are still capable of rapid neurotransmitter release (Spafford et al., 2003). This suggests that the ability of the Ca^{2+} channels to bind Mint1 and/or CASK is more fundamentally relevant than the ability to interact with synaptic proteins. The interaction between Ca^{2+} channels and SNAREs is not necessary for transmitter release in invertebrate neurons, which utilizes mechanisms for optimizing Ca^{2+} entry. Therefore, it seems the release occurs independently of a physical association between Ca^{2+} channels and SNARE proteins. (Spafford et al., 2003) also showed that synaptic transmission is abolished following short RNA interference (sRNAi) knockdown of CASK or after the injection of peptide sequences designed to disrupt the Ca^{2+} channel-Mint1 interactions The interaction between Ca^{2+} channels and CASK and Mint1 are therefore functionally important in transmitter release. Their data suggested that Mint1 and CASK serve to localize the non α_{1C} type channels at the active zone.

All of these observations support the idea that CASK and Mint1 might be important in targeting in neurons. This finding significantly advanced the understanding of the mechanisms involved in the exocytosis process in neurons. However, whether CASK and Mint function in targeting in neuroendocrine cells remains unknown.

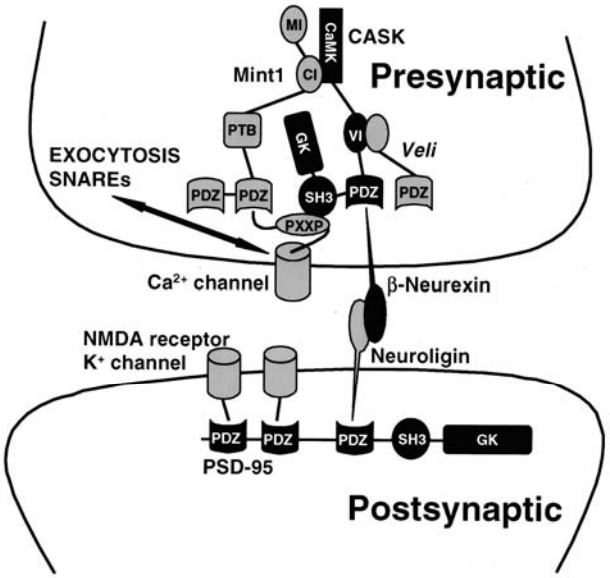

Figure 1.3. The proposed macromolecular structure of VDCC with exocytotic machinery and adaptor proteins CASK and Mint1

Three PDZ domains of postsynaptic density (PSD)-95 bind to the carboxyl termini of K^+ channels, NMDA receptors. Binding of CASK PDZ domain to the cytosolic tail of β-neurexins positions the tripartite complex composed of modular adapter proteins CASK, Mint1, and *Veli* at the synaptic axis. Ca^{2+} channels are recruited to this complex by means of carboxyl-terminal association with Mint1-1 PDZ domain and CASK SH3 domain as described in the present report. The II/III cytosolic loop region of Ca^{2+} channels is involved in interaction with syntaxin and other components of the SNARE complex. The described cascade of molecular interactions results in alignment of fusion-competent synaptic vesicles, presynaptic Ca^{2+} channels, and postsynaptic receptors near a synaptic axis setup by the neurexin-neuroligin association (Maximov et al., 1999).

1.4. BCCs as a model system to study exocytosis

1.4.1. Physiological aspects of BCCs

BCCs derive from the bovine adrenal medulla. They are neuroendocrine cells that secrete catecholamine by exocytosis when needed. These cells, like neurons, fire action potentials and undergo Ca^{2+} dependent exocytosis (Helle and Serck-Hanssen, 1975). They are derived embryologically from the neural crest, sharing a common origin with sympathetic neurons and common subcellular features with many endocrine cells (Aunis, 1998). Some physiologists regard the adrenal medulla as a modified sympathetic ganglion because its secretion is controlled by sympathetic preganglionic fibers (Aunis, 1998). At least three types of chromaffin cells are present in the adrenal medulla: adrenaline cells (85%), noradrenaline cells (14-15%), and cells that only release dopamine (1%). Noradrenaline secreted by chromaffin cells is the same substance secreted as a neurotransmitter by sympathetic neurons and by some neurons in the central nervous system (CNS). Its effects are identical to those of the neurotransmitter but last about ten times longer. Tyrosine is the common precursor for the synthesis of adrenaline, noradrenaline and dopamine. Tyrosine hydroxylase (TH) is the pacemaker of catecholamine synthesis. Dopamine beta hydroxylase (DBH) is a mixed-function oxidase present in chromaffin vesicles that catalyzes the oxidation of dopamine to noradrenaline. Chromogranins are a group of acidic soluble proteins present in the core of catecholamine-storage vesicles (O'Connor and Frigon, 1984). They play a fundamental role in releasing catecholamines and other substances, such as ATP, into the bloodstream. Acute stress causes sympathetic activation and increases catecholamine release many fold, setting the organism into a “fight or flight” state of energy expenditure. Adrenaline floods the bloodstream at 300 times the normal concentration, interacts with cells in

various organs, increasing the heart rate and blood pressure and prompting the release of extra sugar from the liver to fuel muscle work. Under basal conditions, chromaffin cells fire action potentials and release catecholamine at a basal rate set by the sympathetic tone, and contribute to the regulation of the "breed and feed" state of energy storage. In this state, secreted catecholamines regulate homeostatic functions including vascular tone, enteric activity, and insulin release. Physiologically, adrenal catecholamine secretion is driven by activation of nicotinic receptors on the chromaffin cell surface (Aunis and Langley, 1999). Chromaffin vesicles are localized in at least two pools or compartments, the reserve pool that contains the majority of the chromaffin vesicles and the release ready pool that contains 1-4% of the total content under resting conditions (Duncan et al., 2003).

1.4.2. The approaches to monitor the process of exocytosis in BCCs

Exocytosis, the final step in hormone and neurotransmitter release, has been the topic of intense investigation for decades. The introduction of new techniques, such as caged-Ca^{2+} compounds, capacitance recording and amperometric recording, has recently greatly increased our understanding of Ca^{2+} dependent exocytosis.

Among the cell models that have provided insight into the molecular machinery underlying the successive steps of exocytosis, BCCs have taken a prominent place. Some of the advantages of these cells are their spherical shape, large size and large granules. Exocytosis of a single granule can be detected as an abrupt "jump" in membrane capacitance. This measurement traces changes of the cell surface area due to membrane addition by exocytosis of secretory granules. So the exocytotic process can be measured using capacitance recording. Another advantage to use this model is the character of catecholamines. Catecholamines are stored in chromaffin granules at a very high

concentration (0.5-1M). Upon exocytosis, leave the storage vesicles and diffuse into the extracellular space. Catecholamines can be oxidized so that a carbon fiber electrode, maintained at a highly positive potential, can detect it through the generation of an electrical current when the electron is passed from the catecholamine molecules to the fiber electrode (Wightman et al., 1991). Wightman and collaborators developed the technique to the point that release from single cells and single secretory granules can be resolved. This method is called amperometric recording. This technique is so sensitive that release from a single granule can be detected (Graham et al., 2000). The release from single granules can be observed as typical spike-like waveforms of amperometric current (Wightman et al., 1991). These recordings provide considerable information on the release process. The rate at which the spikes occur is a direct measure of the frequency of the exocytotic events occurring at the membrane beneath the electrode.

1.4.3. Indications of Ca^{2+} channel clustering in BCCs

By offering the opportunity to combine the use of recent biophysical techniques allowing single-vesicle resolution and specific biochemical modifications in the protein machinery involved in exocytosis, chromaffin cells remain a powerful model to address new and still open questions in the field of secretion.

1.4.3.1. The heterogeneous distribution of Ca^{2+} entry

The active zone on the presynaptic membrane is essential for synaptic release. Ca^{2+} entry through VDCCs results in a highly heterogeneous distribution of the near membrane Ca^{2+}. Similar Ca^{2+} entry zones or "hotspots" have been identified by recent Ca^{2+} imaging data. Ca^{2+} influx through VDCCs in BCCs is highly localized to a small number of "hotspots".

Pulsed laser imaging is a powerful approach to image rapid changes in Ca^{2+} levels. It overcomes the shortcoming of lacking sufficient spatial and temporal resolution to detect microdomains of high Ca^{2+} by conventional imaging techniques. It is able to offer sub millisecond temporal resolution, as well as spatial resolution of less than a μm. Using this technique, signals were shown to be fast and highly localized in BCCs. Transient opening of VDCCs gave rise to localized elevations of Ca^{2+} that had the appearance of either "hotspots" or partial rings found immediately beneath the plasma membrane (Monck et al., 1994).

Another very promising technique to monitor exocytosis optically with high spatial and temporal resolution is evanescent fluorescence microscopy, or total internal reflection microscopy (TIRF). It is a highly efficient illumination method that illuminates only in a very thin layer (100-300nm), just a few hundred nm above the substrate on which cultured cells are attached (Reichert and Truskey, 1990). It has low fluorescence bleaching and low light toxicity. With this technique, the spike-like changes of fluo-5 (a low-affinity Ca^{2+} indicator) fluorescence as short-lived, submicrometer, near-membrane domains of micromolar (μM) Ca^{2+} resulting from voltage-gated Ca^{2+} influx was monitored in BCCs (Wiegand et al., 2002).

Other evidence comes from immunocytochemical data, in which α_{1B} type Ca^{2+} channels were shown to be clustered on the BCC plasma membrane (Fisher et al., 2000). Another study using bioassay employing Ca^{2+}-activated K^+ channels as Ca^{2+} sensors also demonstrated the existence of microdomains in chromaffin cells (Prakriya et al., 1996). These differences may be relevant for the differential release regulation of each catecholamine under physiological and pathophysiological conditions. This study also found that clustering of Ca^{2+} channels may explain the precise localization of exocytotic

sites in the terminals of neurite-emitting chromaffin cells. By applying Ca^{2+} chelator and by electrophysiology, study also suggest that these vesicles are located in close proximity to Ca^{2+} channels (Moser and Neher, 1997). All these studies suggested that Ca^{2+} influx in a non-homogeneous pattern and Ca^{2+} channels are clustered.

1.4.3.2. Exocytosis occurs at preferential sites of release

Does exocytosis occur evenly or at preferential sites in BCCs? The answer for this question has been provided by different researchers using different techniques.

Combining DBH staining and confocal microscopy, immunofluorescence studies in cultured BCCs giving a distinct punctate pattern of labeling was shown (Hesketh et al., 1981). It reflected the distribution of chromaffin granules and the exocytotic sites, suggesting that exocytosis may occur in specialized area in BCCs. Secretion from cultured BCCs was stimulated to examine the pattern of exocytotic fusion on the plasma membrane. DBH immunofluorescence in intact cells stimulated for 20s with the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium was almost entirely punctate (Wick et al., 1997). In agreement with these data, a preferential deposition of DBH into the neurite terminal plasmalemma after stimulation of intact cells was observed. These studies might be of interest since the differences in the distribution of secretory "spots" between round and neurite-emitting chromaffin cells could be used to study the molecular factors determining active site localization (Gil et al., 2001). This supports that exocytotic release occurs at preferential sites.

Additional support for the idea that exocytotic release occurs at specific area on the BCCs comes from an amperomety study done by Schroeder et al (1994), who mapped exocytotic release site of 2 μm on the cell surface of BCCs .

The tracking of individual granules with TIFR has also indicated that the granules

tend to be docked and fused at specific areas of membrane and that exocytosis is triggered by Ca^{2+} microdomains (Steyer et al., 1997; Becherer et al., 2003).

So these studies all support that the release occurs at preferential sites on the BCC membrane.

1.4.3.3. The Ca^{2+} entry sites colocalize with the release sites

Since both the sites of Ca^{2+} influx and the release sites are heterogeneously distributed, it was of great interest to see if they are colocalized. Previous studies using pulsed laser imaging combined with amperometry recording have shown that the "hot spots" of Ca^{2+} influx on the plasma membrane of BCCs are associated with the preferential sites of exocytotic catecholamine release. They reported overlap of "hotspots" of submembrane Ca^{2+} and secretion in isolated chromaffin cells (Monck et al., 1994; Robinson et al., 1995; Robinson et al., 1996).

By combining Ca^{2+} imaging (as Ca^{2+} influx) with tracking of the movement of single LDCVs (labeled with an acidophilic dye) and amperometric recordings, researchers found the sites of single-vesicle exocytosis were colocalized with the hot-spots of Ca^{2+} entry in BCCs (Becherer et al., 2003).

A very recent research using pulsed-laser Ca^{2+} imaging and membrane capacitance measurement studied the relationship between Ca^{2+} concentration and depolarization-induced exocytosis in adrenal chromaffin cells, showing that Ca^{2+} dependent fusion of vesicles occurs from a small immediately releasable pool (Marengo, 2005). This revealed the colocalization of Ca^{2+} influx with the release sites.

