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ASSEMBLING THE PRESYNAPTIC ACTIVE ZONE: THE CHARACTERIZATION OF AN ACTIVE ZONE PRECURSOR VESICLE

by

RONG GRACE ZHAI

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2001

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ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Ph.D. Program Neurobiology

Name of Candidate Rong Grace Zhai

Committee Chair Craig C. Garner

Title Assembling the Presynaptic Active Zone: The Characterization of an Active Zone Precursor Vesicle

The active zone is a specialized region of the presynaptic plasma membrane where synaptic vesicles dock and fuse. Underlying the active zone is a meshwork of cytoskeletal filaments believed to be important for tethering synaptic vesicles and the machinery of synaptic vesicle recycling. This cortical cytomatrix assembled at the active zone (CAZ) is likely to play an important role in defining the active zone as the site for neurotransmitter release. The assembly of the active zone is an important step in synaptogenesis. To understand the process of the assembly of the CAZ, we investigated the cellular mechanism that underlies the sorting and transport of Piccolo to the active zone. Piccolo is a novel component of the presynaptic CAZ that is tightly associated with synaptic junctions. It is found to be specifically localized to the presynaptic terminals of both excitatory and inhibitory synapses but not to the cholinergic neuromuscular junction. In immature neurons, Piccolo exhibited a punctate pattern that was similar yet distinct to synaptic vesicles. Our characterization of the Piccolo puncta revealed that it was associated with ~80 nm dense core vesicles that were concentrated in axonal growth cones and in association with nascent synapses. These vesicles are termed Piccolo

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transport vesicle (PTV). To ensure precise neurotransmitter release, the machinery of synaptic vesicle exocytosis has to be assembled properly at the active zone. Piccolo transport vesicles carry the components of synaptic vesicle exocytosis machinery. These components have been genetically proven to be involved in either SNARE complex formation, such as syntaxin and SNAP-25, or SNARE complex regulation, such as Munc18, Munc13, complexin, Ca^{2+} channel, and Rab3. In addition, PTV carries all four high molecular weight CAZ proteins: Piccolo, Bassoon, RIM, and Munc13. Furthermore, PTV also contains key adhesion molecules of synaptic junctions, such as N-cadherin, N-CAM, and syndecan2. These results suggest that the PTV is a preformed complex that serves as an active zone precursor. The fusion of such vesicle with the plasma membrane at sites of axonal-dendritic cell-cell adhesion is expected to deposit Piccolo and initiate the establishment of the presynaptic active zone.

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DEDICATION

This dissertation is dedicated to my father, Jie Zhai, my mother, Kangle Yang, and my brother, Dazhi Zhai.

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LIST OF ABBREVIATIONS

Ab	Antibody
AchR	Acetylcholine receptor
AP	Alkline phosphatase
ApCAM	*Aplysia* cell adhesion molecule
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ATP	Adenosine 5'-triphosphate
B	Bound fraction
BDNF	Brain derived nerve growth factor
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CaMKII	Calcium/calmodulin-dependent protein kinase II
CAPs	Clathrin associated proteins
CASK	CaM kinase, SH3, and GK domain containing protein
CAZ	cytoskeletal matrix assembled at active zones
CC	Coiled-coil
CGRP	Cacitonin gene-related peptide
CNS	Central nervous system
CRIPT	Cysteine-rich interactor of PDZ three

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LIST OF ABBREVIATIONS (Continued)

div	Days in vivo
DNQX	6,7-Dinitroguinoxaline
E18 (15)	Embryonic day 18 (15)
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscope or electron microscopy
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FC	Fragment C
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Fz	frizzled
GABA	γ-Aminobutyric acid
$GABA_AR$	GABA receptor α subunit
GAP-43	Growth associated protein of 43 kDa
GAT1	GABA transporter 1
GFP	Green fluorescent protein
GK	Guanylate kinase
GKAP	Guanylate-like kinase domain associated protein
GRIP	Glutamate receptor interacting protein
GluR1	AMPA receptor subunit 1
GSK	Glycogen synthase kinase

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GST	Glutathion-s-transferase
GuK	Guanylate-like kinase
HBSS	Hank's balanced salt solution
IgG	Immunoglobulin G
LAR	Leukocyte common antigen-related receptor
LDCV	Large dense core vesicle
LTP	Long term potentiation
MAGUK	Membrane-associated guanylate-like kinase
mAb	Monoclonal antibody
MAP	Microtubule associated protein
mEPSP	miniature excitatory postsynaptic potentiation
mIPSP	miniature inhibitory postsynaptic potentiation
MuSK	Muscle-specific kinase
NB	Non-bound fraction
N-CAM	Neuronal cell adhesion molecule
NEM	N-ethylmaleimide
NMDA	N-methyl-D-aspartate
NMJ	Neuromuscular junction
nNOS	Neuronal nitric oxide synthase
NP-40	Nonidet P40
NSF	NEM-soluble factor
NT-3	Neurotrophin 3

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PB	Phosphate buffer
PBH	Piccolo Bassoon homology
PBS	Phosphate buffer saline
PDZ	PSD95/DlgA/ZO-1
PRA-1	Prenylated Rab acceptor 1
PSD	Postsynaptic density
PTV	Piccolo transport vesicle
rAb	rabbit polyclonal antibody
RIM	Rab3a interacting molecule
RIPA	Restricted interruption of protein association
RTV	RIM transport vesicle
SAP	Synapse associated protein
SCa	Slow component a
SCb	Slow component b
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
SH3	Src homology 3
SNAP-25	Synaptosome-associated protein of 25 kDa
SNARE	Soluble NSF protein attachment receptor
Stg-lu Ab	Synaptotagmin luminal domain antibody
TBS	Tris buffer saline
TTX	Tetanus toxin

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LIST OF ABBREVIATIONS (Continued)

VAMP	Vesicle associated membrane protein
TGN	Trans golgi network

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INTRODUCTION

The term "synapse" was introduced by Sir Charles Scott Sherrington in 1897, when he "felt the need of some name to call the junction between nerve-cell and nerve-cell, because the place of junction now entered physiology as carrying functional importance" (Fulton, 1938). The "functional importance" of synapses recognized by Sherrington more than a hundred years ago has indeed been crucial to the understanding of the function of the nervous system. Synapses are sites of cell-cell contacts that are highly specialized for neural transmission, which makes up the functional basis of the nervous system. Synapses are the information processors that ensure rapid and efficient communication between neurons; they are the building blocks of learning and memory; they are the functional units that maintain cognition and consciousness. Accurate and regulated neural transmission requires complex and highly ordered structural organization. While generations of neuroscientists in the past century have made tremendous advances in uncovering the myth of neural transmission and synapse structure, our understanding of how this structure is formed with such precision and elegance is largely rudimentary. The analysis of the structure components of synaptic junctions has recently led to the identification of a family of proteins, including Piccolo and Bassoon, with restricted presynaptic localization. These proteins have provided valuable tools to our further understanding of synapse assembly. The general goal of this research is to understand one facet of the process of synapse formation by revealing the mechanism that underlies the assembly of presynaptic terminal in the central nervous

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system (CNS). This dissertation will focus on the characterization of the presynaptic cytomatrix proteins Piccolo and Bassoon during neuronal development and synaptogenesis.

The Structure of Neurons

Like any other organ or tissue, the nervous system is made up of cells. There are two distinct classes of cells: nerve cells (neurons) and glial cells (glia). Neurons are the signaling units in the nervous system, and glial cells are the supporting elements. The supporting roles of glial cells include providing physical protection to neurons, separating and insulating neurons from each other, buffering extracellular ions and removing chemical neurotransmitters released by neurons during synaptic transmission, providing guidance for the migration of neurons and the outgrowth of axons during development, and providing nutritive support for neurons. Although neurons can not survive without glial cells in vivo, the primary functions of the nervous system are mainly carried out by neurons. Therefore, understanding the cell biology of neurons will help us understand the mechanisms of nervous system functioning.

There are many different types of neurons present in the vertebrate nervous system, differing greatly in size, morphology, and physiological properties. Despite the differences, neurons share common structural and biological characteristics that distinguish them from all other cell types.

First, neurons are polarized. A typical neuron has four morphologically defined regions: the cell body (soma), dendrites, axon, and presynaptic terminals (Figure 1; Kandel, 1991). The cell body consists of a nucleus and a perikaryal region that contains the metabolic machinery of the cell, such as Golgi, endoplasmic reticulum (ER), and a

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high concentration of poly-ribosomes. Extending from the cell body are two types of processes called dendrites and axons. A typical neuron usually has several dendrites and one axon. Dendrites are relatively thicker in diameter and usually have extensive branches. They serve as the main apparatus for receiving the input to the neuron from other neurons. An axon is usually thinner, longer, and highly branched. However, unlike the cell body and dendrites, axons do not contain polyribosomes (Bartlett and Banker, 1984; Deitch and Banker, 1993). The main function of an axon is to propagate the information in the form of electrical signals and action potentials over a long distance to the target cell. Situated along the more distal segments of axons at sites of cell-cell contact with dendrites are vericosities or swellings called presynaptic terminals or buttons. The swelling is due to the presence of a large number of small clear vesicles containing neurotransmitters called synaptic vesicles. It is at these presynaptic terminals that electrical signals are converted into chemical signals, through the release of the neurotransmitters from synaptic vesicles and an extracellular space between the presynaptic terminal and the target cell called the synaptic cleft, and received by the postsynaptic target cell.

Second, in each neuron, information flows in a predicted and consistent direction. The information is received by dendrites and the cell body and integrated in the cell body. The region connecting the axon to the cell body, called the axon hillock, is a specialized gate for the subsequent information propagation. An action potential will be generated at the axon hillock if the information received by dendrites and the cell body meets a critical threshold (Figure 1). The action potential then travels along the axon away from the cell body to the presynaptic terminal. There, the action potential triggers the release of the neurotransmitter from the presynaptic terminal, which will diffuse to the postsynaptic

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Figure 1. The Main Structural Features of a Typical Vertebrate Neuron

This neuron is drawn to illustrate its four regions and its points of contact with other nerve cells. The cell body contains the nucleus and perikaryon. Extending from the cell body are two types of processes: dendrites and axons. The axon is the transmitting element of the neuron. The axon hillock, the region of the cell body where the axon emerges, is where the action potential is initiated. Branches of the axon of one neuron (the presynaptic neuron) form synaptic connections with the dendrites or cell body of another neuron (the postsynaptic cell). The branches of the axon of one neuron may form synapses with as many as 1,000 other neurons.

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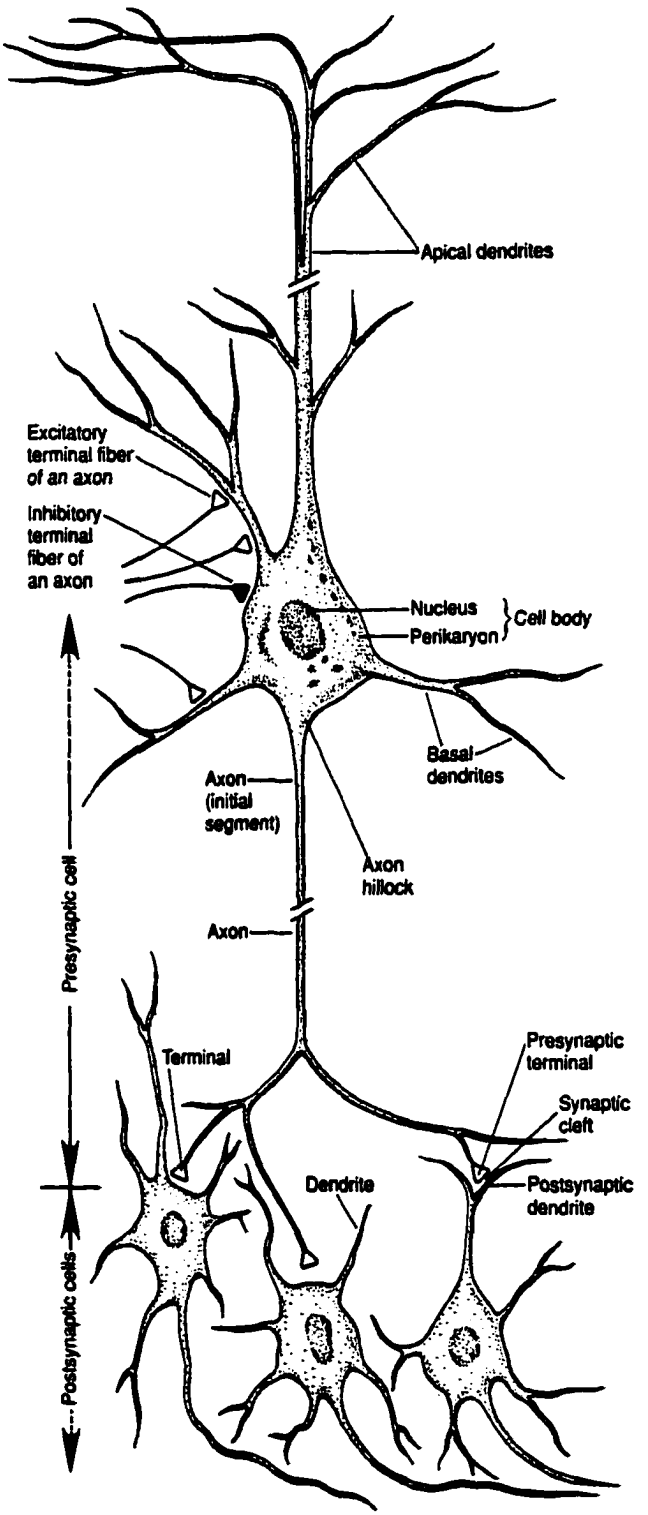

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target cell and therefore deliver the information. The polarity of neurons ensures the unidirectional propagation of signal from the dendrites to presynaptic terminals.

Third, the connection between a neuron and a neuron or between a neuron and an excitable target cell (e.g., a muscle cell) is specific. The nervous system is a network of interconnected neurons. The connections are highly specialized. Each neuron makes specific connections with some target cells, but not others, at contact sites called synapses. Information can only be passed from a neuron to a target cell at these sites. The signal transduction at synapses connects different types of neurons and target cells into a circuit, allowing the signals to be processed in the most efficient and effective way.

Cellular polarization, unidirectional flow, and connection specificity distinguish neurons from other cell types, forming the cellular basis for the function of the CNS.

Synaptic Architecture

Synapses are specialized cell-cell contact sites where signals are transduced from a neuron to its target with speed and precision. Based on the nature of the signal transmitted, synapses can be divided into chemical synapses and electrical synapses (Bennett, 1972). Chemical synapses use a chemical intermediate to propagate the signal, while electrical synapses are gap junctions allowing bidirectional flow of current between two neurons in the form of ions (Leitch, 1992; Bennett, 2000). This dissertation will only focus on chemical synapses, and hereon, the chemical synapse will be referred to as the synapse.