Ca^{2+} microdomains shown on the BCC membranes suggest that the local increase in Ca^{2+} concentration greatly enhances the probability of vesicle fusion and transmitter

release. Furthermore, data showed that the release occurs more rapidly from the resultant hotspots of influx, suggesting that isolated BCCs maintain an organization designed to result in fast release. The colocalization of Ca^{2+} channels and vesicle-fusion zones places the release machinery within domains of high Ca^{2+} in the near vicinity of open Ca^{2+} channels. This is a feature of the rapid release. This indicates a close relationship between the release sites and Ca^{2+} entry site. All the above study leads to the conclusion that the catecholamine release sites are close to the sites of Ca^{2+} entry in BCCs.

1.4.3. Subtypes of Ca^{2+} channels in BCCs

Chromaffin cells are a very powerful system for the study of Ca^{2+}-triggered exocytosis. $\alpha_{1A,}$ $\alpha_{1B,}$ $\alpha_{1C,}$ α_{1D} and α_{1E} Ca^{2+} channels have been found in BCCs. In addition to these Ca^{2+} channels, a recent study revealed the presence of α_{1G} subunits generating low threshold T-type currents in BCCs (Garcia-Palomero et al., 2000). Patterns of Ca^{2+} channel expression in chromaffin cells have been shown to be different in different species (Garcia et al., 1998). As with other chromaffin cells, BCCs possess $\alpha_{1A,}$ α_{1B} and α_{1C} Ca^{2+} channels, which have been identified by patch clamp techniques and various selective blockers.

Channels may to some extent induce differences in coupling efficacy, as in BCCs and mouse pancreatic β cells. Thus, it seems that not all classes of Ca^{2+} channels are necessarily coupled with the same efficacy to exocytosis. Some Ca^{2+} channel subtypes are coupled more tightly with the exocytotic machinery than others (Lara et al., 1998). An α_{1A} blocker omega-agatoxin IVA was able to block 70% of the exocytotic release occurring from the neurites, whereas α_{1C} type blocker furnidipine had a weak effect (Lara et al., 1998). This reveals distinctive functions for α_{1C} type Ca^{2+} channels and non α_{1C}

Ca^{2+} channels.

In summary, Ca^{2+} triggered exocytosis of neurotransmitter or hormone-filled vesicles has developed as the main mechanism of cell-cell communication in animals. Therefore, understanding the mechanism underlying the fast release in BCCs (possibly due to Ca^{2+} channel clustering) is critical in facilitating our understanding of exocytosis. Studies published to date strongly suggest that there is clustering of Ca^{2+} channels in BCCs, but the direct evidence for the differential distribution of different subtypes of Ca^{2+} channels and the mechanisms by which the channels are targeted and/or clustered are still unclear.

1.5. Hypotheses and Objectives

There is evidence for clustering of certain types of Ca^{2+} channels in BCCs, but our understanding is incomplete. All of the studies showing the heterogeneous patterns of release colocalize with the sites of Ca^{2+}entry suggest that Ca^{2+} channel clustering might be important. The Ca^{2+} channel targeting has been studied extensively in heterogeneous expression systems. While Ca^{2+} channel's proximity to exocytotic proteins and vesicle clusters at active zones in neurons has been inferred from biomedical, histological and ultrastructural data, very little is understood about how different subclasses of channels are differentially targeted in BCCs. Furthermore, direct evidence of the relationship between Ca^{2+} channels with CASK and Mint1 in BCCs is also absent, and the underlying mechanisms involved in clustering are still unknown.

As discussed above, currently unanswered questions include: how are different subtypes of Ca^{2+} channels distributed in BCCs? Are they clustered? If they are, what proteins are involved in targeting and clustering? What are the mechanisms for it? The

goal of this study was to explore these questions.

Based on the previous studies, the following hypotheses have been formulated:

Hypotheses:

(1) Subtypes of Ca^{2+} channels are differentially clustered on the plasma membrane of BCCs.

(2) CASK and Mint1 are present in BCCs and function in clustering and/or targeting Ca^{2+} channels to the exocytotic location.

Objectives:

This work was designed to firstly define the Ca^{2+} channel subtypes expression patterns in BCCs. The localization of VDCC α_1 subunits in cultured BCCs was examined by immunocytochemistry, using antibodies against different α_1 subunits. Also, BCCs were transfected with α_1 subunits fused with GFP to visualize the localization patterns.

To gain insight into the roles of CASK and Mint1 in Ca^{2+} channel clustering and targeting, the following experiments were designed: 1. RT-PCR, Western blotting and immunocytochemistry were used to test whether they are present in BCCs; 2. coimmunoprecipitation experiments were used to test whether they interact with Ca^{2+} channels α_1 subunits; and 3. transfection of NC3-GFP was used to determine if disruption of the interaction between Ca^{2+} channels and CASK and Mint1 prevents clustering.

2. Materials and Methods

2.1. Chemicals and Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and fetal calf serum (FCS) were from Gibco, USA. TRIzol and the Super Script First-strand Synthesis System for RT-PCR were from Invitrogen, USA. Mouse monoclonal IgG anti-CASK and mouse monoclonal IgG anti-Mint1 were obtained from BD Transduction Laboratory, USA. Flurolink^{TM} Cy3 labeled goat anti-rabbit IgG (H+L) was from Amersham Pharmacia Biotech Inc, USA. The goat polyclonal anti-DBH antibody, the rabbit poly-clonal anti-α_{1A}, α_{1B} and α_{1C} Ca^{2+} channel antibodies and anti-syntaxin antibody were purchased from Santa Cruz Biotech Inc, USA. The horseradish peroxidase-conjugated secondary antibodies and ECLTM Western blotting detection reagents were from Amersham Pharmacia, USA. Bovine serum albumin (BSA), collagenase, protease and all other chemicals were from Sigma, USA.

The α_{1A}-GFP, α_{1B}-GFP and α_{1C}-GFP plasmids were gifts from Dr. Manfred Grabner of the University of Innsbruck, Austria. The coding sequences of the α_1 subunits of the cardiac muscle α_{1C} (Gene bank access No: X15539), neuronal α_{1A} (Mori et al., 1991), and neuronal α_{1B} (Fujita et al., 1993) were inserted "in-frame" and downstream of the coding region of a modified GFP, cloned in a proprietary mammalian expression vector. The *Hin*dIII to *Eco*RI segment of the polylinker of pSP72 (Promega) was inserted directly after the last GFP codon (Lys-238). GFP was fused with the N-terminus of each α_1 subunit. The plasmids carry an ampicilin resistance gene. Transcription was under cytomegalovirus (CMV) promoter control and the plasmids may be considered high copy plasmids (Grabner et al., 1998).

NC3-GFP was a gift from Dr. IIya Bezprozvanny of the Medical Center, University of Texas Southwestern, USA. NC3-GFP codes for 2021-2339 amino acid of human α_{1B} carboxyl terminal (Maximov and Bezprozvanny, 2002). The constructs were generated on the basis of the pEGFPC3 vector (Clontech, Palo Alto, CA).

2.2. Experimental Procedures

2.2.1. BCC isolation and culture

Bovine adrenal glands were obtained from a local slaughterhouse where they were freed from adhering fat. The adrenal glands were rinsed with ice cold Kreb's buffer (145 mM NaCl, 5.0 mM KCl, 1.2 mM NaH_2PO_4, 10 mM glucose, 20 mM Hepes, adjusted to pH=7.4) and kept on ice while being transported back to the laboratory. The chromaffin cells were isolated following methods modified from the protocol used in the laboratory of Dr. Julio Fernandez's (Robinson et al., 1995). In brief, glands were first rinsed by injecting Kreb's buffer into the veins of the adrenal glands at room temperature until the solution comes out clear. The glands were then injected with 0.08 % protease (from *Streptomyces griserus*; Sigma P-5147), transferred to a beaker and incubated in a water bath for 10 min at 37°C. Then glands were injected with 0.08 % protease and incubated at 37°C for another 10 min. The medullar tissue was then dissected from the cortex, placed in a Petri dish and minced into fine pieces with a scalpel, transferred to a flask and digested with 0.1% collagenase (Type I; Sigma C-0130) at 37°C with shaking at 120rpm for half an hour. The cells were filtered through four layers of cheese cloth and collected by centrifugation at 1000rpm for 10 min at room temperature. The pellet was resuspended in Kreb's buffer. This cell wash was repeated 4 times by centrifugation at 1000rpm, 400rpm, 400rpm and 400rpm, 10 min each time, all at room temperature. In

some of our experiments, the resuspended BCCs were added dropwise on top of 4% BSA and then centrifuged. This procedure was used to remove the cell debris, which was retained in the supernatant.

Freshly isolated cells were suspended in DMEM supplemented with 10% fetal bovine serum (FBS). Penicillin (100 units/ml) and streptomycin (100 g/ml) were added to prevent bacteria growth and 10 μM 5-fluoro-2'-deoxyuridine (Sigma, USA) was added to prevent fibroblast proliferation. BCCs were plated on poly-L-lysine coated 35mm petri dishes and maintained at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. Viability was determined by trypan blue exclusion and the purity was determined by DBH and syntaxin staining.

2.2.2. Expression of plasmids in BCCs

The transfection protocol was taken, with some modification, from (Wilson et al., 1995). Cells were plated at a density of 4×10^5/ml and after 16 hours, the media was replaced with serum free media and the cells were incubated for a further 1-2 hours. Then the cells were transiently transfected with each GFP fused Ca^{2+} channel α_1 subunit coded plasmids, α_{1A}-EGFP, α_{1B}-EGFP, α_{1C}-EGFP (Grabner et al., 1998) or NC3-GFP (Maximov and Bezprozvanny, 2002) by the Ca^{2+} phosphate precipitation method. The transfection buffer was composed of 280 mM NaCl, 40 mM Pipes, 1.5 mM NaH_2PO_4, pH = 7.1. The procedure involved adding the DNA-Ca^{2+} complex drop wise to the cultured cells. After 4 hours, cells were subjected to glycerol shock (12.5% glycerol in DMEM) for 3 minutes at room temperature. Cultures were subsequently maintained for 48 h in DMEM with FBS, and antibiotics in 5% CO_2 at 37 °C before microscopic analysis. Attempts were made to visualize GFP fluorescence after fixation, but the intensity of the

fluorescence after cold methanol and paraformaldehyde was much decreased.

2.2.3. Immunocytochemistry

To determine if Ca^{2+} channel α_1 subunits are differentially distributed in BCCs, immunocytochemical analysis of isolated BCCs was carried out. The characterization of Ca^{2+} channel distribution was visualized by immunofluorescence with antibodies that specifically recognize the $\alpha_{1A,}$ α_{1B}, α_{1C} and α_{1D} Ca^{2+} channels subunits.

Cultured BCCs were washed with phosphate-buffered saline (PBS) three times, then fixed with methanol at room temperature for 15 min, permeabilized by 0.02% triton X-100 and blocked by 0.3% BSA for 30 minutes at room temperature. Then the fixed cells were washed with PBS and probed with antibodies against α_1 subunits Ca^{2+} channels overnight at 4°C. Anti-α_{1A}, anti-α_{1B}, anti-α_{1C}, anti-α_{1D}, anti-DBH, anti-syntaxin, anti-CASK and anti-Mint1 were used at dilutions of 1:250, 1:250, 1:250, 1:250, 1:200, 1:200, 1:250 and 1:250 respectively. Then the primary antibody was washed out by three successive washes in PBS buffer. The BCCs were incubated with secondary antibodies diluted 1:400 for half an hour, then washed and mounted, examined with a Zeiss imaging invert vision system (Zeiss Axiovert 200 Inverted Microscopy). The primary antibodies of anti-α_{1A}, anti-α_{1B}, anti-α_{1C}, anti-α_{1D}, anti-syntaxin and anti-DBH were detected using Cy3–conjugated secondary antibodies. The primary antibodies of CASK and Mint were detected using Cy2-conjugated secondary antibodies.

After a few days in culture, spherical BCCs develop processes. Therefore, immunocytochemistry was done to determine the distribution of Ca^{2+} channel subunits in differentiated BCCs.

In another part of study, to test the NC3-GFP function on Ca^{2+} channel clustering,

BCCs were transfected with NC3-GFP plasmid. 48 hours after transfection, BCCs were fixed and the distribution of Ca^{2+} channels was studied by immunocytochemistry.

2.2.4. RT-PCR

RT-PCR was performed to determine whether the CASK gene is expressed in BCCs.

2.2.4.1. RNA extraction

Total RNA was extracted from BCCs as follows:

1ml TRIzol reagent was added to 100 µl suspension of BCCs. The mixture was incubated for 5 minutes, then 0.2 ml chloroform and 1.0 ml TRIzol were added. It was then shaked vigorously by hand for 15 sec and incubated for 2 min. Finally, it was centrifuged at 10000 rpm for 15 min at 4°C. The RNA was extracted from the aqueous phase, transferred to a fresh tube, mixed with 0.5ml isopropyl alcohol, incubated for 10 min, then centrifuged for 10 minutes at 4°C. The supernatant was removed and the pellet was washed with 0.75 ml 75% ethanol, vortexed and centrifuged at 7500 rpm for 5 min.

2.2.4.2. cDNA synthesis

The total RNA extracted as above was transcribed to cDNA by SuperScript First-strand Synthesis System and the cDNA was used as a template for amplification in PCR.

2.2.4.3. PCR

3 µl PCR buffer, 0.3 µl 10mM dNTP Mix, 1.2 µl 50mM $MgCl_2$, 0.3 µ l forward primer, 0.3 µl reverse primer, 0.2 µl Taq DNA polymerase (5U/µl), 1.5µl cell cDNA template and distilled water was added to make a final volume of 15µl. It was mixed gently and 2 drops of mineral oil was added on top. It was heated at 94°C for 2 min and

33 cycles of PCR were run to amplify the cDNA. Then 7µl of the PCR product was applied to an agarose gel and eletrophoresis was run at 120 V. The bands were visualized by ethidium bromide staining. To determine the molecular weight of the product, a 1-kb ladder marker DNA (Invitrogen, USA) was used.

Specific primers for CASK were designed and synthesized. Forward primer is GTGGCAGGGTAAACTGGAAA and reverse primer is TGTGTCTTCTCCCAACACCA (University Core DNA Services, Calgary, Canada).

Since the Mint1 bovine gene has not been cloned yet, the Mint1 gene expression in BCCs could not be tested.

2.2.5. Western blotting and detection of proteins

The BCCs were resuspended in lysis buffer and samples were boiled (5 min, 100 °C). BCC membrane proteins were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred electrophoretically to nitrocellulose for 1.5h at 0.9A. The nitrocellulose blots were incubated 1 hour with 5% skim milk. The membranes were then rinsed with PBST buffer and subsequently probed with CASK / Mint1 antibody (diluted 1:2500) at 4°C overnight. Thereafter, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary anti-mouse antibody (diluted 1:5000) for 1h at room temperature. Then the bands were visualized by enhanced chemiluminescence (ECL).

2.2.6. *In vitro* binding immunoprecipitation assays

To prepare the membrane protein extract, the freshly isolated BCCs were centrifuged at 10000 rpm for 10 min to remove the buffer. Then the pellet was resuspended in 5.0 ml Tris buffer without NaCl. After three brief homogenizing by Polytron, 15 seconds each

time, the samples were centrifuged at 17000 rpm at 4°C for 15 min. The pellet was then resuspended in 3.5ml Hepes buffer (pH =7.2), and Laemmli buffer was added and boiled. Then it was solubilized by 1% N-cyclohexyl-2-hydroxy-3-aminopropanesulfonic acid (CAPSO) for 1 hour at 4°C. Following centrifugation of the mixture for 30min at 33000rpm, tre pellet was discarded and the supernatant retained.

The beads were prepared using the following protocol. Protein G Sepharose (50µl) was washed in 500µl PBS and then it was centrifuged at 10000rpm for 3 min. The beads were then resuspended in PBS. Then 5µl of one of the primary antibodies (anti-α_{1A}, anti-α_{1B} or anti-CASK) and incubated at 4°C overnight. The beads were centrifuged for 1 min at 13000 rpm and then washed with PBS twice, followed by centrifugation at 10000 rpm for 3 min and resuspended in an 500 µl PBS.

Immunoprecipitations were performed using a standard protocol. The solubilized membrane protein was incubated with protein G-Sepharose beads at 4°C overnight. The following morning, the membrane-antibody-bead complex was centrifuged at 13000rpm for 1 min and the supernatant was removed and boiled with laemmli buffer and stored as "supernatant". After three washings, the equal volume of washing buffer and laemmli was added, boiled, centrifuged at 10000rpm for 3 min. The supernatant was transferred to a new tube and marked as "bound". Then 20 µl of sample was loaded into each well and Western blotting was run using 10% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes at 0.9A for 1.5 hours. Strips of membrane were probed by antibodies directed against CASK, α_{1A}, or α_{1B} and then incubated with horseradish peroxidase-conjugated secondary anti-mouse antibody (for CASK) or anti-rabbit antibody (for α_{1A} or α_{1B}). The bands were visualized by ECL.

3. Results

3.1. Identification of BCCs---DBH staining of BCCs

Chromaffin vesicles contain not only the end products contents (material for export), but also enzymes involved in catecholamine synthesis (e.g. dopamine-beta-hydroxylase, DBH, tyrosine hydroxylase). DBH is therefore a chromaffin vesicle marker, so a polyclonal antibody against DBH was used in our study to identify the BCCs. We followed the protocol from Dr. Fernandez's lab with some small modifications for isolating BCCs (Robinson et al., 1995), which was reported to yield a purity of greater than 95%. Although we didn't quantify the purity of our cultures, the proportions of BCCs appeared similar to the reported 95%.

Figure 3.1. (top row) illustrates typical DBH immuno positive cells. Immunochemical analysis revealed the presence of DBH in the vast majority of cultured BCCs, suggesting very low contamination with other types of cells, such as adrenal cortical cells, fibroblast cells and parenchymal cells. In addition, it is easy to differentiate BCCs from other cells by morphology. The results clearly indicate that highly purified BCCs can be obtained and identified by the isolation method used.

Since BCCs derive from the neural crest, they possess syntaxin, which is a member of SNARE proteins and has been shown to be involved in the final step of exocytosis (Schiavo et al., 2000). In contrast, the other cells that might come with the isolation, like cortical cells and fibroblasts, are not neuronal cells, so they do not possess syntaxin. Therefore, an antibody against syntaxin was used to further identify BCCs. Figure 3.1 (middle row) demonstrates the syntaxin staining of BCCs. Syntaxin is localized more intensely on the plasma membrane (indicated by arrows). As we can see,

the majority of the cells are stained with syntaxin1, which indicates that the vast majority of the cells are BCCs. It seems that the staining of syntaxin is clearer in the junction between the cells. However, we don't know the reason for this.

The control image (bottom row of figure 3.1.) shows cells that were not treated with any primary antibody, but only with a secondary antibody. The exposure time for taking the picture was longer than both of the above images. These cells didn't show any staining. These results are typical of two experiments. It suggests that the antibody-antigen bindings are specific for DBH and syntaxin.

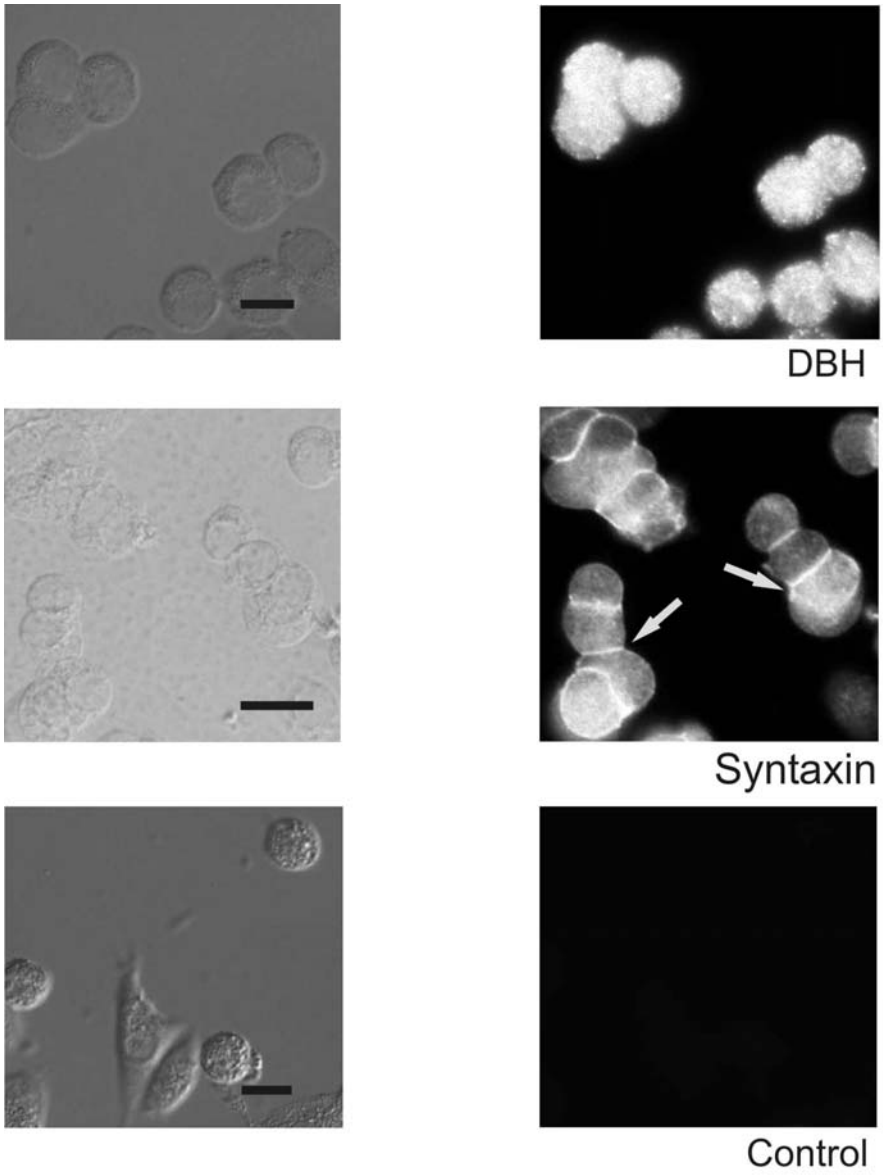

Figure 3.1. Identification of BCCs in primary culture using antibodies directed against dopamine beta hydroxylase (DBH) and syntaxin

The panels in the left column are phase contrast images and the panels in the right column are fluorescence images of isolated BCCs. The right panel of the top row shows the DBH staining. DBH is homogenously distributed throughout the cells with some spots which are likely to reflect individual granules. The right panel of the middle row is the syntaxin staining. The arrows show the distribution of the syntaxin1. Syntaxin is mainly distributed on the membrane of BCCs. Scale bars are 10 μm.

3.2. α_{1A} and α_{1B} Ca^{2+} channels are clustered on plasma membrane of BCCs

To understand the functions of Ca^{2+} channels subtypes in BCCs, it is necessary to know how they are distributed within the cells. The specific localization of VDCC α_1 subunits in BCCs was determined using antibodies against different subtypes of Ca^{2+} channels by immunocytochemistry and was also confirmed by transfection with α_1 subunits fused with GFP plasmids.

The use of two independent methods to determine the distribution of Ca^{2+} channels is important because transfection avoids possible artifacts due to the immunocytochemistry procedure or the process of isolation of cells. Antibodies might precipitate to form clumps in immunocytochemistry and this won't happen in transfection. When the BCCs were isolated in situ, the Ca^{2+} channels adhering to the plasma membrane at sites of presynaptic inputs might come together with the isolated BCCs. So these channels that will show fluorescence by immunocytochemistry, but not with transfection methods. Also, the development of a protocol for Ca^{2+} channels transfection is important for the study of other related exocytotic molecular mechanisms.