The morphology of the synapse has been extensively studied by electron microscopy (EM). Synapses formed between different types of neurons in different organisms and in different regions of nervous systems have a variety of different forms.

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For example, based on the origin of pre- and postsynapstic elements, synapses can be divided into axosomatic, axodendritic, axoaxonic, and even somatodendritic synapses (Figure 2A, 2B, and 2G; Gray, 1974). In the hippocampus and cerebral cortex, many synapses have a distinct spine like a postsynaptic structure projecting from the dendritic shaft. These synapse are called spine synapses (Figure 2A). At retinal photoreceptor synapses and at the squid giant synapse, ribbon like structures can be seen within the presynasptic terminal (Figure 2O). In the thalamus and olfactory bulb, a special type of synapses called serial synapses can be observed. Here, both the pre- and postsynaptic elements have the morphological features of dendrites that display "reciprocal synapses," in which one process is presynaptic to another at one point and postsynaptic to that process at an adjacent site (Figure 2C, 2D; Gray, 1974).

Despite these differences in size and shape and the origin of pre- and postsynapstic elements, the basic structure of all synapses share common characteristics. For example, a typical chemical synapse in vertebrate CNS can be dissected into three parts: a presynaptic terminal, a postsynaptic terminal, and a narrow gap between the pre- and postsynaptic terminals, called the synaptic cleft (Figure 3; Landis et al., 1988). A key feature of the presynaptic terminal is that it is filled with a large number vesicles (from 100 to 1,000). Generally, two types of vesicles can be observed. The first, called synaptic vesicles, is approximately 30-50 nm in diameter, appears to have clear centers, and contains fast-acting neurotransmitters like amines and amino acids. The second type, called large dense core vesicles, is larger in diameter (from 100 to 200 nm), has an electron dense core, and contains slow-acting neurotransmitters and peptides (Burns and Augustine, 1995). Synaptic vesicles are found clustered at a specialized region of presynaptic plasma membrane, termed the active zone. The cytoplasmic face of the

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Figure 2. Various Types of Synapses Seen in the Electron Microscope

(A) Axosomatic, axondendritic, and axoaxonic contacts on a cortical pyramidal cell. Type I synapses are excitatory spiny synapses, mostly axodendritic. Type II synapses are shaft synapses.
(B) Axoaxonal contacts of the type thought to be involved in presynapsic inhibition of the spinal cord.
(C) Serial synapses seen in the thalamus.
(D) Serial synapses seen in the olfactory bulb.
(E) Synapses between amacrine (i.e., axonless) cells.
(F) En passant synapses at a node of Ranvier.
(G) A somatodendritic synaspe.
(H) An electronic contact in the brain of a fish.
(I) A combined electrical and chemically transmitting synapse.
(J–M) Various forms of gap junction synapse.
(O) A photoreceptor synapse in the retina, showing the typical presynaptic ribbon.
Abbreviations: sp, spine; per, perikaryon; is, initial segment; my, myelin; den, dendrite; msf, mossi fiber; sv, synaptic vesicle.

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plasma membrane associated with active zones is electron dense as compared to the perisynaptic plasma membrane, indicating the presence of underlying cytoskeletal matrix. Synaptic vesicles docked at the active zone are surrounded by this meshwork of fine filaments (Landis et al., 1988). The synaptic cleft also contains a denser concentration of material relative to the surrounding extracellular space, indicating the presence of a high amount of extracellular matrix (ECM) proteins and adhesion molecules (Landis et al., 1988; Vaughn, 1989). Based on the morphology of the postsynapse, synapses in the CNS can be divided into two types (Figure 2A; Gray, 1959). Type I, or asymmetric synapses, has a thick electron dense region associated with the postsynaptic membrane, called postsynaptic density (PSD), which is aligned with the active zone (Figure 3). The PSD is enriched in receptors, adhesion molecules, and cytoskeleton components (Garner and Kindler, 1996; Kennedy, 1997). Type II, or symmetric synapses, has a less pronounced PSD, though it does have a similar presynaptic electron thickening as asymmetric synapses (Vaughn, 1989).

Synaptic Vesicle Exocytosis

Neurotransmission at chemical synapses is mediated by a variety of chemical neurotansmitters released from the presynaptic neuron. Independent of the neurotransmitter type, neurotransmission is achieved by synaptic vesicle exocytosis triggered by the depolarization of the plasma membrane in presynaptic terminals. The arrival of an action potential at presynaptic nerve terminals induces the influx of calcium ions, which then triggers the fusion of synaptic vesicles with the presynaptic plasma membrane and therefore the release of the neurotransmitters stored in synaptic vesicles. After exocytosis,

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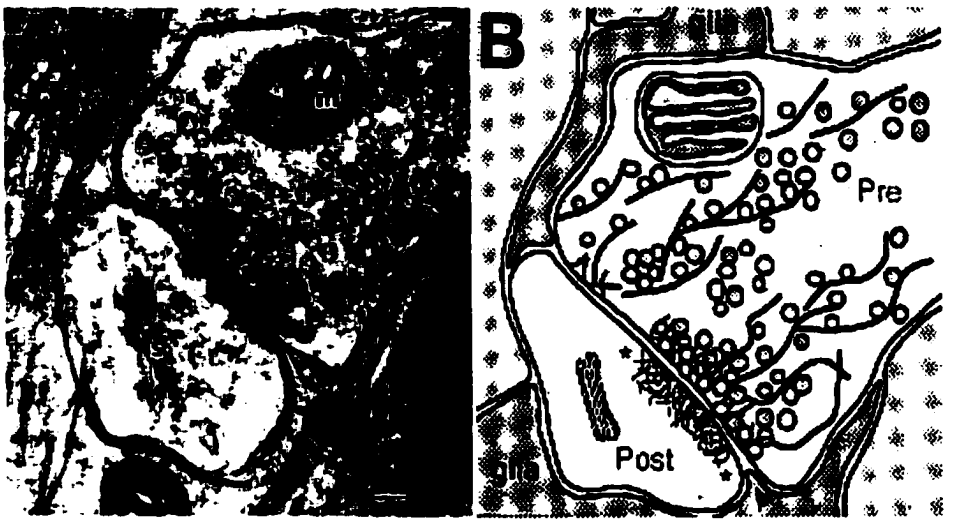

Figure 3. The Structure of a Glutamatergic Synapse

(A) An EM micorgraph of a cerebellar glutamatergic synapse. The presynaptic bouton (*) is filled with synaptic vesicles and contains a mitochondrion (m). Opposed to the presynapse is the postsynaptic spine (s). Between the pre- and postsynaptic elements is the synaptic cleft (arrow). There is an electron dense region across the synaptic cleft. The postsynaptic side of this region is the PSD, and the presynaptic side is the active zone.
(B) Line drawing of this synapse. The presynaptic nerve terminal (pre) contains multiple pools of synaptic vesicles, with the readily releasable vesicles clustered at the active zone. The PSD is depicted by a meshwork of filaments (*). Glial cells are also part of synapse structure (Glia).

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synaptic vesicles are rapidly retrieved by endocytosis and recycled for subsequent release (Burns and Augustine, 1995).

Synaptic vesicles in the presynaptic terminal can be divided into three pools: readily releasable pool, proximal pool, and reserve pool (Pieribone et al., 1995; Garner et al., 2000a; Südhof, 2000). The readily releasable pool refers to a layer of synaptic vesicles, usually 5 to 10 vesicles, that attached to the active zone plasma membrane (Schikorski and Stevens, 1997; Figure 3). During any given cycle of activity, these vesicles are released. Synaptic vesicles that reside in the terminal, about 200 nm away from the active zone, comprise the reserve pool. Situated between the reserve pool and readily releasable pool is the proximal pool, usually around 17 to 20 vesicles per active zone (Südhof, 2000). Synaptic vesicles in this pool have to replace the readily releasable pool and become associated with the plasma membrane in a docked state before they can be released (Südhof, 1995). After docking, a prefusion or priming step is required to prepare the vesicle for the calcium triggered neurotransmitter release. Adenosine 5'-triphosphate (ATP) is required in the priming process (Südhof, 1995). Once the synaptic vesicle is primed, it will fuse with the active zone plasma membrane within 0.1 msec of calcium influx through voltage-gated calcium channels embedded in the active zone (Sabatini and Regehr, 1999). Each of the three steps of synaptic vesicle exocytosis is tightly regulated.

Surprisingly, synaptic vesicles docking and fusion within the presynaptic boutton occur only at the active zone. This suggests that synaptic vesicles specifically recognize the active zone. The molecular analysis of the proteins involved in docking and fusion of synaptic vesicles has revealed the existence of a core protein complex called the soluble N-ethylmaleimide (NEM) sensitive fusion (NSF) protein attachment receptor (SNARE)

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complex (Rothman and Warren, 1994; McMahon et al., 1995; Schiavo et al., 1995). The SNARE complex is formed by the synaptic vesicle protein vesicle associated membrane protein (VAMP)/synaptobrevin and the plasma membrane proteins syntaxin and SNAP-25. VAMP/synaptobrevin contains three defined regions: a hydrophobic carboxyl terminus that is anchored in synaptic vesicle membrane, a proline-rich amino terminus, and a 70 amino acids central region that forms an α-helical structure (Südhof et al., 1989). Syntaxin also has a hydrophobic carboxyl terminus that is anchored to the plasma membrane rather than synaptic vesicles. SNAP-25, synaptosomal protein of 25 kDa, is anchored to the plasma membrane through multiple palmitoylated cysteines situated in the middle of the protein (see Rizo and Südhof, 1998). Recently, the three-dimensional structure of the minimal SNARE core complex has been solved (Sutton et al., 1998). The complex is formed via a four-helix bundle, one α-helix each from VAMP/synaptobrevin and syntaxin and two α-helixes from SNAP-25. The helical bundle brings these three proteins very close together, reducing the distance between the synaptic vesicle and the plasma membrane, allowing the two membrane bilayers to merge. Additionally, in the middle of this four-helix bundle, two glutamine residues from SNAP-25 and an arginine residue from VAMP/synaptobrevin form salt bridges that strengthen the compact structure. The hydrophobic residues present along each helix are buried within the core of the complex. These features result in a very stable structure with an unfolding temperature of 95°C. When this stable structure is formed, a large amount of free-energy is released, which is thought to drive membrane fusion (Hayashi et al., 1994).

The assembly of this core complex is a multistep and highly regulated process. In the first step, the two plasma membrane proteins, syntaxin and SNAP-25, form a dimeric complex. In the second step, the synaptic vesicle protein, VAMP/syuaptobrevin, binds

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the dimeric complex (Nicholson et al., 1998). Syntaxin plays a critical role in the assembly of the core complex. Syntaxin contains four α-helixes, three in amino terminus and one in carboxyl terminus. The carboxyl terminal helix participates in SNARE complex formation, called the SNARE motif. In its "resting" state, the N-terminal three helixes fold back and bind the SNARE motif, resulting in a closed conformation. The formation of a SNARE complex requires the open conformation in which the SNARE motif is exposed (Dulubova et al., 1999). In the close conformation, synatxin binds a presynaptic soluble protein Munc18 or nsec1, which is the mammalian homologue of the *Caenorhabditis elegans* unc13 and the yeast sec1 (Hata et al., 1993; Pevsner et al., 1994). To initiate the formation of core complex, syntaxin has to be freed from Munc18 and change its conformation into an open state. The exposed SNARE motif can then interact with SNAP-25 and form the dimeric complex. Although the transition mechanism is not entirely clear, the closed-Munc18 binding state and the open-SNAP-25 binding state of syntaxin are mutually exclusive (Misura et al., 2000). The structural and biophysical analyses of the complexes provide a clear picture of its critical role in membrane fusion.

The Cytoskeletal Matrix at Active Zones

The presyanptic active zone has unique morphological characteristics and functional importance. Morphologically, the plasma membrane at the active zone is parallel to and in close register with the postsynaptic plasma membrane. When analyzed by EM, an electron dense thickening is observed lying just underneath the active zone plasma membrane, interspersing between the docked vesicles. This observation suggests the presence of underlying cytoskeletal matrix (Landis, 1988). Functionally, the active zone is the only region of the plasma membrane where synaptic vesicles will dock and

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fuse in an activity-dependent manner (Landis, 1988; Matthews, 1996). Given the unique properties of the active zone, several specialized proteins must be localized at the active zone and involved in the synaptic vesicle recycling. The two SNARE proteins, syntaxin and SNAP-25, are localized in the active zone plasma membrane where they participate in the formation of the exocytotic fusion complex. However, neither of them is selectively concentrated in the presynaptic membrane at the active zone (Garcia et al., 1995). This suggests that other proteins present in the cytoskeletal matrix at active zones are responsible for the selective tethering and exocytosis of synaptic vesicles at the synapse.