3.2.1 The Ca^{2+} channel distribution in spherical cells revealed by immunocytochemistry

The cultured BCCs were fixed and incubated with antibodies against $\alpha_{1A,}$ α_{1B} and α_{1C} Ca^{2+} channels. They were then washed, incubated with secondary antibodies labeled with Cy3, mounted and observed with a fluorescence microscope. For α_{1A} Ca^{2+} channels (the top row of figure 3.2.), the immunoreactivity was shown as puncta on the plasma membrane of BCCs. For α_{1B} Ca^{2+} channels (the second row of figure 3.2.), they yielded a

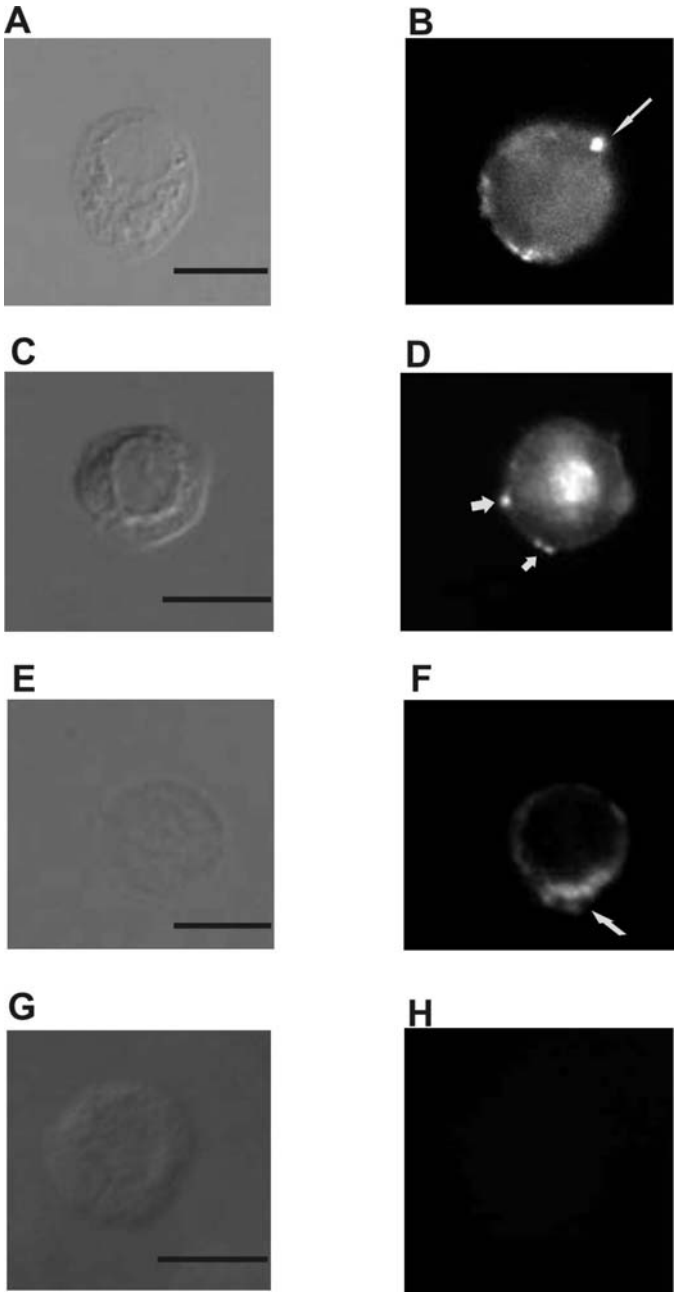

Figure 3.2. Immunolocalization of Ca^{2+} channel α_1 subunits in spherical BCCs

The panels in the left column are phase contrast images and the panels in the right column are fluorescence images of isolated BCCs. α_{1A} immunoreactivity (panel B) is demonstrated as spots on the BCC plasma membrane. α_{1B} immunoreactivity (panel D) is preferentially present as spots on the plasma membrane and also staining of nuclei in BCCs. Whereas α_{1C} (panel F) is mainly present as a diffuse patch pattern on the plasma membrane of BCCs. A control cell (panel H, without primary antibody incubated) is shown on the bottom row. The exposure time for the control cell was longer than any of the above ones. As shown, there is no staining on the control cell. Scale bar is 10μm.

punctate staining pattern on the plasma membrane (shown with the arrows). These results are typical of at least three experiments. Note the spots indicate the localization of α_{1B} Ca^{2+} channels. Also, we can consistently see staining in the perinuclear area for α_{1B} subunits. That might be because that are targeted to perinuclear organelles to perform some unknown functions or it might be that the ER and Golgi normally contain high concentrations of α_{1B} Ca^{2+} channels, perhaps because of a high turnover rate.

In contrast, α_{1C} Ca^{2+} channels (the third row of figure 3.2.) lack punctate staining and appear as diffuse patches indicated by arrow, which suggests that they are not clustered. The control cell is shown on the bottom row of figure 3.2. When the primary antibody was omitted, BCCs lack staining.

3.2.2 The Ca^{2+} channel distribution in differentiated cells revealed by immunocytochemistry

Although BCCs do not have processes in situ or for a short time in culture (2-3 days), when cultured more than 3 or 4 days, they become flattened and develop varicose, long processes ending in growth-cone-like structures similar to neurons (Unsicker et al., 1980). In order to test if the distribution of Ca^{2+} channels will be changed after long periods of culturing, immunocytochemistry was done on differentiated BCCs. Most of the other immunocytochemistry studies in this thesis were done on BCCs after short periods in culture before they had developed processes.

Stronger staining was found along neurite-like structures (panel D and E on figure 3.4.). Fluorescence was most intense in the ends of the processes in α_{1B} Ca^{2+} channels (panel D in figure3.3.). This experiment was done once. This result suggests that the clustering of Ca^{2+} channels in non differentiated cells may be analogous to the

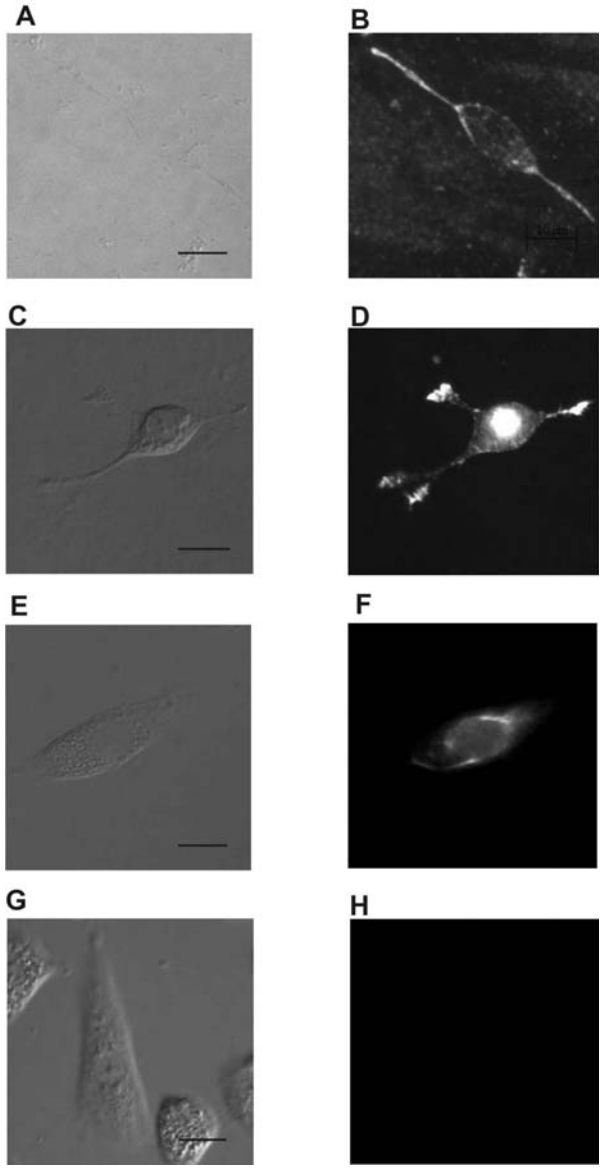

Figure 3.3. Immunocytochemical localization of α_{1A}, α_{1B}, and α_{1C} Ca^{2+} channels in differentiated BCCs

The panels in the left column are phase contrast images and the panels in the right column are fluorescence images of isolated BCCs. Top, middle, third, bottom rows show α_{1A}, α_{1B}, α_{1C} channels and control distribution individually. α_{1A} channels are clustered along the plasma membrane to the processes and on the processes (panel B); α_{1B} channels show strong staining at the end of the processes and the nuclei (panel D). In contrast, α_{1C} channels show a diffuse patch pattern of staining (panel F). In control, there is no staining with the highest exposure time of the above three. Scale bar is 10μm.

localization in neurites in differentiated cells.

3.2.3. The Ca^{2+} channel distribution revealed by transfection

The expression of EGFP labeled protein is a strong tool to track protein localization. We used GFP fused Ca^{2+} channels to look at Ca^{2+} channel localization. Fourteen to sixteen hours after plating of BCCs, α_{1A}, α_{1B} and α_{1C} plasmids with GFP attached were transfected into the cells. Then BCCs were allowed to have enough time (48 hours) to syntheses new proteins. The EGFP fluorescence was observed after fourty-eight hours.

The results are shown in figure 3.4. EGFP signals, representing the channel distribution, are shown in the right column of figure 3.4. Clusters of α_{1A} and α_{1B} Ca^{2+} channels were observed (panels B and D). The punctate distribution pattern indicated by the arrows suggests the clustering of α_{1A} and α_{1B} Ca^{2+} channels. In contrast, the fluorescence associated with the expression of α_{1C} Ca^{2+} channels appears as a patch. In control experiment, the same procedure was performed but with EGFP plasmid transfection (panel H) or with no plasmid (panel J). Control cells which express EGFP alone displayed fluorescence staining throughout the cell (panel H).

The results from transfection of BCCs with plasmids coded for Ca^{2+} channels fused with GFP (figure 3.4) are essentially in agreement with those obtained by immunocytochemistry. This result is typical of more than three experiments. The observation of a different distribution points to the possibility that different targeting mechanisms exist for the different Ca^{2+} channel subtypes. The mechanisms by which Ca^{2+} channels are clustered and targeted will be discussed next.

We also attempted to combine transfection and immunocytochemistry methods to

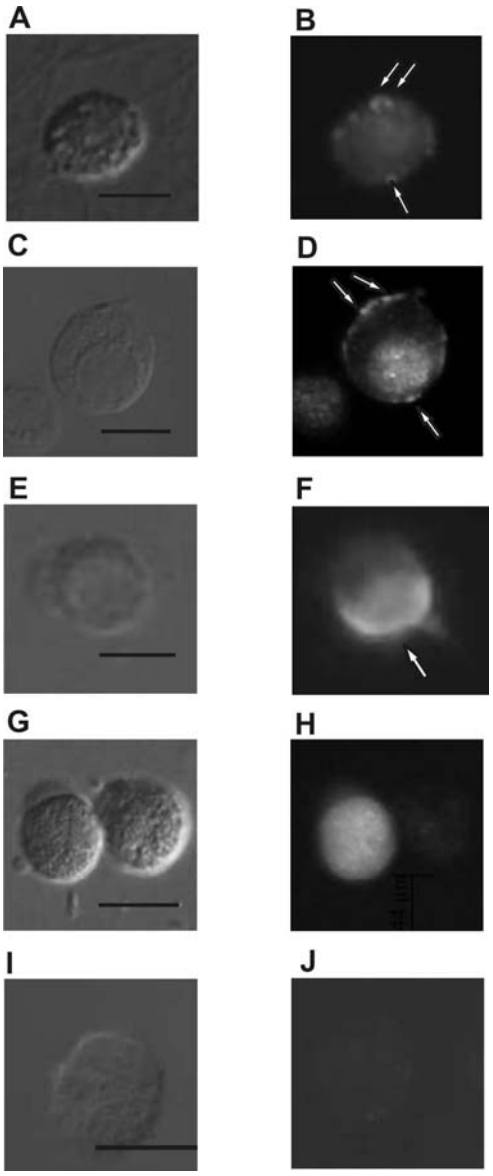

Figure 3.4. The distribution of Ca^{2+} channel α_1 subunits shown by transfection

The panels in the left column are phase contrast images and the panels in the right column are fluorescence images of isolated BCCs. α_{1A} and α_{1B} exhibit a heterogeneous appearance. With both of these constructs, there are several clusters on the membrane (indicated by the arrows in panel B and D. α_{1B} exhibits a punctate staining on the plasma membrane and also a predominantly perinuclear distribution. In contrast, α_{1C} was found to localize as diffuse patches without clusters on the plasma membrane (indicated by arrow in panel H). The fourth row are control cells transfected with EGFP plasmid. They exhibit relatively homogeneous cytoplasmic fluorescence. The bottom row is the negative control cell. The cell went through the same procedure of transfection, but was not

transfected with any plasmid. Scale bar is 10μm.

study the co-clustering of Ca^{2+} channel subtypes, but GFP fluorescence faded much after fixation by cold methanol and paraformaldehyde. Therefore, we were not able to look at the colocalization of Ca^{2+} channel subtypes.

3.3. The presence of CASK and Mint1 in BCCs

CASK and Mint1 have been shown to be important in targeting Ca^{2+} channels in hippocampal neurons (Maximov and Bezprozvanny, 2002), but there have been no previous studies on the role of CASK and Mint1 in neuroendocrine cells. The observation of Ca^{2+} channel clustering in BCCs led to the hypothesis that CASK and Mint1 are present and important in targeting and clustering of Ca^{2+} channel. The present study was sought to test this hypothesis and to determine how the Ca^{2+} channels in BCCs are targeted/anchored to the proper subcellular localization for function, if CASK and Mint1 play roles in it.

3.3.1. RT-PCR shows the presence of CASK gene in BCCs

To address the question of whether CASK and Mint1 play roles in Ca^{2+} channel targeting and /or anchoring, we attempted to demonstrate their presence in BCCs. Since the bovine CASK gene has been partially cloned, primers were designed to amplify the partial codes (gene bank access No. AB098958). RNA from BCCs was isolated and specific primers were used to amplify the RT-PCR product. A single band was observed at about 400 base pairs (bp), which is consistent with the predicted size (393 bp) of the partial CASK gene. This result demonstrated that the CASK gene is expressed in BCCs. Results are shown in figure 3.5. This experiment was done once. The bovine Mint1 gene has not been cloned, so the RT-PCR to test Mint1 gene expression in BCCs could not be

tried.

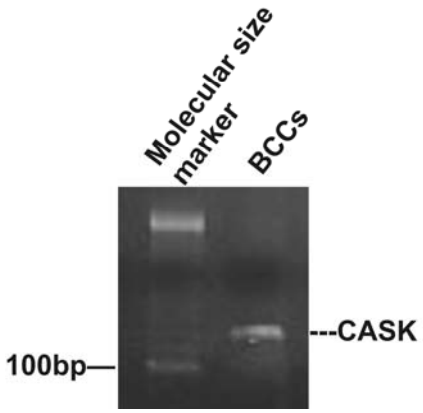

Figure 3.5. mRNA expression of CASK in BCCs demonstrated by RT-PCR

RT-PCR was performed using total RNA extracted from BCCs and amplified by primers specific for CASK. Molecular weight standards are shown in the left lane (marker=100 bp DNA ladder). The PCR product for the CASK gene is shown on the right lane (around 400 bp), which is close to the expected size of the partial CASK gene that has been cloned: 393bp.

3.3.2. Western blotting shows the presence of CASK protein in BCCs

Western blotting was run to determine the presence of the proteins CASK and Mint1 in BCCs. The total rat brain membrane was used as a positive control (figure 3.6. lane 1) From that, we can see that CASK is expressed in rat brain membrane. Lanes 2 and 3 are BCC membrane proteins solubilized from two different preparations. The Western blotting results indicate the existence of CASK protein expression in BCCs (panel A, figure 3.6.). There are two specific CASK bands, one is about 120kDa, the other is about 60kDa (Hata et al., 1996). That's what is shown from figure 3.6. These results confirm that CASK is present in BCCs.

CASK migrates as a doublet because of alternative splicing. There are two alternatively spliced isoforms of CASK present in the CNS (Laverty and Wilson, 1998). The longer one, CASK-B (Swiss-Prot: 070589) is about 120 kDa, binds to cell-surface proteins (including APP, neurexins, and syndecans) and may mediate a link between the extracellular matrix and the actin cytoskeleton via its interaction with syndecan and with the actin/spectrin-binding protein 4.1. The shorter isoform, CASK-A (Swiss-Prot: 070589-2) is about 60 kDa.

3.3.3. Western blotting shows the presence of Mint1 protein in BCCs

To investigate the expression of the protein Mint1 in BCCs, BCCs membrane lysates were loaded for protein analysis. The molecular size for Mint1 protein is 120 kDa (Biederer and Sudhof, 2000). The band at 120 kDa (figure 3.6 B) demonstrates that Mint1 protein is present in BCC membranes. This results is typical of more than three experiments.

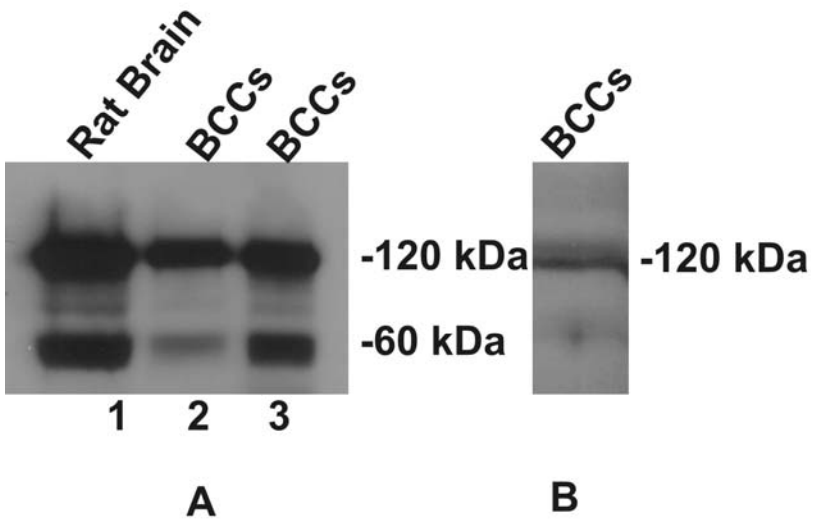

Figure 3.6. CASK and Mint1 proteins are identified in BCCs by Western blotting

Solubilized BCC homogenates were added to the gel and identified by Western blotting. Lane1 is the rat brain membrane used as a positive control, lanes 2 and 3 are BCC membrane protein from two different cell preparations. The left panel shows that CASK is present in BCCs in both the 120 kDa and 60 kDa forms. Mint1 immunoblotting was performed using a monoclonal antibody against Mint1. The molecular weight is shown on the right. A protein corresponding to the expected size of Mint1 (120 kDa) was detected in BCC membranes (Figure 3.6B).

3.3.4. Cellular localization of CASK and Mint1

To address the question of how CASK and Mint1 are distributed in BCCs, the endogenous cellular localization of CASK and Mint1 in BCCs was investigated using immunocytochemistry.

BCCs were probed with a mouse monoclonal antibody against CASK. CASK immunoreactivity is mainly shown on the plasma membrane with some spots (indicated by arrows) and it is also found in the nucleus (top row of figure 3.7.). This result is typical of two experiments. The bottom row of figure 3.7 shows the control cells that were incubated without primary antibody, only with secondary antibody. It demonstrated that there is no staining in control cells. Mint1 immunoreactivity was also tested in BCCs. However, the immunoreactivity was very weak and no clear staining was observed.

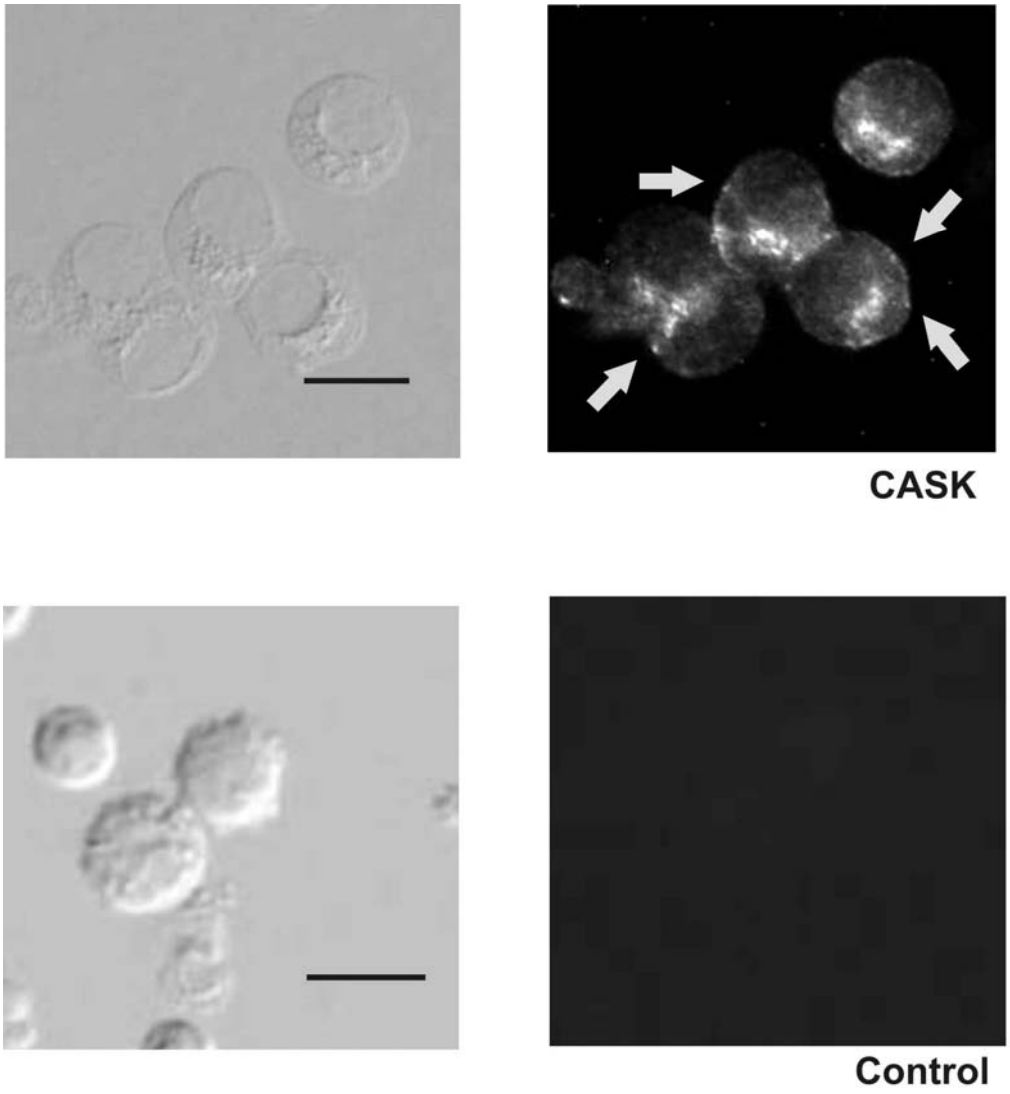

Figure 3.7. The localization of CASK in BCCs

The panels in the left column are phase contrast images and the panels in the right column are fluorescence images of isolated BCCs. Within individual cells, CASK (the right panel of the top row) is present with some spots on the plasma membrane and nuclei. The arrows point to the distinct staining of CASK on the plasma membrane of BCCs. The bottom row shows the control cells which were fixed and incubated with secondary antibody (primary antibody was omitted). Scale bar is 10 μm.

3.4. Co-immunoprecipitation results indicate that there is a biochemical association between α_{1A} and α_{1B} Ca^{2+} channel subunits and CASK

In order to prove that the expression of CASK and Mint1 is necessary to Ca^{2+} channel targeting, we must demonstrate an interaction between endogenous proteins expressed by BCCs. To determine if α_{1A} and α_{1B} Ca^{2+} channels physically bind to CASK and Mint1, studies were performed using coimmunoprecipitation. BCCs were homogenized, solubilized and incubated with beads linked to antibodies against CASK or α_{1A} or α_{1B} Ca^{2+} channels. Then the bound proteins were isolated from beads and separated as supernatant fraction and bound fraction., run on Western blots and probed with antibodies to see if there is an association between CASK and α_{1A} or α_{1B} Ca^{2+} channels.

Since CASK is proposed to associate not only with α_{1A} and α_{1B} Ca^{2+} channels, but also with other proteins, we expected to see that α_{1A} and α_{1B} Ca^{2+} channels were able to pull down only a fraction of total CASK. The lanes 3 and 5 on figure 3.8. demonstrate that both α_{1A} and α_{1B} Ca^{2+} channels are able to precipitate with only a fraction of CASK.

On the other hand, if most or all of the α_{1A} and α_{1B} Ca^{2+} channels are associated with CASK, then when CASK is pulled down, most of the α_{1A} and α_{1B} Ca^{2+} channels should be pulled down together with CASK. This hypothesis was confirmed by our results. Antibodies against CASK are able to coimmunoprecipitate most α_{1A} Ca^{2+} channels (Figure 3.8., lanes 6 and 7). This result is typical of two experiments. The coimmunoprecipitation experiment to test if CASK is able to precipitate with α_{1B} Ca^{2+} channels was also done, but the signal of α_{1B} Ca^{2+} channels are not consistent.

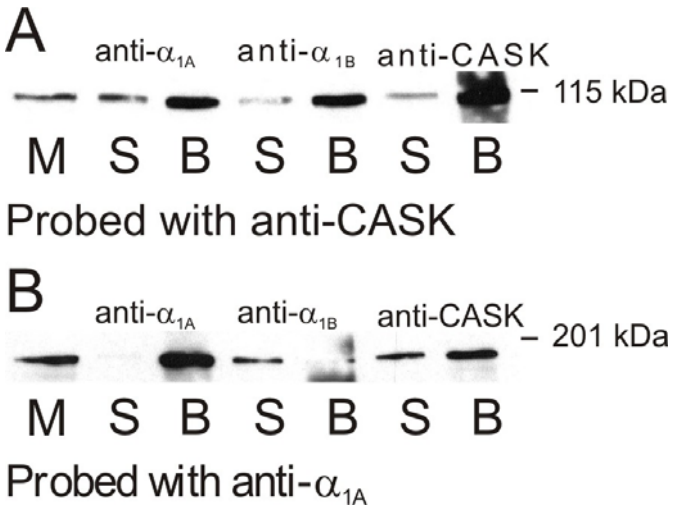

Figure 3.8. Coimmunoprecipitation of CASK with α_{1A} and α_{1B} channels from BCC membranes show CASK interacts biochemically with α_{1A} and α_{1B} Ca^{2+} channel subunits

BCC membranes were solubilized using CAPSO and incubated with antibodies conjugated to Protein Sepharose beads. The supernatant was then removed and Western blots were performed on the proteins bound to the beads (B), theproteins in the supernatants (S), and BCC membrane fractions (M). The antibodies conjugated to the bands are shown above the bands. Numbers at the right indicate the positions of molecular weight markers (in kiloDaltons).