In a search for proteins that are involved in the assembly of synaptic junctions, Garner and colleagues (1993) took a combined molecular and immulogical screening approach to identify proteins that are specifically enriched at synaptic junctions. Two very interesting high molecular weight proteins, Piccolo and Bassoon, were found to be specifically localized at the presynaptic cytoskeletal matrix assembled at the active zones, also referred to as CAZ (Cases-Langhoff et al., 1996; Langnaese et al., 1996; Garner et al., 2000a). More recently, two other proteins, Rab3a interacting molecule (RIM) and Munc13-1, have been identified to be localized to the CAZ (Wang et al., 1997; Betz et al., 1998). Piccolo, Bassoon, RIM, and Munc13-1 share several common features that are characteristic of the CAZ proteins. First, they exhibit a restricted presynaptic localization. Second, they are large, multidomain proteins, with molecular weights of 530 kDa, 420 kDa, 180 kDa, and 200 kDa, respectively (Cases-Langhoff et al., 1996; Wang et al., 1997; Betz et al., 1998; Dieck et al., 1998; Figure 4). Large size, multidomain structure, and specific localization suggest involvement in protein-protein interactions that promote the tethering of either synaptic vesicles and/or the machinery that directs synaptic vesicle

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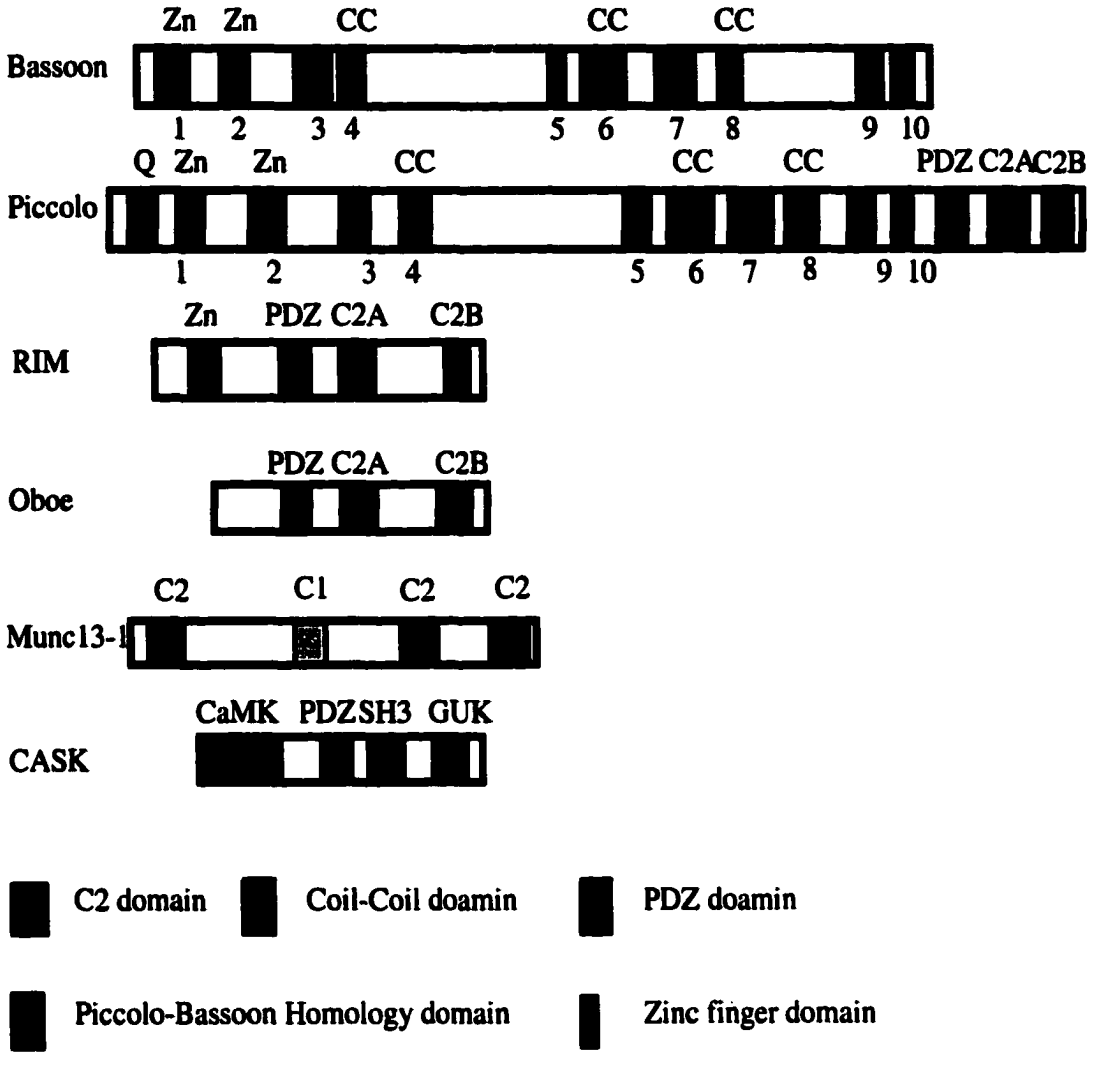

Figure 4. Domain Structure of CAZ Proteins

Schematic diagram of the Piccolo and Bassoon homology domains as compared to other CAZ proteins: RIM, Oboe/RIM2, Munc13-1, and CASK. Bassoon and Piccolo are stucturally related CAZ proteins composed of at least 10 regions of homology called Piccolo Bassoon Homology domains (PBHs, numbered 1-10). These PBHs included two zinc fingers (Zn), three coiled-coil domains (CC), and several proline-rich sequences. Piccolo also contains a PDZ domain and two C2 domains at the carboxyl terminus. RIM contains a single zinc finger domain, a PDZ domain, and two C2 domains. These domains share limited homology with the analogous domains in Piccolo. Oboe/RIM2 contains a PDZ domain and two C2 domains. Munc13-1 contains three C2 domains and a C1 domain involved in phorbol ester binding. CASK is a member of the MAGUK superfamily and contains a CaMKII-like domain, a PDZ, an SH3, and a GUK domain.

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docking and fusion. Studies on RIM have shown that it may regulate neurotransmitter release through its interaction with Rab3, which is a small GTPase (Wang et al., 1997). Genetic analyses on mouse Munc13-1 and its *C. elegans* homologue unc-13 and *Drosophila* homologue dunc-13 have shown that disruption of these genes dramatically suppress both spontaneous and evoked release of neuro-transmitter (Aravamudan et al., 1999; Augustin et al., 1999; Richmond et al., 1999). Piccolo and Bassoon are the two largest members in this family, and they share 50% sequence homology with over 10 homology domains (Fenster et al., 2000). Piccolo and Bassoon do not appear to interact directly with synaptic vesicles, rather the zinc finger domains of Piccolo have been found to bind the prenylated Rab acceptor PRA1 (Fenster et al., 2000). PRA1 is a soluble protein that can interact with both VAMP/synaptobrevin II and Rab3 (Martincic et al., 1997), suggesting that Piccolo may regulate synaptic vesicle trafficking and maturation indirectly via its interaction with PRA1.

In addition to the CAZ proteins, several members of the membrane-associated guanylate kinase (MAGUK) family may also be involved in the assembly of the cytoskeletal matrix at active zones. MAGUK proteins contain PDZ (PSD-95/SAP90/DLG/ZO1), SH3 (src-homology 3), and GUK (guanylate kinase-like) domains. These domains are well-characterized sites actively involved in protein-protein interactions. MUGUKs are thought to function as adapter proteins involved in the localization and assembly of synaptic membrane-associated signaling complexes (Garner and Kindler, 1996; Craven and Bredt, 1998; Garner et al., 2000b). Among many family members, SAP90 (synapse-associated protein 90), SAP97 (synapse-associated protein 97), and CASK (CaM kinase/SH3/guanylated kinase protein) have been found in presynaptic terminals (Kistner et al., 1993; Muller et al., 1995; Hsueh et al., 1998; Koulen

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et al., 1998). CASK directly interacts with syndecan and neurexin, which are cell adhesion molecules, neuronal calcium channels, and Mint1, which is a Munc18 interacting protein (Hata et al., 1996; Butz et al., 1998; Hsueh et al., 1998; Maximov et al., 1999). These interactions suggest that CASK may have a potential in coupling the calcium-dependent synaptic vesicle exocytosis with the neuronal adhesion.

The Synaptic Cell Adhesion

The pre- and postsynaptic terminals are separated by the synaptic cleft. EM studies has revealed that synaptic cleft has an average width of 20 nm and is filled with undefined electron-dense material (Gray, 1959; Akert et al., 1972). In synaptosome preparations where nerve endings are pinched off from the intact brain during the homogenization process, the PSD is still attached to the presynaptic active zone, and the synaptic junction is maintained (Whittaker, 1988). The fact that the pre- and postsynaptic sides of synapses are tightly bound to each other suggests that there are cohesive forces mechanically linking the two sides together and holding the active zone and PSD in close register. The adhesive force linking the pre- and postsynaptic sides is likely due to adhesion molecules. Like other cell-cell adhesion sites, the three classical cell adhesion molecules, cadherins, integrins, and immunoglobulin-domain proteins, appear to be involved in synaptic adhesion (Takeichi, 1990; Leahy, 1997; Takeichi et al., 1997). Unlike most other cell-cell adhesion sites, synaptic junctions require asymmetric cell-cell recognition. Recently, neuronal specific cell adhesion molecules, neurexins and neuroligins have been characterized and found on the pre- and postsynaptic plasma membrane, respectively (Ichtchenko et al., 1995; Butz et al., 1998). They may be

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involved in the specific pre- and postsynaptic adhesion. Additionally, syndecans may also play a role in synaptic junction assembly (Carey, 1997).

Cadherins are calcium-dependent, homotypic cell adhesion molecules (for review see Takeichi, 1990). Classical cadherins consist of an extracellular region containing five tendem repeats of cadherin domains, a transmembrane domain, and a highly conserved cytoplasmic tail (for review see Takeichi, 1990). The extracellular cadherin domains mediate calcium-dependent homophilic interactions. The cytoplasmic tail of cadherins binds β-catenin, and the latter binds α-catenin, which in turn associates with the actin cytoskeleton. Therefore, catenins link the actin cytoskeleton to the cell-cell adhesion sites (for review see Yap et al., 1997). There are at least 10 different isoforms of cadherins. Each has a distinct, characteristic distribution pattern in different brain regions (for review see Takeichi et al., 1997). Since cadherins form homophilic interaction, during brain development, the axon expressing a distinct isoform of cadherin is expected to only contact and form synapses with target cells that express the same isoform. These observations suggest that cadherins contribute to the establishment of synaptic specificity and pattern formation (for review see Takeichi et al., 1997). Interestingly, detailed analysis of the precise localization of cadherins and catenins by immunoelectron microscopy has revealed that these proteins are localized at the borders of active zone rather than in the synaptic junction itself (Uchida et al., 1996). This observation suggests that cadherins are peri-synaptic and that they are perhaps involved in establishing the initial contact site between an axon and a dendrite that leads to the formation of synaptic junctions. This is supported by temporal expression studies showing that N-Cadherin appears very early during nascent synapse formation (Benson and Tanaka, 1998).

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A second class of adhesion molecules belongs to the immunoglobulin family of cell adhesion molecules. This superfamily has several hundred members. Most of them have several extracellular immunoglobulin domains with intradomain disulfide bonds. They are typically attached to the cell plasma membrane by a single transmembrane domain or a glycolipid anchor (for review see Leahy, 1997). Neuronal immunoglobulin cell adhesion molecules include vertebrate L1 and neuronal cell adhesion molecule (N-CAM), *Drosophila* fasciclin II, and *Aplysia* cell adhesion molecule (ApCAM). L1 and N-CAM mediate calcium-independent homophilic or heterophilic cell adhesion. They are involved in cell migration, axonal outgrowth, and path finding (for review see Rutishauser and Landmesser, 1996; Brümmendorf et al., 1998). Their function in synaptogenesis is as yet unclear, because neither is enriched in synapses. Furthermore, mice lacking N-CAM have morphologically normal synapses, albeit a loss of mossy fiber long term potentiation (LTP) in the CA3 region of the hippocampus (Cremer et al., 1998).

Recently, the neurexin and neuroligin families have been identified and suggested to be candidate synaptic cell adhesion molecules (Ichtchenko et al., 1995; Butz et al., 1998). Neurexins are neuron-specific proteins with more than 1,000 isoforms that arise via alternative splicing (Missler et al., 1998). Vertebrates contain at least three neurexin genes (neurexin 1, 2, and 3), and each gene has two independent promoters that direct transcription of the larger α-neurexins and the shorter β-neurexins (Ushkaryov et al., 1992; Ushkaryov and Südhof, 1993; 1994). Structurally, α-neurexins and β-neurexins are very similar: they are both composed of a relatively large N-terminal extracellular domain, a transmembrane region, and a short C-terminal cytoplasmic domain. The extracellular region of α-neurexins is much longer than that of β-neurexins, and the

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transmembrane region and cytoplasmic tail of α-neurexins are identical to those of β-neurexins (Missler et al., 1998). Differences in their extracellular domains result in different binding specificities: α-neurexins bind to neurexophilins, and β-neurexins bind to neuroligins (Missler and Südhof, 1998a). Neurexophilin is a neuropeptide-like, 29 kDa protein that forms a tight complex with α-neurexins (Petrenko et al., 1996). Neurexophilins contain an N-terminal signal peptide, a proteolytically processed N-terminal region, and two C-terminal conserved domains. They are synthesized, processed, and secreted from neurons, but their exact functions are not clear (Missler and Südhof, 1998b). Neuroligins were dicovered as endogenous ligands for β-neurexins (Ichtchenko et al., 1995). Similar to neurexins, neuroligins are neuron-specific cell surface proteins that contain an N-terminal extracellular domain, a transmembrane region, and a short cytoplamic tail (Ichtchenko et al., 1996). The binding of neuroligins to β-neurexins is calcium-dependent, and the binding results in cell-cell adhesion (Nguyen and Südhof, 1997). The developmental expression of neuroligins tightly parallels synaptogenesis, with a peak during the second postnatal week in mice. Immuno-EM studies revealed that neuroligin 1 is highly enriched in synaptic junctions, with a postsynaptic localization, and seemed to be excluded from GABAergic synapses (Song et al., 1999). Like neurexins, neuroligins also have several alternative spliced variants (Ichtchenko et al., 1996). The presence of a large number of isoforms suggests that the binding of neuroligins to neurexins may be isoform-specific, which may mediate a recognition event between neurons at the synapse and establish the type of synapses being formed.

The cytoplasmic tails of neurexins and neuroligins have both been found to interact with intracellular scaffolding proteins, suggesting a possible mechanism to

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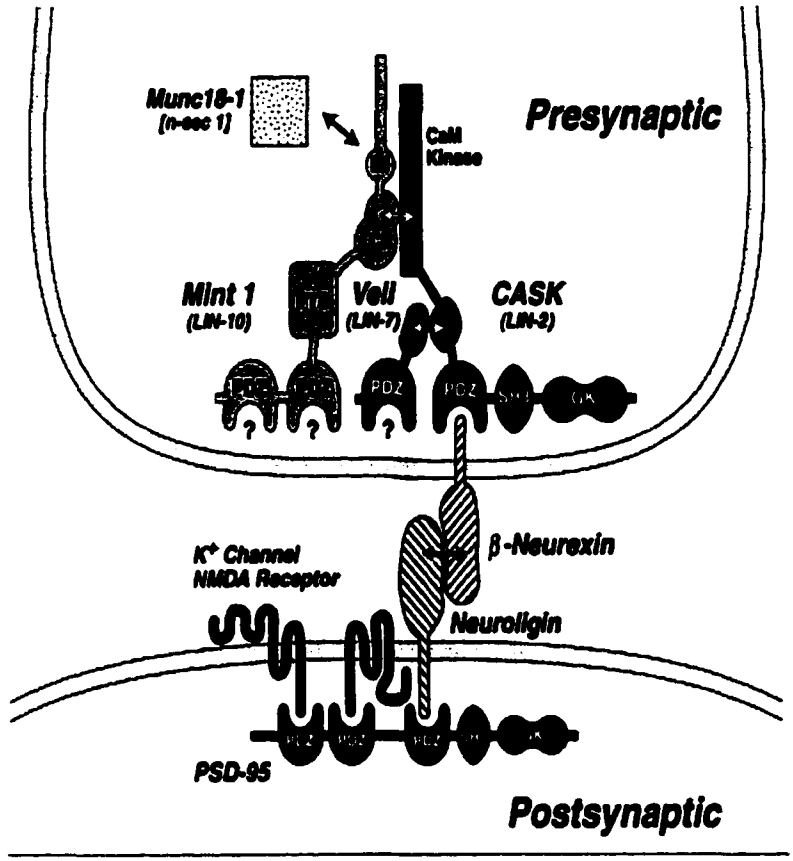

Figure 5. Model of the Intercellular Junctions Formed by the Neurexin-Neuroligin Interaction

The adhesion interaction between neurexin and neuroligin is coated by CASK, Mint 1, and veli on the presynaptic side and by SAP90/PSD95 on the postsynaptic side. Reprinted from Cell, Vol. 94, Butz S., Okamoto, M., and Sudhof, T. C., A Tripartite Protein Complex with the Potential to Couple Synaptic Vesicle Exocytosis to Cell Adhesion in Brain, pp773-782 (1998), with permission from Elsevier Science.

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couple neuronal adhesion and synaptic assembly (Figure 5). In the presynaptic plasma membrane, the cytoplasmic tail of β-neurexin binds to the PDZ domain of CASK (Hata et al., 1996). At the postsynaptic side, the cytoplasmic tail of neuroligin interacts with the PDZ domains of SAP90/PSD95, SAP97, and SAP102 (Irie et al., 1997; Hirao et al., 1998). CASK, SAP90/PSD95, SAP97, and SAP102 are all members of the MAGUK superfamily of adapter proteins, which are thought to be involved in clustering channels and receptors at synapses (see below). The distinct protein complexes interacting with the cytoplasmic tails of either neurexins or neuroligins at both sides create an intrinsic polarization of the synaptic junction. Thus, the adhesion formed between neurexins and neuroligins can act to nucleate the establishment of the asymmetric synapses.

The Postsynaptic Specialization

The postsynaptic plasma membrane is highly specialized for reception of the neurotransmitter signals released from the presynaptic terminal and its transmission to the rest of the postsynaptic neuron. Morphologically, the most pronounced structural feature of postsynapse is the PSD (Figure 3). It was originally defined ultrastructurally as the electron-dense thickening associated with the cytoplasmic face of the postsynaptic plasma membrane of excitatory synapses. In recent years, the PSD has been studied intensively due to its functional importance and technical feasibility. The PSD contains a high concentration of receptors, ion channels, and scaffolding proteins, as well as many other proteins of regulatory or enzymatic functions. Biochemically the PSD is rather insoluble in nonionic detergents, making it possible to isolate the PSD with considerable purity. A large number of molecules have been identified as components of the PSD. These molecules belong to several different categories, including (a) receptors, mainly

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ionotropic and metabotropic glutamate receptors; (b) scaffolding proteins, such as MAGUKs; (c) cytoskeletal elements, such as actin, tubulin, and brain spectrin; (d) kinases, such as calmodulin-dependent protein kinase II (CaMKII), protein kinase C, protein kinase A, and receptor tyrosine kinases; (e) adhesion molecules, such as N-cadherin, syndecan-2, and neuroligins (Langnaese et al., 1996; Ziff, 1997).

At excitatory synapses, glutamate receptors are the key functional component of the PSD. Glutamate receptors can be classified as ionotropic receptors or metabotropic receptors. Among the ionotropic receptors, N-methyl-D-aspartate (NMDA) receptors and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors are the best studied receptors. While both NMDA receptors and AMPA receptors are concentrated in the PSD, NMDA receptors have a highly consistent presence in excitatory synapses, while a significant fraction of excitatory synapses lack AMPA receptors, especially early during development (Nusser et al., 1998; Petralia et al., 1999; Takumi et al., 1999). It has recently been suggested synapses that contain NMDA receptors but lack AMPA receptors correlate to the functionally "silent" synapses that have NMDA receptor-mediated response but lack AMPA receptor-mediated response (Malenka and Nicoll, 1997; Petralia et al., 1999). Unlike NMDA receptors and AMPA receptors, metabotropic receptors are not localized to the PSD but rather to the periphery region surrounding the PSD (Nusser et al., 1994; Luján et al., 1997). The subsynaptic segregation of metabotropic and ionotropic glutamate receptors suggests the presence of specific intracelluar scaffolding proteins that are important for immobilization and clustering different classes of glutamate receptors at specialized postsynaptic sites.

Recent effort in dissecting the components of the PSD has revealed a partial picture of the molecular architecture within the PSD. Among the identified molecules,

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MAGUK family proteins play a pivotal role in the assembly of the PSD and the clustering and targeting of glutamate receptors to their synaptic sites. MAGUK family proteins are characterized by the presence of PDZ domains, an SH3 domain, and a guanylate kinase-like (GK) domain (Garner and Kindler, 1996). PDZ, SH3, and GK domains are all actively involved in protein-protein interactions. Four members, PSD95/SAP90, SAP97, SAP102, and PSD93/Chapsyn-110, have been shown to be components of the PSD and to be associated with NMDA or AMPA receptors in synapses (Cho et al., 1992; Kistner et al., 1993; Kornau et al., 1995; Müller et al., 1995, 1996; Kim et al., 1996).

NMDA receptors are heterometic complexes composed of NR1 and NR2 subunits (Dingledine et al., 1999). The C-termini of NR2 subunits, including NR2A, 2B, 2C, and 2D, bind to the first two PDZ domains of PSD95/SAP90 (Niethammer et al., 1996). The third PDZ domain and the GK domain of PSD95/SAP90 bind to cysteine-rich interactor of PDZ three (CRIPT) and microtubule associate protein 1A (MAP1A) respectively, both of which are microtubule-binding proteins (Brenman et al., 1998; Niethammer et al., 1998; Passafaro et al., 1999), suggesting that PSD95/SAP90 may serve as a linker coupling NMDA receptors with the postsynaptic tubulin-based cytoskeleton. Another GK domain binding protein GK domain associate protein (GKAP) interacts with the Shank/ProSAP family of proteins, which in turn binds to an actin-binding protein cortactin (Figure 6; Kim et al., 1997; Boeches et al., 1999; Naisbitt et al., 1999). Additionally, the cytoplasmic tail of NR1 subunits binds to α-actinin, which is an actin-binding protein of the spectrin superfamily (Wyszynski et al., 1997). Thus, NMDA receptors are linked indirectly with the actin cytoskeleton through intermediate scaffolding proteins. The multidomain PSD95/SAP90 protein can also assemble a

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signaling complex around the NMDA receptors by binding to a variety of cytoplasmic molecules that are involved in downstream signaling of NMDA receptors. These signaling molecules include neuronal nitric oxide synthase (nNOS), SynGAP (a GTPase-activating protein for Ras), Fyn, and Citron (Sheng and Pak, 2000). It has been shown that nNOS binds to the second PDZ domain of PSD95/SAP90 (Brenman et al., 1996). Neuronal NOS is a calcium/calmodulin-regulated enzyme that is selectively activated by calcium influx through NMDA receptors. This interaction can bring nNOS close to NMDA receptors (Sattler et al., 1999). All three PDZ domains of SAP90/PSD95 have been shown to interact with the C-terminus of SynGAP, which is an abundant PSD protein that may be involved in Ras modulation after its activation by tyrosine kinase (Chen et al., 1998; Kim et al., 1998). The third PDZ domain of SAP90/PSD95 also binds to a Rho-type GTPase Citron, which may be involved in NMDA receptor-dependent modulation of postsynaptic actin (Furuyashiki et al., 1999; Zhang et al., 1999). As discussed previously, protein kinases are enriched in the PSD. SAP90/PSD95 and its family members are also involved in localizing cytoplasmic protein kinases and phosphatase to the PSD. For example, an Src family tyrosine kinase Fyn is implicated in NMDA receptor modulation, and Fyn is brought to the vicinity of NMDA receptors via its interaction with the third PDZ domain of PSD95 (Salter, 1998; Tezuka et al., 1999).

Like NMDA receptors, the cytoplamic tails of AMPA receptor subunits also interact with intracellular PDZ domain-containing proteins. The difference is that they interact with a distinct set of molecules. AMPA receptors are formed by heteromeric or homomeric combinations of GluR1-4 subunits (Hollmann and Heineman, 1994; Dingledine et al., 1999). The C-termini of GluR2 and GluR3 interact with the fifth PDZ domain of GRIP (Figure 6; Wyszynski et al., 1997; Dong et al., 1999). The C-terminus of

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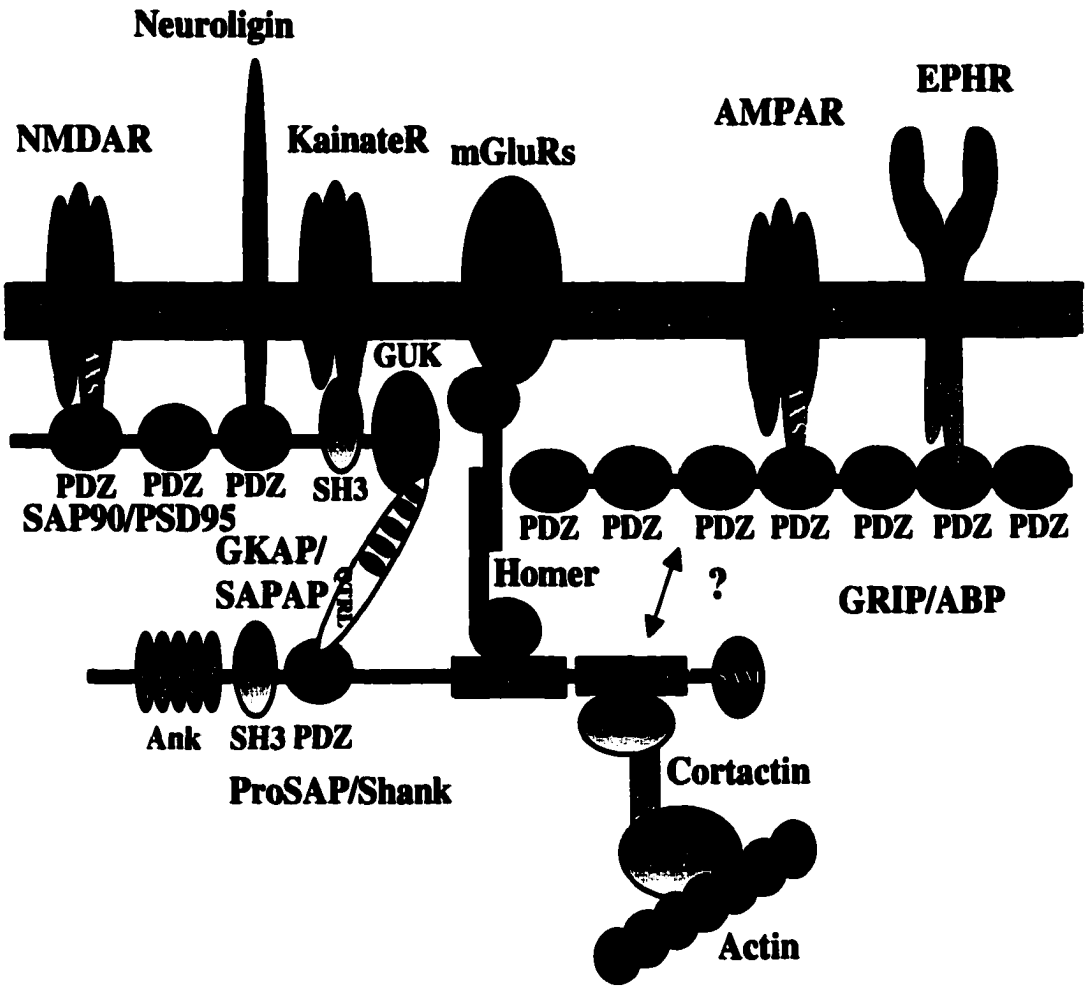

Figure 6. The Clustering and Localization of Postsynaptic Glutamate Receptors Mediated by Multidomain Adapter Proteins

MAGUKs are the best characterized adaptor proteins. The PDZ domains in SAP90/PSD95 interact with the C-terminus of NMDA receptor subunits (NMDAR) and with the cell adhesion molecule neuroligin. Kainate receptors interact with the SH3 domain of SAP90/PSD95, and GKAP interact with the GK domain of SAP90. GKAP in turns binds through its C-terminus to the PDZ domain in ProSAP/Shank family members. ProSAP/Shank also contain serveral ankyrin (Ank) repeats, an SH3 domain, a SAM domain, and a proline-rich region. The latter interacts with the EVH domains in Homer, which is a metabotropic glutamate receptor (mGluR)-binding protein, and also interacts with F-actin-binding protein cortactin. Another major component of PSD is the protein GRIP/ABP, which contains seven PDZ domains and interacts with both AMPA receptors (AMPAR) and ephrin receptors (EPHR).

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GluR1 interacts with the first PDZ domain of SAP97 (Leonard et al., 1998; Valtschanoff et al., 2000).

Although further studies are needed to characterize the functional significance of each protein-protein interaction, it is clear that the MAGUK family of adapter proteins is important for the assembly of the PSD. These multidomain proteins not only cluster and localize glutamate receptors to the PSD but also scaffold specific signaling and regulatory protein complexes in the vicinity of NMDA receptors and AMPA receptors, which allow the regional and rapid synaptic modulation.

Synaptogenesis

Neuronal morphogenesis is characterized by a period of neurite outgrowth, the establishment of neuronal polarity, and synaptogenesis (Tucker, 1990). Synaptogenesis is a crucial step in development of the nervous system to establish proper circuits and wiring. Currently, much of our understanding about synapse formation arises from studies of the neuromuscular junction (NMJ), due to the easy accessibility of this system. In mature NMJs, acetylcholine receptors (AChRs) are highly concentrated at the postsynapses. The synaptic localization of AChRs during synaptogenesis is an inductive event. Basic fibroblast growth factor (bFGF) and three neuronal-derived molecules, agrin, ARIA (neuregulin), and cacitonin gene-related peptide (CGRP), have been identified as regulators of muscle AChR's expression and clustering (Hall and Sanes, 1993). Among these factors, agrin appears to be the key player in AChR clustering (Nastuk and Fallon, 1993). Although both motor neurons and muscle cells synthesize agrin, only nerve-derived agrin is essential for most synaptic differentiation (Sanes et al., 1998). The action of agrin is believed to be mediated by muscle-specific kinase (MuSK;

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Glass et al., 1996). MuSK is a component of agrin receptor complex localized at the postsynapse in muscle cells. Moreover, MuSK knock out mice exhibit neuromuscular defects that are similar or more severe than agrin mutant mice (Glass et al., 1996). A downstream effector protein of agrin and MuSK is Rapsyn (43 kDa), which can cluster AChRs. Myristoylated rapsyn can interact directly with AChRs with 1:1 stoichiometry at synaptic sites (Froehner et al., 1981; Burden et al., 1983; Apel et al., 1997). Agrin induces the clustering of rapsyn in cultured myotube, and the clustering of AChRs occurs coincidentally with rapsyn (Apel et al., 1997). In rapsyn knock out mice, no AChR clusters were observed (Gautam et al., 1999). Taken together, the process of NMJ synaptogenesis is believed to consist of the following steps: (a) Upon initial motor neuron and muscle cell contact, agrin is secreted from motor neuron and deposited into the extracellular matrix, where it binds to MuSK agrin receptor complex; (b) activation of MuSK induces the clustering of rapsyn, which in turn clusters AChRs to the plasma membrane site juxtaposed to presynaptic terminal.