Figure 3.8A shows a Western blot probed with anti-CASK. Anti-CASK was able to pull down most of the CASK since the band for CASK protein in the bound fraction (lane 7) is much darker than that in the supernatant fraction (lane 6). Both anti-α_{1A} and anti-α_{1B} were able to pull down a fraction of the CASK (see lanes 3 and 5, respectively), suggesting CASK interacts biochemically with both channels.

Figure 3.8B shows a Western blot probed with anti-α_{1A}. Anti-α_{1A} is able to pull down most of the solubilized α_{1A} (compare lanes2 and 3). Anti-α_{1B} was not able to pull down α_{1A} (compare lanes 4 and 5), suggesting that non-specific binding to the beads was small. Lanes 6 and 7 show that anti-CASK is able to pull down a fraction of Ca^{2+} channels. Lanes 6 and 7 show that anti-CASK is able to pull down a fraction of α_{1A} Ca^{2+} channels, suggesting again that there is a biochemical interaction between CASK and α_{1A} Ca^{2+} channels.

Similar experiments were attempted with an antibody directed against Mint1. The antibody that we used was unable to immunoprecipitate Mint1 itself, however. We were therefore unable to test the hypothesis. That might be due to the lack of interaction of the Mint1 antibody with solubilized protein.

These results show a direct biochemical interaction between CASK and α_{1A} Ca^{2+} channels, and also between CASK and α_{1B} Ca^{2+} channels. The biochemical association is proposed to colocalize Ca^{2+} channels and presynaptic release sites, thus supporting rapid and efficient initiation of exocytosis.

3.5. Disruption of the interaction of Ca^{2+} channels with CASK and Mint1

3.5.1. Transfection of BCCs with NC3-GFP plasmids

To investigate if CASK and Mint1 play roles in Ca^{2+} channel targeting, the plasmid NC3-GFP was obtained from Dr. IIya Bezprozvanny. NC3-GFP encodes for amino acids 2021-2039 of the human α_{1B} Ca^{2+} channel, which contains a number of potential SH3-domain binding motifs (which have the sequence PXXP) and a motif at the carboxyl terminus (DXXC-COOH), which is the specific site to bind to Mint1-1. This peptide includes the interaction sites for CASK and Mint1. When NC3-GFP was expressed in hippocampal neurons, fluorescence signal was distributed in a punctate fashion along the axons in rat hippocampal neurons (Maximov et al., 1999; Maximov and Bezprozvanny, 2002). This is likely to reflect the binding of NC3-GFP to clusters of CASK and/or Mint1.

Therefore similar experiments was performed in BCCs in our study. BCCs were transfected with NC3-GFP plasmid 14-16 hours after plating. Fourty eight hours later, BCCs were analyzed using flurorescence microscopy. The NC3-GFP peptide also appeared to be clustered in BCC membranes (panel B on figure 3.9). The pattern of NC3-GFP suggests that CASK and/or Mint1 are clustered on BCC membranes.

3.5.2. Immunocytochemistry of NC3-GFP transfected cells shows that α_{1B} Ca^{2+} channels are not clustered on the plasma membrane

The effect of NC3-GFP on the functional interaction of Ca^{2+} channels with CASK and/or Mint1 was investigated by determining the distribution of Ca^{2+} channels in transfected BCCs using immunocytochemistry. Immunofluorescence of the cells after transfection was compared with the non-transfected BCCs. Note that α_{1B} Ca^{2+} channels were localized primarily as punctate staining on the plasma membrane in non-transfected cells (shown by arrows, panel B on figure 3.10.). However, the distribution of α_{1B} Ca^{2+} channels was much more diffuse after transfection (panel D on figure 3.10.). This might result from the disruption of α_{1B} Ca^{2+} channel interactions with CASK and Mint1. These results indicate that the clustering /targeting of Ca^{2+} channels can be altered by transfection with NC3-GFP and this provide further evidence for a role of CASK and Mint1 in α_{1B} Ca^{2+} channel clustering in BCCs. This result is typical of three experiments. These results are similar to what the researchers in Dr IIya Bezprozvanny's lab have found: the clusters of α_{1B} Ca^{2+} channel on the axons in rat hippocampal neurons were abolished by overexpression of NC3-GFP (Maximov and Bezprozvanny, 2002).

Taken together, these findings support our hypotheses that CASK and/or Mint1 interact with α_{1A} and α_{1B} Ca^{2+} channels and they are important in Ca^{2+} channels targeting and/ or clustering.

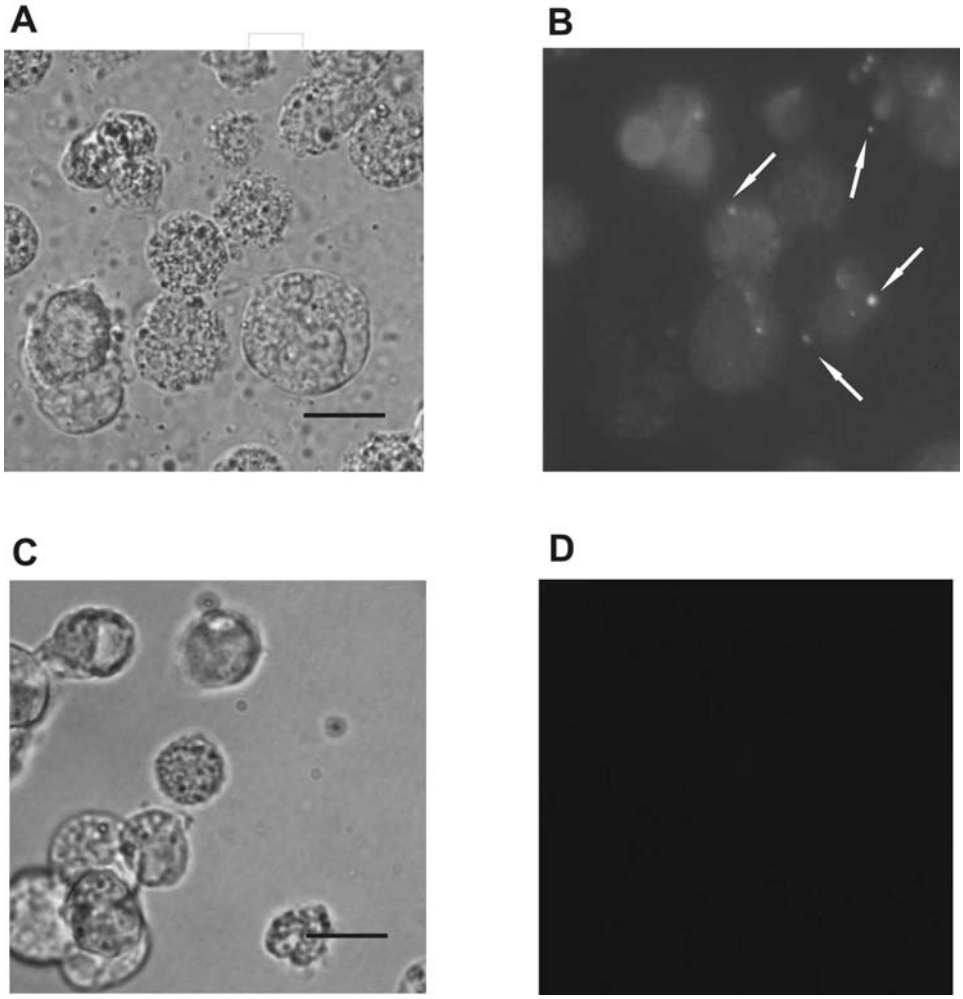

Figure 3.9. Overexpression of NC3-GFP in BCCs

The panels in the left column are phase contrast images and the panels in the right column are fluorescence images of isolated BCCs. Panel B is the image of BCCs transfected with NC3-GFP. Note that there are some spots on the plasma membrane, which suggests that CASK is clustered. Panel D is the negative control, in which BCCs were transfected with empty plasmid. The exposure time for panel D is longer than for panel B. Scale bar is 10 μm.

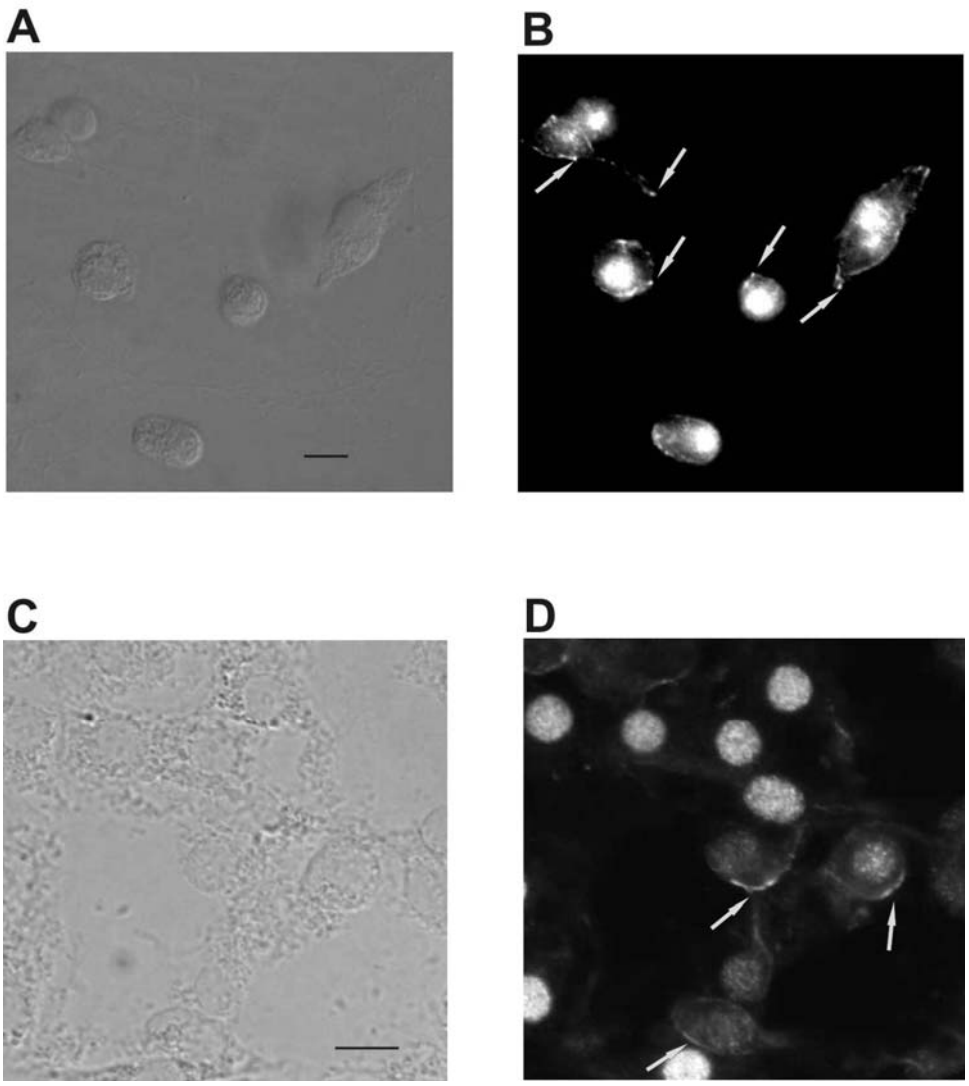

Figure 3.10. The disruption of α_{1B} channels after overexpression of GFP-NC3

The panels in the left column are phase contrast images and the panels in the right column are fluorescence images of isolated BCCs. Panels B and D show the α_{1B} localization in non-transfected cells and cells transfected with NC3-GFP. The punctate staining is much less for α_{1B} Ca^{2+} channels in cells with NC3-GFP compared with the untransfected BCCs. The arrows in panel B shows the punctuate staining of α_{1B} Ca^{2+} channels. In contrast, the distribution of α_{1B} Ca^{2+} channels on the plasma membrane following transfection is much more diffuse (arrows in panel D). Scale bar is 10 μm.