Much less is known about synaptogenesis in the CNS. Most of our current knowledge is revolved around the receptor clustering at the postsynaptic terminals. As introduced above, several classes of proteins have been identified as potential synapse organizing molecules in postsynaptic terminals. At glutamatergic synapses, PDZ domain-containing proteins, like SAP90/PSD95 and SAP102 have been shown to have the ability to cluster NMDA receptors, while GRIP has been shown to bind AMPA glutamate receptors (for review, see Kornau et al., 1997). At inhibitory synapses, gephyrin has been shown to be essential for glycine receptors clustering at glycinergic synapses in spinal cord (Kirsch et al., 1993). More interestingly, gephyrin localizes to synapses earlier than glycine receptors during development, indicating that gephyrin

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could serve as a clustering molecule, like rapsyn at the NMJ (Vannier and Triller, 1997). In searching for the CNS parallel of agrin, a presynaptically secreted protein Narp has been identified (O'Brien et al., 1999). Narp is a neuronal activity-regulated pentraxin. Upon secretion at the cell surface, Narp has the ability to induce the aggregation of AMPA receptors on spinal cord neurons. However, Narp is only found localized to shaft excitatory synapses in spinal and hippocampal cultures but is not found at spiny excitatory synapses between pyramidal neurons. This suggests that Narp may be a specialized factor inducing the receptor clustering at a subset of synapses. Other factors that function at spiny synapses await discovery. Recent work by Scheiffele and collegues (2000) has shed new light on the potential role of synaptic adhesion molecule neuroligin-neurexin complex in triggering presynaptic specializations during synaptogenesis. As introduced previously, neuroligin is localized on the postsynaptic membrane of glutamatergic synapses (Song et al., 1999). When neuroligin is expressed on the surface of heterologous cells, it seems to be sufficient to induce the clustering of synaptic vesicles in axons at forming contact with these cells. This process seems to be mediated by the presynaptic adhesion molecule β-neurexin. These findings suggest that the establishment of neuronal adhesion may be one of the first steps during synapse formation.

Synaptogenesis is a multistep process, including the clustering of synaptic vesicles, the assembly of the vesicle recycling machinery in presynaptic terminals, and the recruitment of receptors and scaffolding molecules to the PSD. The whole process occurs through a period of time. At nerve-muscle contacts it takes nearly a month between the first synaptic contacts and the attainment of a mature NMJ (Sanes and Lichtman, 1999). In CNS, however, mature synapses may be established in a much

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shorter time, although the increase of the number of synaptic vesicles in nerve terminals may last for up to a month (Blue and Parnavelas, 1983a, 1983b). A series of studies using cultured hippocampal neurons suggest that synaptic components are sequentially recruited to synaptic sites during a period of several days (for review, see Lee and Sheng, 2000). In contrast, studies using novel imaging approaches suggest that functional synapses may form within a few hours (Dailey and Smith, 1996; Ziv and Smith, 1996; Maletic-Savatic and Malinow, 1998; Toni et al., 1999; Jontes et al., 2000). Recently, an elegant study by Vardinon-Friedman and colleagues (2000) provided a detailed picture of the formation of functional presynaptic boutons and the recruitment of the core components of synapses. In this study, a combination of time-lapse microscopy and retrospective immunohistochemistry allowed the real-time labeling of newly appearing presynaptic boutons and the subsequent detection of the recruited synaptic components. Using this approach, they have shown that at presynaptic terminal the capacity of synaptic vesicles to dock, fuse, and recycle can be acquired around 20 to 40 min after the initial axon-dendrite contact. Similarly the presynaptic CAZ protein Bassoon is recruited to the active zone 10 to 25 min after contact (Figure 7). At postsynaptic terminal, SAP90/PSD95 starts to accumulate around 70 min, and glutamate receptors accumulate between 85 and 110 min after initial contacts at postsynaptic sites (Figure 7). Although this study confirms the notion that the assembly of synapses and the recruitment of synaptic components occur in a sequential manner, the duration of the whole process requires only a couple of hours in culture hippocampal neurons (Vardinon-Friedman et al., 2000). These results are clearly different from studies showing that synaptogenesis of the NMJ and CNS synapses occurs over a period of several weeks. One explanation for their different results may lie in the difference in the condition or statuse of axons and

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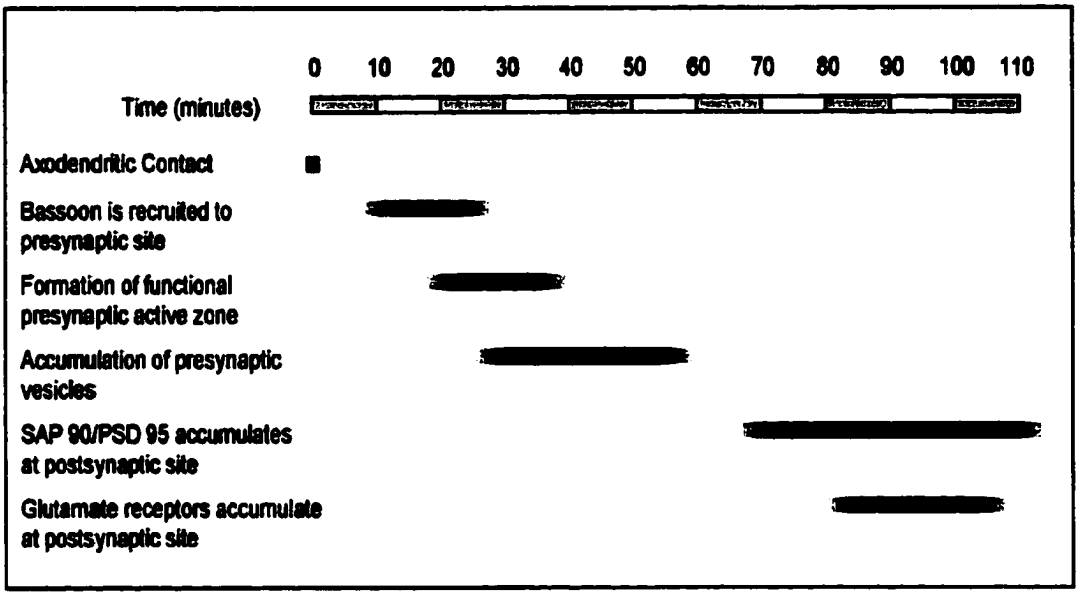

Figure 7. Proposed Time Line of Glutamatergic Synapse Assembly

The putative timing of key events during the assembly of individual glutamatergic synapses is displayed.

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dendrites in each study. For example, when the axon and dendrites are both "competent" for synaptogenesis, synapses can be established within hours upon axodendritic contact. However, it may take days for the neurons to "get ready" for synaptogenesis. The "preparing" process includes the synthesis and transport of all the molecules needed for the synapse formation, which may take a long time. When synaptic molecules are abundant, synapses can be established very quickly.

Summary

Synapses are highly asymmetric structures, which are composed of specialized subsynaptic compartments that have distinct morphological and functional features. We have come a long way in understanding the physiology of synaptic transmission and dissecting the ultrastructure of synapses. The more we understand about synapses, the more complex it has become and the more we are intrigued about the mechanisms that govern the formation and maintenance of such apparatus with high functional and structural complexity. The recent molecular characterization of PSDs has led to the discovery of many key components that are involved in the clustering of receptors and channels, as well as signaling molecules in the postsynatpic terminal. These studies are providing a detailed but rather static picture of the molecular structure of postsynaptic terminal. At present, we know very little about the assembly of presynaptic nerve terminal. The identification of the CAZ proteins Piccolo, Bassoon, RIM, and Munc 13 shed some light on the presynaptic side of the story of the synapse assembly. The active zone-restricted localization of these proteins suggests that they may be involved in organizing the structure of active zones, while the mechanisms await elucidation. The objective of this dissertation is to understand the mechanism of presynaptic terminal

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assembly in CNS neurons by studying the sorting and trafficking of Piccolo to the nerve terminal and defining its role in the assembly of active zones during synaptogenesis.

Aims of Dissertation

The general hypotheses of this dissertation are (a) the CAZ protein Piccolo is transported to axon terminals as a part of a preformed vesicular complex; and (b) this vesicular complex serves as a precursor for the active zone, and it is involved in the assembly of synapses.

The aims of this dissertation are to test these two main hypotheses. These aims include (a) characterizing the synaptic localization of Piccolo in different types of synapses, including excitatory and inhibitory synapses, as well as the neural muscular junctions; (b) determining the temporal distribution of the CAZ proteins Bassoon and Piccolo during neuronal development; (c) defining the mechanism underlying the sorting and transport of Piccolo to nerve terminals during the early stage of development; and (d) examining the mechanism that underlies the assembly of active zones by characterizing the components carried in/on the Piccolo transport vesicle (PTV).

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PICCOLO IS SPECIFICALLY LOCALIZED TO THE PRESYNAPTIC NERVE TERMINALS OF BOTH EXCITATORY AND INHIBITORY SYNAPSES BUT NOT TO THE NMJ

Summary

Piccolo is a novel component of the presynaptic cytoskeletal matrix assembled at the active zone of neurotransmitter release. Previous studies have shown that Piccolo is a large protein (~530 kDa) tightly bound to the presynaptic cytoskeletal matrix, requiring harsh conditions for extraction (Cases-Langhoff et al., 1996; Fenster et al., 2000). Additionally, immunogold EM studies indicate that Piccolo is part of the amorphous material situated between synaptic vesicles located just proximal to the active zone (Cases-Langhoff et al., 1996). In this study, I have shown that Piccolo is found to be specifically localized to the presynaptic terminals of both excitatory and inhibitory synapses but not to the cholinergic NMJ. Biochemically, Piccolo is very tightly associated with synaptic junctions, and it is highly enriched in the synaptic junctional preparation. Thus, Piccolo is a general component of the cytomatrix of the active zone at different types of synapses in CNS.

Introduction

Piccolo was originally identified in a broad immunoscreen for synaptic junctional proteins (Garner et al., 1993; Cases-Langhoff et al., 1996; Langnaese et al., 1996). Rabbit antisera raised against rat brain synaptic junctional proteins prepared from 20- and 30-day-old rats were used to screen a λgt11 postnatal day 25 rat brain cDNA expression

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library (Garner et al., 1993). Among the cDNA clones identified in this screen, there are 47 clones encoding novel potential SAPs, and 44 encoding known proteins. Almost half of the identified proteins fall into the category of structural and mechanochemical proteins (Langnaese et al., 1996). Piccolo and Bassoon are two large cytomatrix proteins identified in this screen sharing 50% sequence homology with over 10 homology domains (Fenster et al., 2000). Studies of the spatial distribution of Piccolo and Bassoon in adult rat brain revealed that they are present at high levels in most synaptic-rich regions. These regions include the cerebellum, olfactory bulb, superior colliculus, hippocampus, and spinal cord (Cases-Langhoff et al., 1996; Dieck et al., 1998). Immunohistochemistry and immuno-EM studies further revealed that Piccolo is highly concentrated at presynaptic nerve terminals opposing the PSD of neighboring dendritic spines (Figure 8A). Additionally, EM immunogold analysis of isolated synaptosomes showed that Piccolo antibodies decorated the amorphous material surrounding synaptic vesicles within presynaptic terminals (Figure 8B). These studies suggest that Piccolo is localized at the presynaptic terminal of asymmetric type I synapses (Cases-Langhoff et al., 1996). However, Bassoon has been shown to be localized at both asymmetric type I and symmetric type II synapses in brain (Dieck et al., 1998; Richter et al., 1999). Subcellular fractionation studies have shown the specific enrichment of Piccolo protein in synaptic junctions (Cases-Langhoff et al., 1996). Piccolo is found highly enriched in synaptosomes and in synaptic junction preparations but is not found in microsomal, mitochondria, or synaptic vesicle fractions of adult brain. Furthermore, Piccolo is tightly associated with synaptic junctions and is resistant to detergent extraction. It can only be solublized by 2% Sodium dodecyl sulfate (SDS) or 8 M guanidine HCl (Cases-Langhoff

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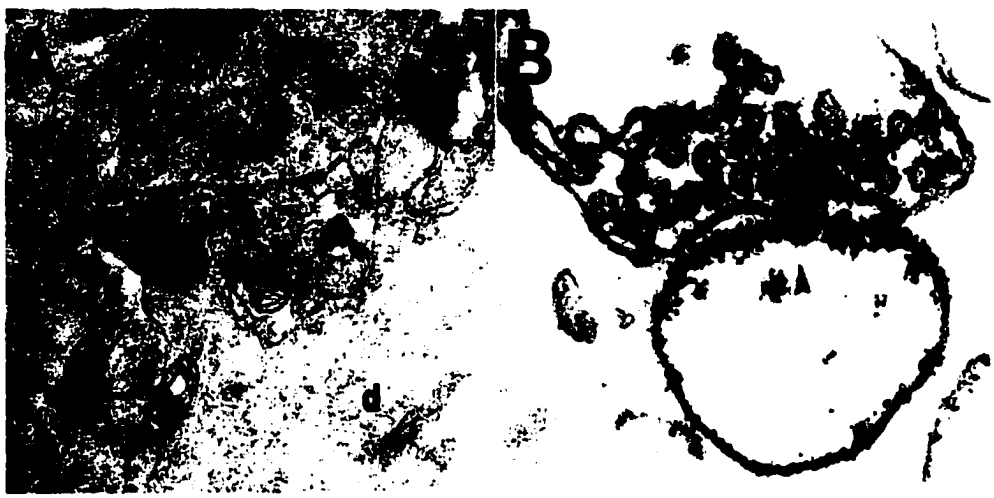

Figure 8. Piccolo is Specifically Localized at the Presynaptic Nerve Terminal

(A) EM micrograph of rat brain cerebellum stained with Piccolo antibody. Piccolo staining is specifically localized to the presynaptic nerve terminal (*) opposing to the postsynaptic spines extending from a dendrite (d).
(B) EM micrograph of isolated synaptosome stained with Piccolo antibody followed by 5 mm gold conjugated secondary antibody. Gold particles decorated presynaptic cytomatrix surrounding clustered synaptic vesicles. PSD (arrow) is also visible, juxtaposed to the active zone (arrow).

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et al., 1996). The strong and specific interaction between Piccolo and synaptic junctions indicates that Piccolo is a structural component of the CAZ and thus may play a role in defining the presynaptic active zone.

To gather further information about the detailed localization of Piccolo and Bassoon in synapses of different types, double labeling immunofluorescent microscopy of cultured hippocampal neurons was used to determine their distributions. Colocalization studies reveal that Piccolo and Bassoon are shared components of both glutamatergic and GABAergic synapses but not the NMJ.

Results

Piccolo and Bassoon are Localized to Synapses in Cultured Hippocampal Neurons

Our previous studies on the subsynaptic localization of Piccolo and Bassoon in adult rat brain showed that they are highly restricted to the presynaptic cytomatrix assembled at the active zone of asymmetric type I synapses (Cases-Langhoff et al., 1996; Dieck et al., 1998; Richter et al., 1999). The homology observed between Piccolo and Bassoon (Fenster et al., 2000) suggests that they may perform analogous functions at different synapses or distinct functions at identical synapses. To test this hypothesis, I have first examined whether Piccolo colocalized with Bassoon at synapses. Primary hippocampal cultures have been well established in the past two decades as a model system for localization studies of synaptic proteins due to the great spatial resolution that cultured neurons have offered (Goslin and Banker, 1998). When cultured for more than 2 weeks, ~14 days in vitro (div), neurons establish mature synaptic contacts with similar biological and physiological properties as they do in vivo (Goslin and Banker, 1998). In

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this study, 24 div neurons were used. The distribution of Piccolo was first compared with that of a synaptic vesicle protein synaptotagmin by double-label immunofluorescent microscopy using antibodies against Piccolo and synaptotagmin. Approximately 95% of the synaptotagmin immuno-positive clusters were observed to co-localize with Piccolo-containing clusters situated along dendritic profiles (Figure 9A-9C). This pattern suggests that Piccolo has in these cultures become localized to synaptic boutons. Double labeling neurons with Piccolo and Bassoon antibodies revealed near one-to-one codistribution patterns (Figure 9D-9F), indicating that both CAZ proteins are present at identical synapses. Thus, Piccolo and Bassoon appear to localize to synapses in cultured neurons at mature stage.

Piccolo and Bassoon are Present in Both Excitatory and Inhibitory Synapses in CNS

EM studies on mature brain cerebellum suggested that Piccolo and Bassoon are present in excitatory spiny synapses (Figure 8; Cases-Langhoff et al., 1996; Dieck et al., 1998), but it is not known if they are restricted to spiny synapses. Although Piccolo colocalizes with synaptotagmin, which is a general synaptic vesicle protein that is present at all synapses, it is still not clear whether the distributions of Piccolo and Bassoon are specific to certain types of synapses. To investigate whether Piccolo and Bassoon are present at both excitatory and inhibitory synapses in cultured hippocampal neurons, their distributions were compared to those of AMPA receptor subunit 1 (GluR1) and γ-Aminobutyric acid (GABA) receptor α subunit ($GABA_AR$) by double-label immunofluorescent microscopy. GluR1 is a subunit of the AMPA receptor, which

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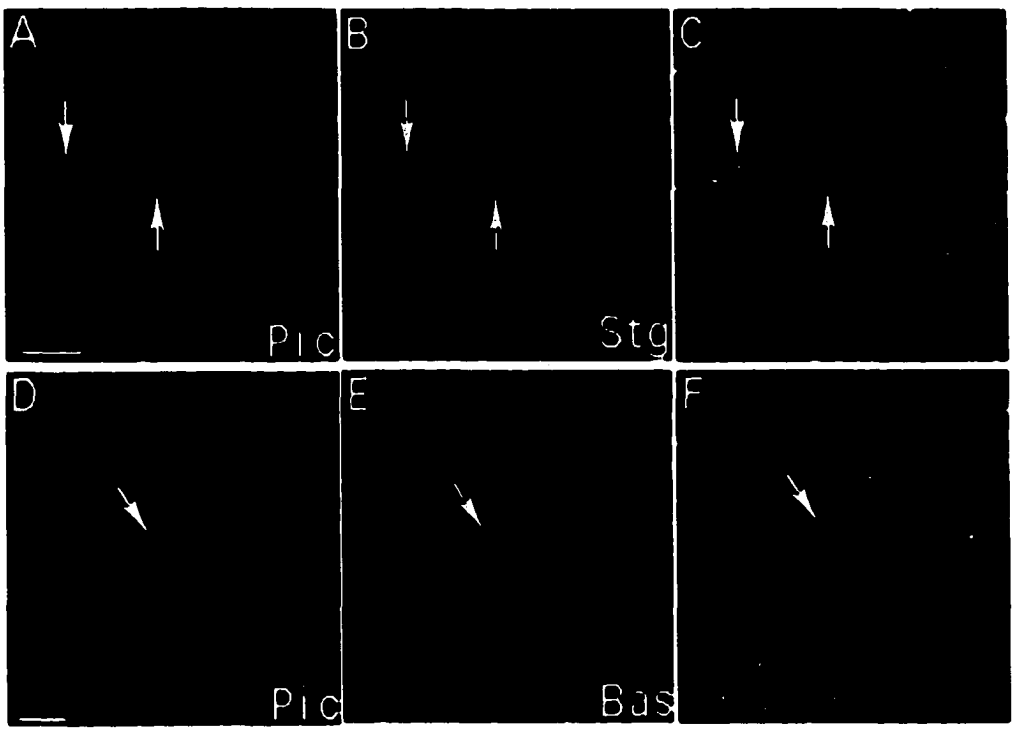

Figure 9. Piccolo is Localized to Synapses in Cultured Hippocampal Neurons

Rat hippocampal neurons cultured for 24 div were double stained with anti-Piccolo (A, D) and anti-synaptotagmin (B) or anti-Bassoon (E) antibodies. Merged images for each pair are shown in (C) and (F), respectively. Arrows point to examples of colocalizaing clusters. Scale bars, 20 μm.

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conducts excitatory postsynaptic responses, and $GABA_A$ is an isoform of GABA receptor, which conducts inhibitory response (Nusser et al., 1996). In general, about 80% and 20% of the Piccolo clusters colocalized with GluR1 and $GABA_A$ receptor clusters, respectively (Figure 10A-10C and Figure 10D-10F). Interestingly, the immunofluorescent staining also revealed the morphological differences between excitatory synapses and inhibitory synapses. GluR1 positive clusters are mostly localized along the dendrites and showed a "spiny" shape (diagrammed in Figure 10G). GluR1 antibodies stained the tip of the spines and the Piccolo clusters were observed directly opposed to GluR1 clusters with a thin line of colocalization in between suggested by the yellow color between the green and red clusters (arrows in Figure 10A-10C, diagrammed in Figure 10G). On the contrary, $GABA_A$ antibodies labeled shaft synapses along dendrites and soma (diagrammed in Figure 10H). Piccolo clusters were also observed directly opposed to $GABA_A$ clusters with "yellow" colocalization in between (arrows in Figure 10D-10F, diagramed in Figure 10H). A similar approach was used to examine the presence of Bassoon in excitatory and inhibitory synapses. As shown in Figure 11, Bassoon co-localizes with either GluR1 receptors (Figure 11A and 11B) or $GABA_A$ receptors (Figure 11C and 11D). These results clearly demonstrate that Piccolo and Bassoon are shared components of both excitatory and inhibitory synapses, suggesting that these CAZ proteins perform a complementary role at the active zone of CNS synapses.

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Figure 10. Piccolo is Localized at Both Excitatory Glutamatergic and Inhibitory GABAergic Synapses

(A-F) Rat hippocampal neurons cultured for 24 div were double stained with anti-Piccolo (A, D) and anti-GluR1 (B) or anti-$GABA_AR$ (E) antibodies. Merged images for each pair are shown in (C) and (F), respectively. Arrows point to examples of co-localizing clusters. Scale bars, 20 μm.

(G-H) Schematic diagram of a spiny synapse and a shaft synapse, depicting the staining of Piccolo and GluR1 or $GABA_AR$. GluR1 antibody stained the tip of the spine (green in G), $GABA_AR$ antibody stained the postsynaptic terminal of shaft synapse (green in H), and Piccolo antibody stained the active zone region of the opposing presynaptic terminal (red in G and H). The overlapping region is shown in yellow (G and H). Abbreviations: d, dendrite; pre, presynaptic terminal; sm, soma; sp, spine.

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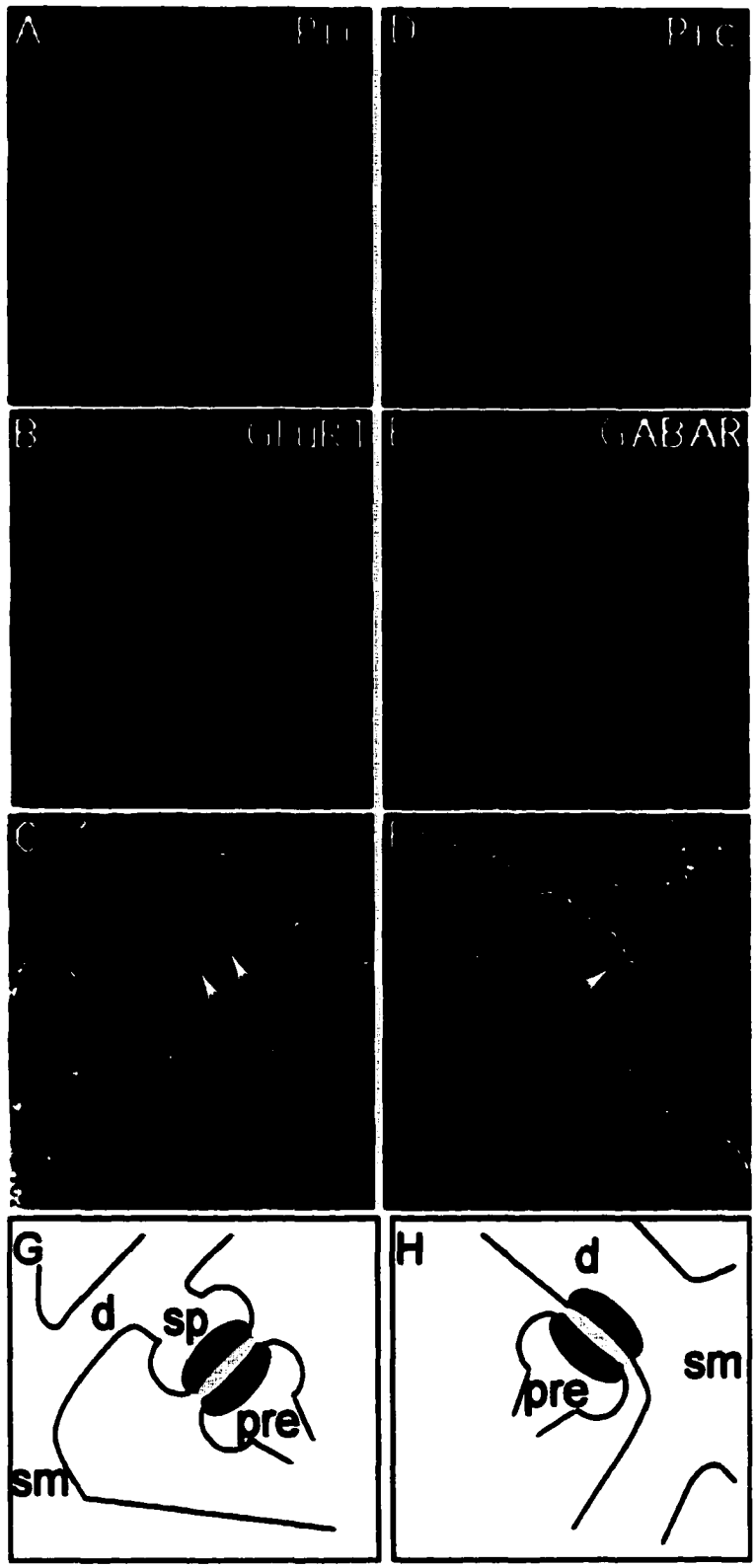

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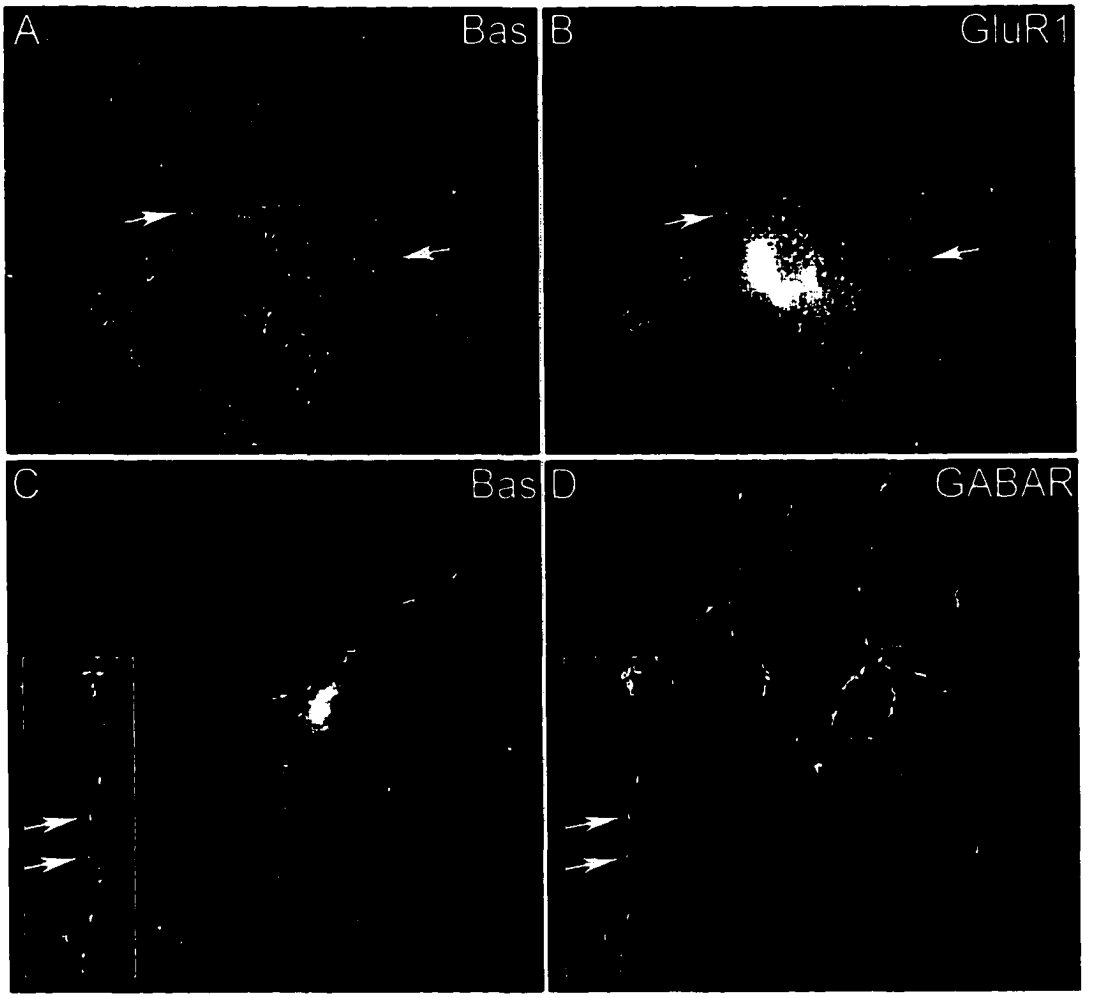

Figure 11. Bassoon is Localized at Both Excitatory Glutamatergic and Inhibitory GABAergic Synapses

Rat hippocampal neurons cultured for 21 div were double stained with anti-Bassoon (A, C) and anti-GluR1 (B) or anti-$GABA_AR$ (D) antibodies. Arrows point to examples of colocalizing clusters. Scale bar, 20 μm.