3.6. α_{1D} Ca^{2+} channel distribution

We also investigated the α_{1D} Ca^{2+} channel distribution in BCCs by immunocytochemistry. From figure 3.11 (indicated by arrows on panels B and D), we can see the punctate staining of α_{1D} Ca^{2+} channels, which suggest that they are also clustered.

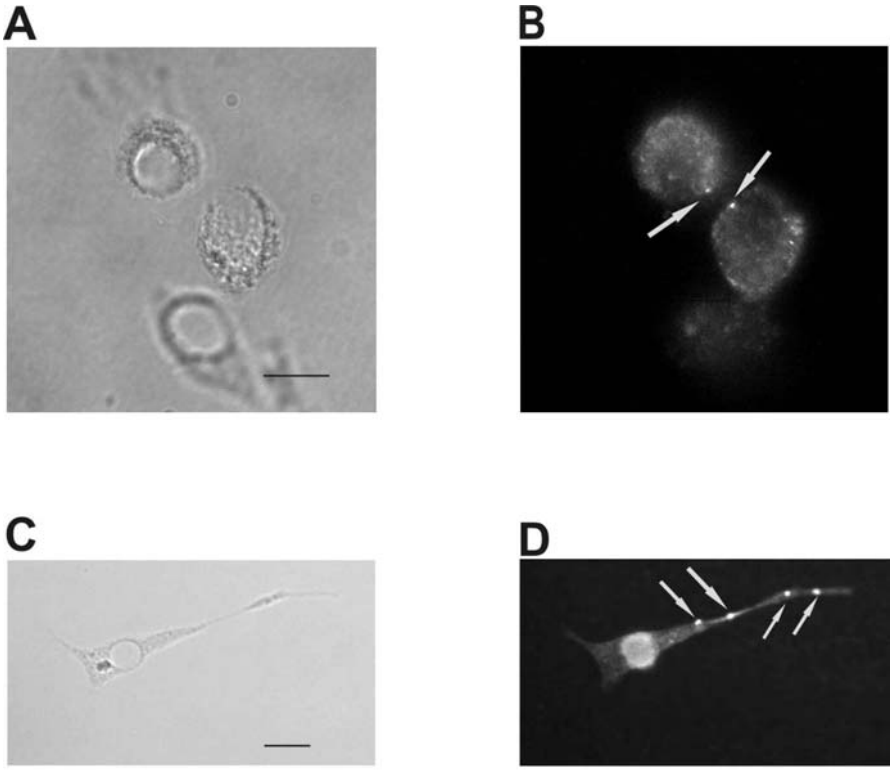

Figure 3.11. The distribution of α_{1D} Ca^{2+} channels in BCCs revealed by immunocytochemistry

The panels in the left column are phase contrast images and the panels in the right column are fluorescence images of isolated BCCs. Antibodies directed against α_{1D} Ca^{2+} channels were used to determine the localization of α_{1D} Ca^{2+} channels. The top row (panel A and B) shows the distribution of α_{1D} Ca^{2+} channels in spherical BCCs, while the bottom row (panel C and D) indicates the α_{1D} Ca^{2+} channels in differentiated BCCs. The arrows suggest that Ca^{2+} channels are also clustered. The punctate pattern of distribution for α_{1D} Ca^{2+} channels is also apparent in differentiated BCCs (panel D), as we can see the bright spots along the process (shown by arrows). Scale bar is 10 μm.

4. Discussion

4.1. The distribution of Ca^{2+} channels

In this study, we firstly set out to gain insight into the distribution of Ca^{2+} channels within BCCs. To obtain this goal, immunocytochemistry with antibodies against Ca^{2+} channel subtypes was performed. Also, transfection of GFP-fused subtypes of Ca^{2+} channels was used to verify the distribution of Ca^{2+} channels. Both the immunocytochemistry and transfection studies demonstrated that α_{1A} and α_{1B} Ca^{2+} channels exist in clusters on the plasma membrane in BCCs while α_{1C} Ca^{2+} channels display a diffuse patch pattern of distribution. These results support the hypothesis that subtypes of Ca^{2+} channels are clustered in BCCs.

4.1.1. Why is there Ca^{2+} channel clustering in BCCs?

Since intracellular Ca^{2+} concentration falls off steeply as a function of distance away from the Ca^{2+} channels, the brief rise in Ca^{2+} concentration to the level necessary for exocytosis in neurons (Llinas et al., 1992) is likely to occur only in proximity to the Ca^{2+} channels. Flash photolysis of caged Ca^{2+} provides a way to elevate Ca^{2+} concentration uniformly and to measure rates of release as a function of cytosolic Ca^{2+}. Studies with caged-Ca^{2+} compounds indicated that the Ca^{2+} requirement for exocytosis is substantially greater than that suggested by conventional methodologies (Kasai and Takahashi, 1999). Indeed, the presynaptic Ca^{2+} channels of vertebrate neurons display a highly restricted subcellular distribution in the active zone, and colocalize with the vesicular release machinery (Westenbroek et al., 1998). This suggests that the channels are close to the release sites. In endocrine cells, release might occur at sites distant from

Ca^{2+} channels.

In neuroendocrine cells, like single adrenal chromaffin cells, two phases of release have been demonstrated by capacitance measurement: a fast phase release is followed by a sustained slow release phase (Moser and Neher, 1997). The release of readily releasable vesicles is strongly hindered by the fast high affinity chelator BAPTA, but much less by the slow, low affinity chelator EGTA. This suggests that these vesicles are localized in close proximity to the Ca^{2+} influx. The exact mechanisms underlying the two phases of release are still unclear. Moser and Neher (1997) proposed two possible explanations for fast release in neuroendocrine cells, one is that Ca^{2+} channels are clustered and that fast release occurs near the clusters. The second explanation is that a subset of vesicles are physically associated with Ca^{2+} channels.

Clustering of α_{1A} and α_{1B} channels was observed in BCCs in this study. Our data therefore confirm previous immunocytochemistry work done on α_{1B} Ca^{2+} channels in BCCs (Fisher et al., 2000) and show that α_{1A} Ca^{2+} channels are also clustered. But why is the clustering of Ca^{2+} channels important? A prior study using pulsed laser imaging showed microdomains of Ca^{2+} influx and served as indirect evidence of Ca^{2+} channel clustering (Monck et al., 1994; Robinson et al., 1995). The heterogeneous distribution of release sites in BCCs has been detected using various methodological approaches, like amperometry (Schroeder et al., 1994), granule trafficking (Steyer et al., 1997) and DBH immunocytochemistry (Hesketh et al., 1981).

Studies have suggested sites of Ca^{2+} influx colocalize with the sites of release. This has been revealed morphologically (Becherer et al., 2003), as docked vesicles were preferentially located around sites of Ca^{2+} entry. A study which combined amperometric measurement of catecholamine release and pulsed laser imaging showed evidence that

exocytosis occurs preferentially near the sites of influx (Robinson et al., 1995). Additional evidence for the colocalization of Ca^{2+} entry with release sites is provided by studies that use Ca^{2+} imaging combined with granule trafficking (Aunis, 1998; Becherer et al., 2003).

All of these studies favor the interpretation that Ca^{2+} channel clustering is critical for rapid transmission. This led us to propose that the clusters of Ca^{2+} channels might be important and necessary for the fast release phase in BCCs. Both the rapid exocytotic release of the catecholamines from docked vesicles and the refilling of the readily releasable pools are dependent on the local rise of the cytosolic concentration of Ca^{2+}. Ca^{2+} influx through the clustered channels results in a Ca^{2+} build up to high concentration in a Ca^{2+} microdomain (a region of elevated Ca^{2+} formed around open Ca^{2+} channels). This is responsible for the synchronization of vesicle fusion with an action potential, which is a requirement for fast release (Llinas et al., 1995). The clustering of α_{1A} and α_{1B} channels in BCCs may result in local large increases in Ca^{2+}, which may be responsible for the fast release phase in these cells. Moreover, catecholamines released from BCCs regulate homeostatic processes, such as cardiac output and vascular tone. Catecholamine release is very important under both basal "breed-and-feed" conditions and acute stress "fight-or-flight" conditions.

4.1.2. Why do α_{1A} and α_{1B} Ca^{2+} channels cluster while α_{1C} Ca^{2+} channels do not?

Neurons express multiple types of VDCCs, which are associated with specific cellular functions. α_{1A} and α_{1B} type channels are predominantly found along the apical

dendrites and axon terminals where they control the release of neurotransmitters. By contrast, α_{1C} type Ca^{2+} channels, predominantly located in cell body and proximal dendrites are associated with non-exocytotic functions such as the regulation of gene expression and enzyme activity. Previous immunocytochemistry data revealed the similar punctuate labeling pattern of α_{1A} and α_{1B} channels in several types of neurons (Westenbroek et al., 1992; Westenbroek et al., 1995). The α_{1A} and α_{1B} Ca^{2+} channels are particularly important in mediating fast Ca^{2+} influx into the presynaptic terminal and in triggering vesicle fusion and transmitter release. Many studies suggest that α_{1A} and α_{1B} channels are targeted to the presynaptic terminals.

The identification of Ca^{2+} channel $\alpha_{1A,}$ α_{1B}, α_{1C} and α_{1D} subtypes in BCCs in this study is consistent with the pharmacological and biophysical experimental results in BCCs from other labs (Lukyanetz and Neher, 1999; Mansvelder and Kits, 2000). One report showed that non α_{1C} or α_{1D} type Ca^{2+} channels contribute about 80% of the total current in BCCs, but α_{1C} and α_{1D} only contribute about 20% (Albillos et al., 1996). In a recent study, Ca^{2+} current components in mouse adrenal tissue slices under conditions that match the dynamic native cell behavior action potential was measured under pharmacological conditions. They analyzed and found that α_{1A} channels contribute significantly to exocytosis under short durations of AP (Chan et al., 2005). Previous data provided by Lara et al showed that α_{1A} channels are coupled more tightly with exocytotic active sites as compared to α_{1C} type Ca^{2+} channels. In this experiment, K^{+} evoked catecholamine release was measured by electrochemical detector and the contribution of different Ca^{2+} channels to secretion was determined by the deduction of release by blocking some specific subtypes (Lara et al., 1998).

The present study has shown the different distribution patterns of these channels,

which implies specific functions. The priority of α_{1A} and α_{1B} type Ca^{2+} channels are demonstrated by their clustering while α_{1C} type are not. These data are consistent with a previous study that showed that α_{1B} Ca^{2+} channels are predominant in chromaffin cells in controlling catecholamine release stimulated by nerve (O'Farrell et al., 1997). Opioids and ATP control Ca^{2+}-dependent neurotransmitter release from chromaffin cells (Currie and Fox, 1996). By focal amperometry, it was found that opioids and ATP could inhibit α_{1A} and α_{1B} type Ca^{2+} channels in BCCs, which resulted in a inhibition of catecholamine secretion and it seems that neither α_{1C} or α_{1D} type Ca^{2+} channels are involved in catecholamine secretion (Carabelli et al., 1998). This indicated that the release is more controlled by α_{1A} and α_{1B} Ca^{2+} channels compared with α_{1C} or α_{1D} types of Ca^{2+} channels.

This indicates that release is activated more by α_{1A} and α_{1B} Ca^{2+} channels than by α_{1C} or α_{1D} Ca^{2+} channels.

The structure and function of α_{1A} and α_{1B} channels are similar. In many types of neurons, both types are abundantly expressed and play vital roles on controlling secretion. The functions of these channels, however, may be subtly different because of differences in biophysical properties, differences in modulation of these properties of differences in the functional interaction of these channels with other proteins.

From the above, we can see that the subtypes of Ca^{2+} channels that are involved in fast release are likely to be those that are clustered.

4.2. The role of CASK and Mint1 in Ca^{2+} channel clustering and targeting

How Ca^{2+} channels cluster and localize in BCCs is of great interest. CASK and Mint1 have been proposed to act as adaptor proteins and suggested to be important in

membrane protein targeting in neurons (Hata et al., 1996; Biederer and Sudhof, 2000). Specifically, they have been shown to function in Ca^{2+} channel targeting in neurons (Maximov and Bezprozvanny, 2002). However, it is not known whether these proteins are involved in Ca^{2+} channel targeting in neuroendocrine cells. The clustering of Ca^{2+} channels in BCCs led us to hypothesize that CASK and Mint1 could play roles in it.

4.2.1. The role of CASK and Mint1 in neurons

Among the possible mechanisms for explaining the fast release phase, Ca^{2+} channels clustering is supported by many studies. Coupling between Ca^{2+} influx through Ca^{2+} channels and release depends on the trafficking and recruitment of Ca^{2+} channels by other molecules. It is intriguing to find out how the trafficking is accomplished by other proteins, including CASK and Mint1.