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Piccolo is Present in Glycinergic Synapses but not in NMJ

All studies on Piccolo and Bassoon thus far have been restricted to the CNS. To understand their roles in synapses, it is important to obtain a rather comprehensive picture of the general distribution of Piccolo and Bassoon throughout the nervous system. Therefore, the distribution of Piccolo in glycinergic synapses of cultured spinal neurons and in NMJ was also examined. Cultured spinal cord neurons were double stained for Piccolo and glycine receptors. As shown in Figure 12A-B, Piccolo is co-localized with glycine receptors. The distribution of Piccolo in NMJ was examined by staining longitudinal sections of the rat diaphragm with FITC-conjugated α-bungarotoxin and antibodies against synaptotagmin or Piccolo. NMJs are cholinergic synapses and acetylcholine receptors are highly concentrated at the postsynaptic terminal of the NMJ (Hall and Sanes, 1993). α-bungarotoxin is a high affinity antagonist of acetylcholine receptor. FITC-conjugated α-bungarotoxin can specifically label the acetylcholine receptor in the plasma membrane of NMJs. As shown in Figure 12, synaptotagmin co-localizes with α-bungarotoxin-labeled acetylcholine receptors (Figure 12A-12B), but Piccolo was not detected at NMJs (Figure 12C-12D). Interestingly, Bassoon was not found in NMJ either (data not shown).

Piccolo is Enriched in Synaptosomes and Synaptic Junctional Preparations

Immunofluorescent studies demonstrated that Piccolo is localized to the synapses of different types; however, these studies did not reveal whether Piccolo is associated with the CAZ. To assess the biochemical property of Piccolo and to confirm the previous findings, brain membrane fractionations were analyzed. The partitioning of Piccolo into

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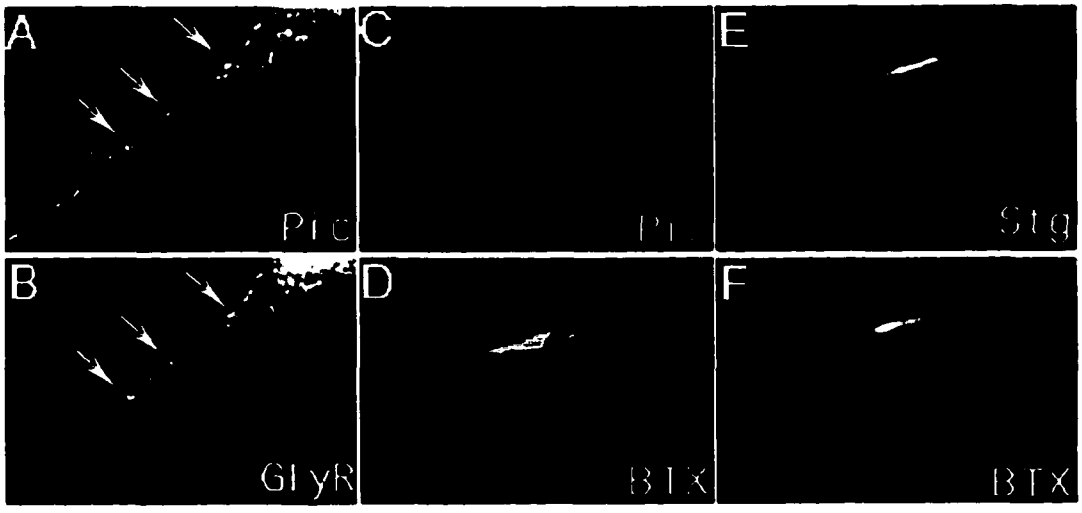

Figure 12. Piccolo is Present in Glycinergic Synapses but not at NMJs

(A-B) Spinal cord neurons cultured for 14 div were double stained with anti-Piccolo (A) and anti-glycine receptor (B) antibodies. Arrows point to examples of colocalizing clusters.
(C-F) Longitudinal sections of P7 rat diaphragm stained with FITC-conjugated α-bungarotoxin (BTX) (D and F) and anti-Piccolo (C) or anti-synaptotagmin (E) antibodies.
Scale bars, 20 μm.

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synaptosomal, synaptic plasma membrane, and detergent-extracted synaptic junctional preparations was analyzed and compared with other known synaptic proteins. Synaptosomes can be purified from crude brain membrane by sucrose density gradient centrifugation. After synaptosomes are hypnotically lysed, synaptic vesicles and synaptic plasma membrane can be further separated by differential centrifugation. When synaptic plasma membranes are extracted by detergent, only the cytoskeletal matrix of the synaptic junction and the proteins strongly associated with it will remain. In this preparation, Piccolo is found highly enriched in synaptosomes, along with the synaptic vesicle protein synaptophysin, suggesting that both proteins are enriched in synaptic nerve terminals (Figure 13). However, Piccolo is also concentrated in the PSD preparation where synatpophysin is not present, indicating that Piccolo is associated with synaptic junctions, which are biochemically distinct from synaptic vesicles preparations (Figure 13).

Discussion

To obtain a comprehensive and detailed picture of the distribution of Piccolo and Bassoon in different types of synapses, doule-labeling immunofluorescent microscopy was used to assess their localization in glutamatergic, GABAergic, and NMJs. Piccolo and Bassoon colocalize in both glutematergic and GABAergic synapses, but neither were present at the NMJs.

Previous studies suggested that the general distribution of Piccolo is strikingly similar to Bassoon. They are colocalized in the same brain regions and to the same types of synapses, including excitatory spiny synapses, inhibitory synapses, and ribbon

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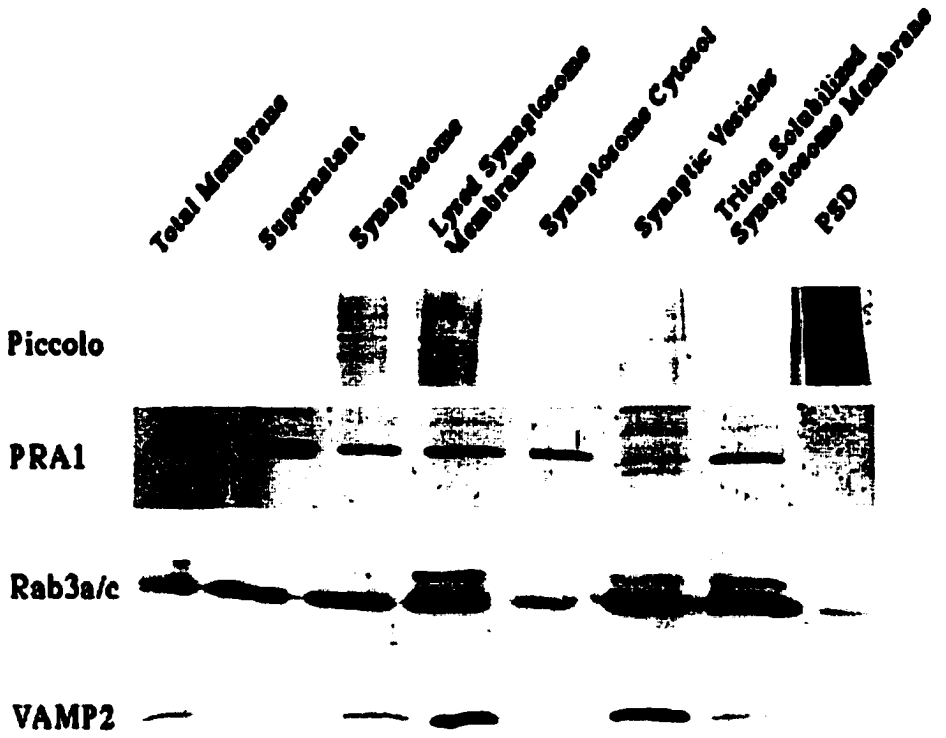

Figure 13. Piccolo is Strongly Associated With PSD

P21 rat brain was homogenized, and membranes were separated by differential centrifugation and sucrose gradient centrifugation. Each fraction, as indicated on the top of the figure, was resolved by SDS-PAGE and Western blotted for Piccolo, PRA1, Rab3a, and Synatpobrevin/VAMP2. Equal amounts of protein were loaded in each lane.

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synapses (Case-Langhoff et al., 1996; Dieck et al., 1998; Brandstatter et al., 1999; Richter et al., 1999). Here, the double-labeling analysis clearly demonstrated that Piccolo and Bassoon are colocalized to the same synapses. The colocalization suggests that Piccolo and Bassoon are playing either the same or partially overlapping roles at a variety of stucturally and functionally diverse synapses. The protein structure analyses revealed that Piccolo and Bassoon share at least 10 homology domains with overall 50% sequence homology. Although they are very similar at the structural level, Piccolo has a PDZ domain and two C2 domains that are not present in Bassoon, suggesting that Piccolo may perform different functions in nerve terminals. This combination of domains is also observed in another CAZ protein RIM, which has been shown to be involved in synaptic vesicle cycling (Wang et al., 1997). The protein structure similarities among Piccolo, Bassoon, and RIM, as well as their nearly identical distribution pattern, indicate that these CAZ proteins may orchestrate the activities and functions of presynaptic nerve terminals in a complementary manner.

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TEMPORAL APPEARANCE OF THE CAZ PROTEINS BASSOON AND PICCOLO DURING NEURONAL DEVELOPMENT

Summary

Bassoon and Piccolo are both high molecular weight cytomatrix proteins and colocalize in a variety of structurally and functionally diverse synapses. Both proteins are expressed at early stage of brain development and are potentially involved in the structural organization of presynaptic active zones. To investigate a possible role for Bassoon and Piccolo in synaptogenesis and in defining synaptic vesicle recycling sites, their temporal appearances in neurons and synapses were examined. Both Bassoon and Piccolo are selectively sorted into axons during early stage of neuronal differentiation. As synaptogenesis begins, clusters of Bassoon and Piccolo appear along dendritic profiles simultaneously with synaptotagmin I and sites of synaptic vesicle recycling. A role for Bassoon in the assembly of excitatory and inhibitory synapses is supported by the colocalization of Bassoon clusters with clusters of GKAP and AMPA receptors, as well as GABA receptors. Therefore, the synaptic recruitment of Bassoon and Piccolo is an early step in the formation of synaptic junctions.

Introduction

Synapse formation is accurate and precise. Defining the time or developmental stage at which synaptic proteins are localized to synapses provides clues to the mechanisms underlying synapse assembly. As discussed earlier, the observation that

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gephyrin is localized to synapse before glycine receptors strongly supports the hypothesis that gephyrin is a structural molecule that clusters glycine receptors to type II symmetric synapse during synaptogenesis (Vannier and Triller, 1997). In the CNS, the molecular mechanisms by which neurons control the localization of synaptic components during development are not well understood, partially due to a lack of suitable experimental models. In recent years, primary hippocampal cultures have been thoroughly characterized and established as a model system for the study of neuronal development and synaptogenesis (Matteoli et al., 1995; Goslin and Banker, 1998). This approach will be used as a major experimental model system in this and the following chapters. When cultured under specific conditions, hippocampal neurons extend axons and dendrites and eventually form physiologically active synaptic contacts. Based on their morphology, the development of neurons in culture can be divided into five stages (Figure 14; Dotti et al., 1988; Goslin and Banker, 1998). Stage 1, characterized by lamellipodia surrounding the cell body, is when neurons are just attached to the culture substrate surface. Usually neurons at stage 1 can be observed within 0.5 to 1 hr after plating. At stage 2, around 1 to 2 div, neurons send out several processes (usually more than one) equal in length toward different directions. Around 2 to 3 div, stage 2 cells develop into stage 3 neurons. Here one process elongates and exhibits the features of an axon. Starting from 3 to 4 div, the minor processes become longer and thicker with multiple branches, exhibiting the dendritic characteristics. Stage 4 begins when presynaptic specialization starts to form on the surface of the somato-dendritic region. At stage 5, neurons are fully matured. Starting from 5 to 7 div, the density of axonal and dendritic networks increase and dendritic arbors become elaborate and highly branched. The density of synaptic contacts increases

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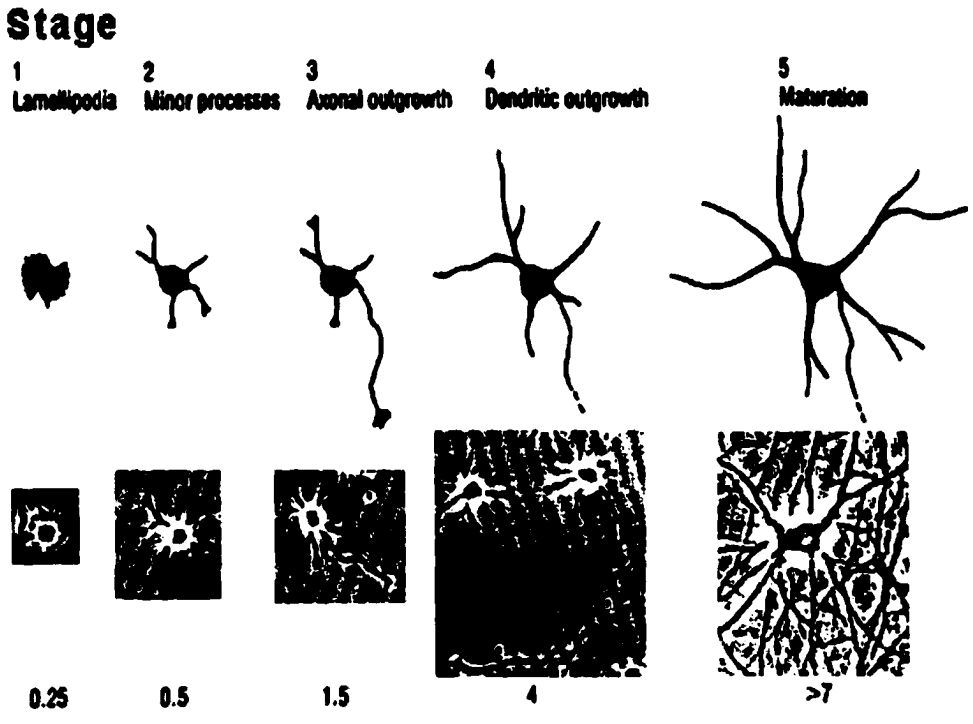

Figure 14. Developmental Stages of Hippocampal Neurons in Culture

Based on morphological and physiological characteristics, the development of cultured hippocampal neurons can be divided into five stages, as indicated at the top of the figure. For each stage, a drawing is used to illustrate the morphology, and a DIC image of a neuron is used as an example. The time (in days) of each stage is indicated at the bottom of the figure.