MAGUKs assemble a specific protein complex by binding to a set of membrane and cytoplasmic proteins. In this way, they are believed to play roles in the functional and structural organization of the plasma membrane (Fanning and Anderson, 1996). Organization around a PDZ-based scaffold allows the stable localization of interacting proteins and enhances the rate and fidelity of signal transduction within the complex. PDZ-containing proteins may play an important role not only as localized scaffolds, but also as mediators of the trafficking of their binding partners within the cell. They also target proteins to specific membrane compartments and assemble them into the supramolecular complexes (Sheng and Sala, 2001).

Both CASK and Mint1 contain PDZ domains, which make them adaptor proteins and allow them to play roles in targeting other proteins. Mint1 and CASK have been implicated in function in protein targeting (Okamoto and Sudhof, 1997; Tabuchi et al.,

2002). Mint1 was shown to bind to Munc18-1, an essential component of the synaptic vesicle fusion apparatus, and to recruit Munc18-1 to neurexin-containing regions of the plasma membrane via an association of the N-terminal with the membrane associated protein CASK (Okamoto and Sudhof, 1997; Biederer and Sudhof, 2000). Mint1 was proposed to transport N-methyl-D-asparate (NMDA) type glutamate receptor to synapses in vertebrates (Ho et al., 2003). The importance of Mint1 in the regulation of exocytosis has been demonstrated in Mint1-deficient mice, which displays impaired dopamine release as well as impaired GABAergic synaptic transmission and roles in targeting and localization of dopamine transporters (Mori et al., 2002; Ho et al., 2003). Studies also revealed that knocking down of CASK via sRNAi (short RNA interference) prevented synaptic transmission in cultured *Lymnaea* neurons (Spafford et al., 2003).

4.2.2. The interaction of CASK and Mint1 with Ca^{2+} channels

Mint1 and CASK can both interact with the C-terminus region of Ca^{2+} channels. The E/D-X-W-C/S-COOH, on the C-terminal tail of the long C-terminal isoforms of α_{1A} and α_{1B} Ca^{2+} channels, specifically associates with the first PDZ domain of Mint1 (Maximov et al., 1999). This interaction site does not exist in α_{1C} or α_{1E} Ca^{2+} channels. Furthermore, a proline-rich region present in the C-terminal sequence of α_{1A} and α_{1B} binds to the SH3 domain of CASK. This could also serve as an explanation of why α_{1A} and α_{1B} Ca^{2+} channels are clustered while α_{1C} Ca^{2+} channels are not. The evolutionary analysis of presynaptic Ca^{2+} channels and their ancestors done by Spafford and coworkers indicated that the Mint1 and CASK binding sites evolved at a much earlier stage than the synprint site (Spafford and Zamponi, 2003). This suggests that the interaction of Ca^{2+} channel with CASK and Mint1 is more fundamental than the interaction with the synprint site. Expression of NC3-GFP plasmids, which code for a

peptide that mimics the interaction site for CASK and Mint1 in the rat hippocampal neurons (see Introduction), led to a displacement of the channels (Maximov and Bezprozvanny, 2002). In addition, injection of a peptide, that mimics the interaction site and thereby competitively inhibits CASK and Mint1 binding to the α_{1A} and α_{1B} channels in *Lymnaea* neurons, acutely blocks synaptic transmission (Spafford et al., 2003).

Previous studies showed that Mint1 is only detectable in the brain. Mint1 and Mint2 have been reported to be exclusively expressed in neurons, containing N-terminal domain that specifically interact with Munc-18-1 (Okamoto and Sudhof, 1998). This study is the first to demonstrate that CASK and Mint1 are also present in BCCs. Immunoprecipitation of CAPSO solubilized proteins from BCC membranes using anti-CASK antibodies provided direct evidence that CASK biochemically interacts with α_{1A} and α_{1B} Ca^{2+} channels.

How do the clusters occur? The mechanism underlying the location and clustering of channels in BCCs is less well understood. While α_{1B} Ca^{2+} channels in cultured hippocampal neurons were redistributed (Maximov and Bezprozvanny, 2002), we were interested to see whether expression of a peptide mimicking the interaction site could change the distribution of Ca^{2+} channels in BCCs. So we did experiments similar. NC3-GFP was overexpressed in BCCs. Since NC3 interacts with CASK and Mint1, the sites of their interaction will show fluorescence after transfection with NC3-GFP. The hypothesis that CASK and Mint1 are involved in Ca^{2+} channel clustering implies that CASK and Mint1 themselves should be clustered on the BCC plasma membrane. Our data demonstrated that NC3-GFP displays a punctate pattern of fluorescence, suggesting that CASK and/or Mint1 are clustered on the plasma membrane of BCCs. The size and number of puncta that we saw in these experiments were similar to what we saw for Ca^{2+}

channel clustering, which is consistent with the idea that Ca^{2+} channels cluster at the same places that CASK and Mint1 clusters. When the CASK and Mint1 binding domain is overexpressed in BCCs as a dominant-negative construct, the NC3-GFP could competitively uncouple the binding of CASK and Mint1 to endogenously expressed Ca^{2+} channels. This would be expected to cause a change in distribution of Ca^{2+} channels. Immunostaining of BCCs with antibodies against α_{1B} Ca^{2+} channels after transfection of NC3-GFP displayed diffuse staining. The disruption of α_{1B} punctate distribution after overexpression of NC3 supported our hypothesis that CASK and Mint1 function in clustering of Ca^{2+} channels.

Another possible function of CASK and Mint1 interaction with Ca^{2+} channel is that CASK and/or Mint1 may play an important role in targeting Ca^{2+} channels to the plasma membrane. It is assumed that the α_1 subunit folds to form an immature channel in the endoplasmic reticulum (ER). If newly synthesized channel proteins are stored in the ER without CASK and Mint1, they may be unavailable for transport to the plasma membrane. This hypothesis could be tested by measurement of Ca^{2+} current. If channels on the plasma membrane are reduced after transfection of NC3-GFP, the Ca^{2+} influx will be decreased, which could be demonstrated by the reduction of Ca^{2+} current.

Hu et al., (2005) did recently performed experiments similar to those of Bezprozvanny's group (Maximov and Bezprozvanny, 2003) except that they studied α_{1A} Ca^{2+} channel targeting in cultured mouse hippocampal neurons instead of α_{1B} Ca^{2+} channels. They reported that mutation of the interaction site for CASK and Mint1 did not effect the distribution of α_{1A} channels, suggesting that CASK and Mint1 are not important in targeting of α_{1A} Ca^{2+} channels. (Yap et al., 2003; Hu et al., 2005). One possible explanation is that α_{1A} Ca^{2+} channels and α_{1B} Ca^{2+} channels are targeted by different

mechanisms. Recent work by Brice and Dolphin (1999) provides support for this possibility. In their experiment, a polarized epithelial cell line was used to study the targeting of Ca^{2+} channel α_1 subunits and it was shown that although expressed α_{1B} channels were always targeted to a particular membrane, the targeting of α_{1A} channels depended on the coexpressed β subunit (Brice and Dolphin, 1999). We did not see any change in the distribution pattern of α_{1A} Ca^{2+} channels in NC3-GFP transfected cells (data not shown). This could be because CASK and Mint1 are not important in targeting α_{1A} Ca^{2+} channels in BCCs either or it could be that it is more difficult to observe an effect on α_{1A} channels because they are turned over more slowly than α_{1B} Ca^{2+} channels.

A recent paper reported that CASK and Mint are not colocalized with the long splice variant of α_{1B} Ca^{2+} channels in the isolated chick ciliary ganglion presynaptic calyx terminal, suggesting that CASK and Mint1 are not important in anchoring Ca^{2+} channels in these cells (Khanna et al., 2006). Although they reported that CASK and Mint1 interact with Ca^{2+} channels biochemically using coimmunoprecipitation experiments, the absence of colocalization suggested that Ca^{2+} channels are not bound to CASK and Mint1 in these terminals. This may indicate that the interaction between CASK and Mint1 and Ca^{2+} channels is not important in this particular terminal. Alternatively, the results described by Bezprozvanny may indicate a role for CASK and Mint1 in Ca^{2+} channel targeting rather than anchoring. Thus, disruption of the interaction between BCCs and Ca^{2+} channel may have prevented the targeting of α_{1B} Ca^{2+} channels to the axon terminals in cultured hippocampal cells rather the anchoring of the channels. Since we did not show colocalization of CASK and/or Mint1 with the α_{1B} Ca^{2+} channel, it is possible that they are important in targeting rather than anchoring Ca^{2+} channel in BCCs.

4.3. Ca^{2+} channels in BCCs might interact with the exocytotic machinery

We showed that CASK and Mint1 are important in clustering of α_{1B} Ca^{2+} channels in BCCs**,** but it is possible that exocytotic proteins might also be involved. The loop between domain II and domain III of α_{1A} and α_{1B} Ca^{2+} channels interacts with the synprint site (Mochida et al., 1996). SNAREs function in Ca^{2+} channel clustering and exocytosis in BCCs is still to be elucidated.

4.4. The clustering of α_{1D} Ca^{2+} channels

It was originally thought that α_{1D} Ca^{2+} channels were not clustered (Hell et al., 1993). Unexpectedly, our study found the clustering of α_{1D} channels in BCCs. Since α_{1D} channels do not have interaction sites with CASK and Mint1, they must be clustered by some other mechanism, one that is different from that of α_{1A} and $\alpha_{1B.}$ channels.

Studies showed that the interactions with PDZ domains are required for α_{1C} and α_{1D} type Ca^{2+} channels to efficiently activate CREB (Weick et al., 2003). An interaction between the PDZ domain and a novel site in the carboxy-terminal domain of the α_{1C} was reported (Zhang et al., 2005). Bezprozvanny's group used cultured rat hippocampal neurons, expressing epitope-tagged recombinant α_{1D} subunit to demonstrate that the Shank-binding motif in the α_{1D} subunit is both necessary and sufficient for synaptic clustering of α_{1D} Ca^{2+} channels (Zhang et al., 2005). Shank proteins are modular adaptor proteins that play a role of "master scaffold" in the postsynaptic specializations at glutamatergic synapses (Sheng and Kim, 2000). This suggests that α_{1D} clustering might be due to an interaction with Shank.

5. Conclusions and Future Studies

The following conclusions can be made from this study:

1. α_{1A}, α_{1B} and α_{1D} Ca^{2+} channels are clustered on BCC membranes

2. CASK and Mint1 are present in BCCs

3. CASK and Mint1 interact biochemically with α_{1A} and α_{1B} channels in BCCs

4. CASK and Mint1 play roles in clustering of α_{1B} Ca^{2+} channels

Future experiments:

Transfection of GFP-tagged Ca^{2+} channels provides a very valuable tool in studying Ca^{2+} channel physiology. The ability to visualize the localization of Ca^{2+} channel clustering in live cells makes it possible to correlate the location of channels with release sites and to correlate changes in distribution with alterations in the channels themselves. For example, we can correlate clusters with release sites using amperometry or pulsed laser Ca^{2+} imaging, or labeled granules to track the movement of granules to the release sites.

The clustering of Ca^{2+} channels was demonstrated in this study. However, the functional importance of Ca^{2+} channel clustering and the role of CASK and Mint1 in regulation of exocytosis still need to be verified. We propose that the Ca^{2+} channel clustering is important for the fast release phase in BCCs. If this is true, then disruption of clustering by overexpression of NC3-GFP would be expected to inhibit exocytosis. This should be detectable by evoked capacitance measurement. If there is a change in evoked capacitance in the fast release, then the conclusion could be made that the channel

clustering is important in fast release phase. If Ca^{2+} current is decreased after overexpression of NC3-GFP, it will suggest that CASK and Mint1 may be important in targeting channels from the ER to the plasma membrane.

Interesting questions remain: Are the channels clustered alone by CASK and Mint1 or together within a signaling complex? Are there interactions with other proteins that are important in targeting? The model developed in our study provides a good system to test the importance of other mechanisms of targeting (e.g, the role of synaptic proteins and the Ca^{2+} channel β subunit). We can express mutant channels lacking the specific interaction sites or overexpress other proteins (e.g, β subunits or synprint peptide) to see if channel distribution could be altered.

The clustering of α_{1D} Ca^{2+} channels observed in our study is interesting. Shank has been shown to be important in the targeting of α_{1D} Ca^{2+} channels in rat cultured hippocampal neurons (Zhang et al., 2005). It would be of interest to determine if Shank is expressed in BCCs, if Shank plays a role in targeting of α_{1D} Ca^{2+} channels, and the functional importance of α_{1D} Ca^{2+} channel clustering in BCCs.

The answers to these questions and others await investigation in this exciting and evolving field.

6. References

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