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dramatically. The approximate time of each stage is based on previous experimental observations in our laboratory, which differs slightly from the time shown at the bottom of Figure 14. This difference may be due to variations in culture densities and slight changes in culture condition.

The synapses formed in culture have all the features of synapses in vivo, including the presynaptic accumulation of synaptic vesicles (Dotti et al., 1988; Goslin and Banker, 1998) and the clustering of postsynaptic receptors (Craig et al., 1993). Studies of glutamate synapse in cultured hippocampal neurons show that neurons control the localization of synaptic components in a timely manner (Rao et al., 1998). Utilizing antibodies against synaptic vesicle proteins and PSD components, it was shown that synaptic vesicles and structural components of the PSD such as SAP90/PSD95 and GKAP cluster at newly forming synapses at about the same time (Rao et al., 1998). In contrast, synaptic recruitment of AMPA receptor subunits appears to be a later event (Rao et al., 1998). This indicates that synaptic vesicle clustering is an early event in the assembly of CNS synapses. This hypothesis is supported by earlier studies showing the accumulation of synaptic vesicles in axonal growth cones and observation of neurotransmitter secretion from growth cones (Kidokoro and Yeh, 1982; Young and Poo, 1983; Matteoli et al., 1992). However, at what time Bassoon and Piccolo localize at nascent synapses and whether they are involved in synapse assembly are not known.

To examine whether the CAZ proteins Bassoon and Piccolo may play a role in early synaptogenesis and in defining the presynaptic active zone as the site of neurotransmitter release, we have examined the temporal appearance of Bassoon and Piccolo in differentiating neurons and compared the time of their appearance in synapses

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with that of other synaptic proteins as well as the acquisition of the synaptic vesicle recycling. Defining the stage of development in which Bassoon and Piccolo are involved will greatly expand our understanding of the potential role of Bassoon and Piccolo during synaptogenesis.

Results

Expression of Bassoon and Piccolo in Differentiating Hippocampal Neurons

Utilizing hippocampal cultures as a model system to study synaptogenesis (Matteolli et al, 1995; Goslin and Banker, 1998), I next examined the temporal and spatial appearance of Bassoon in differentiating neurons by double label immuno-fluorescent microscopy. Antibodies against MAP2, a somatodendritic microtubule associated protein (Matus et al., 1986), were used to visualize dendrites at different stages of neuronal differentiation (Goslin and Banker, 1998). In stage 2 neurons, when minor processes first appear (~2 div), Bassoon immunoreactivity was observed as fine puncta in all MAP2 positve processes (Figure 15A and 15B). At 3 div, as axonal outgrowth is initiated (stage 3), the distribution of Bassoon and MAP2 became polarized. Fine Bassoon puncta were primarily found in the distal part of the axon and the growth cone (Figure 15C and 15I). The axonal localization of Bassoon was confirmed by double labeling 3 div neurons with the axonal marker protein tau (Figure 15I and 15J). In contrast, MAP2 is present in the dendrites and proximal axonal segments (Figure 15D). By 10 div, Bassoon aggregated into larger clusters along dendritic profiles (Figure 15E). The number of Bassoon clusters increased steadily during neuronal maturation (10-26 div; Figure 15E, G), which is indicative of an association of Bassoon with new synapses.

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Figure 15. Spatial Distribution of Bassoon in Differentiating Hippocampal Neurons

Cultured hippocampal neurons were fixed at 2 (A, B), 3 (C, D, I, J), 10 (E, F), 15 (G, H) div and immuno-stained for Bassoon (A, C, E, G, I) and the dendritic marker MAP2 (B, D, F, H) or axonal marker tau (J). In stage 2 cells (2 div), Bassoon was present in all processes as fine puncta (A); MAP2b was also present in all neurites but in a diffuse pattern (B). In stage 3 (3 div) neurons as neurites began to differentiate, MAP2b immuno-reactivity became restricted to the somatodendritic regions and proximal segment of the axon (D), and Bassoon puncta became concentrated in axons (C). To confirm the axonal staining, neurons at this stage were also stained for tau. Tau staining is restricted in axon and displays a diffuse pattern (J). At later stages (E, F, G, H), Bassoon immuno-reactivity was clustered along dendritic profiles and the cell somata, suggesting a synaptic localization. Scale bars: 10 μm in all panels.

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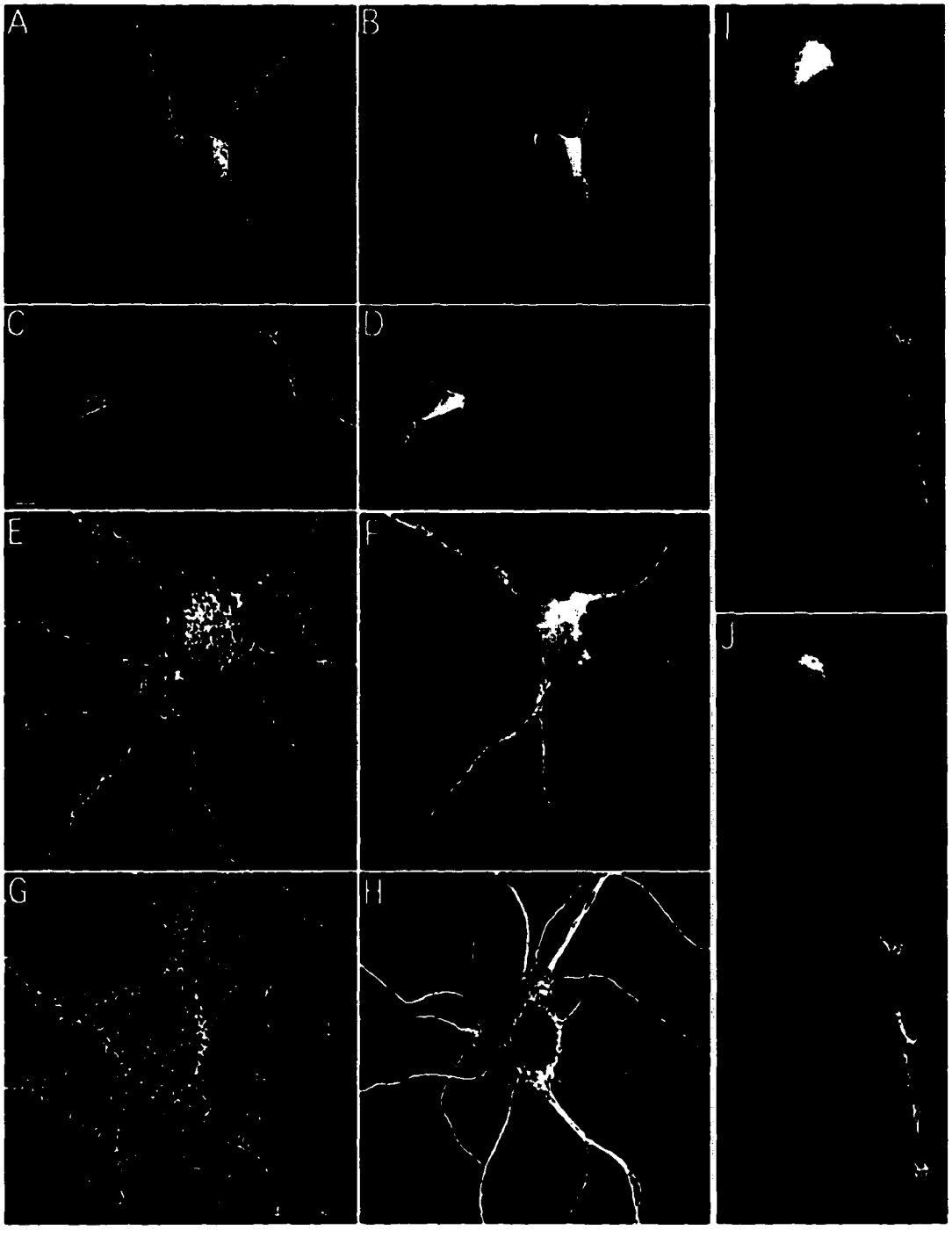

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When Piccolo antibodies were used in the double labeling of neurons at different stages, similar results were obtained. At stage 3, Piccolo immuno-reactivity was found primarily in the distal part of the axon and the growth cone as fine puncta (Figure 16D and 16F), while MAP2 immuno-reactivity was detected in the dendrites and in the proximal axonal segments (Figure 16E and 16F). As at stage 2, a perinuclear punctate staining pattern for Piccolo was observed, indicating that Piccolo is synthesized and packaged into discrete particles in the cell soma (Figure 16A and 16D) which are then sorted and transported into axons. By 10 div, Piccolo immuno-reactivity was observed as larger clusters along MAP2-containing dendritic profiles (Figure 16G and 16I). Such clusters were also observed as early as 4 div (arrowheads in Figure 16F). The number of these immuno-positive clusters increased steadily during neuronal maturation (4-26 div).

Synaptic Vesicles Accumulate and Recycle at Sites Where Bassoon is Clustered

To assess whether Bassoon or Piccolo clusters along dendritic profiles represented synapses, we compared the temporal appearance of Bassoon and Synaptotagmin I clusters. In mature neurons (14 div), most of the Synaptotagmin I clusters colocalize with Bassoon clusters (Figure 17C and 17D) indicating that Bassoon accumulates at synaptic boutons. In stage 3 neurons, the majority of both Bassoon and Synaptotagmin I labeling is found in axonal growth-cones (stars in Figure 17A and 17B). However, a few Bassoon and Synaptotagmin I coclusters can be detected on cell somata and along proximal dendrites (inserts in Figure 17A and 17B). These data indicate that during neuronal differentiation Bassoon and synaptic vesicle clusters form at the same time and place. To examine whether these newly formed Bassoon clusters represent sites of

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Figure 16. Spatial Distribution of Piccolo in Differentiating Hippocampal Neurons

Piccolo is expressed in immature neurons and becomes synaptically localized at later stages. Cultured hippocampal neurons were fixed at 2 (A, B, C), 4 (D, E, F), and 10 (G, H, I) div and immuno-stained for Piccolo (A, D, G) and the dendritic marker MAP2 (B, E, H). In stage 2 cells (2 div), Piccolo was present in all processes as fine puncta (A); MAP2b was also present in all neurites but in a diffuse pattern (B). In stage 3 and 4 neurons as neurites began to differentiate, MAP2b immuno-reactivity became restricted to the somato-dendritic regions and proximal segment of the axon (E) and Piccolo puncta became concentrated in axons (asterisks in D-F). From stage 4, Piccolo immuno-reactivity started to be clustered along dendritic profiles and the cell soma (arrowheads in D and F), and the number of clusters greatly increased in 10 div neurons (G). Interestingly, a peri-nuclear punctate staining of Piccolo was observed in stage 2 to 4 neurons (arrows in A and D). Scale bars: 10 μm in all panels.

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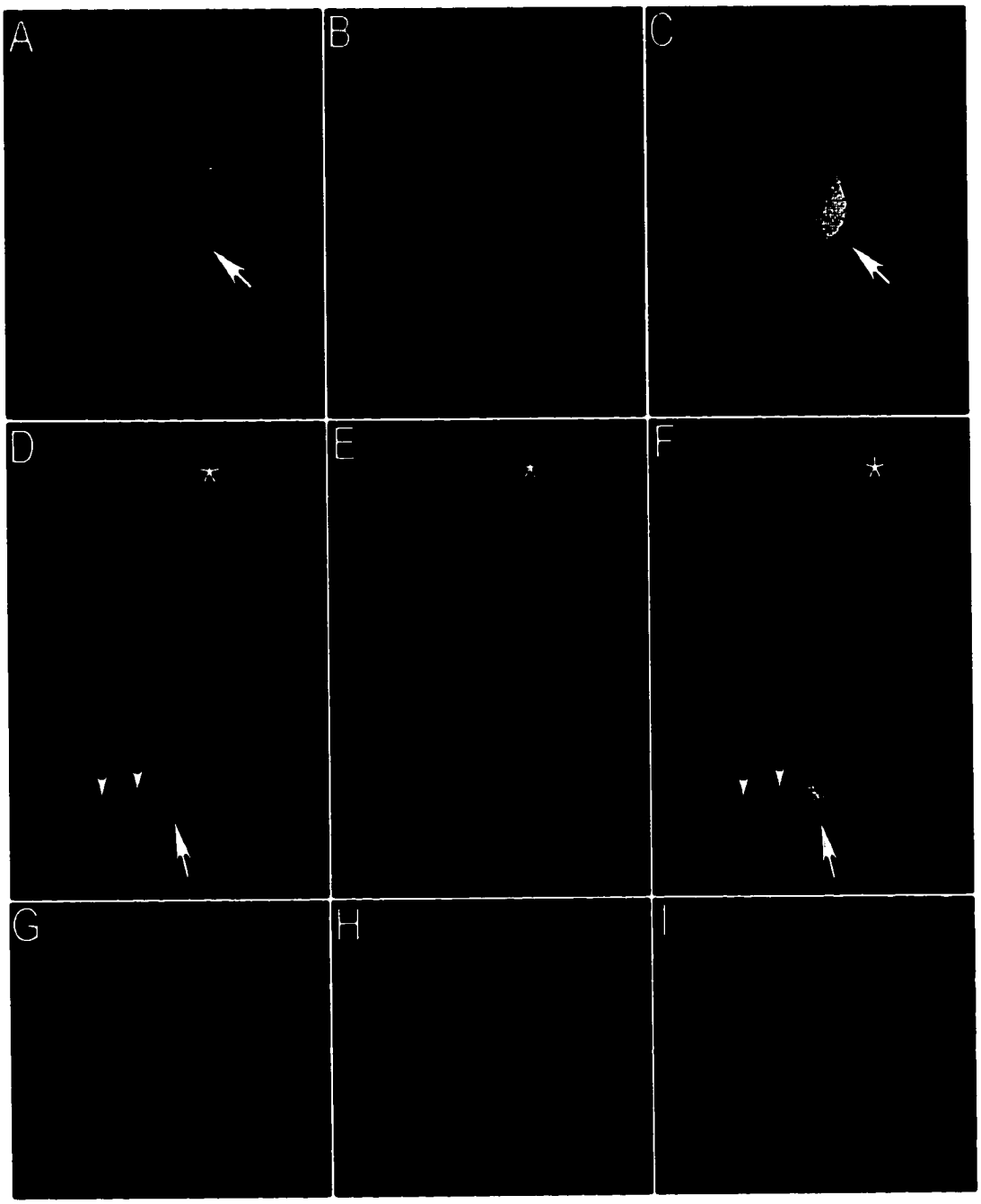

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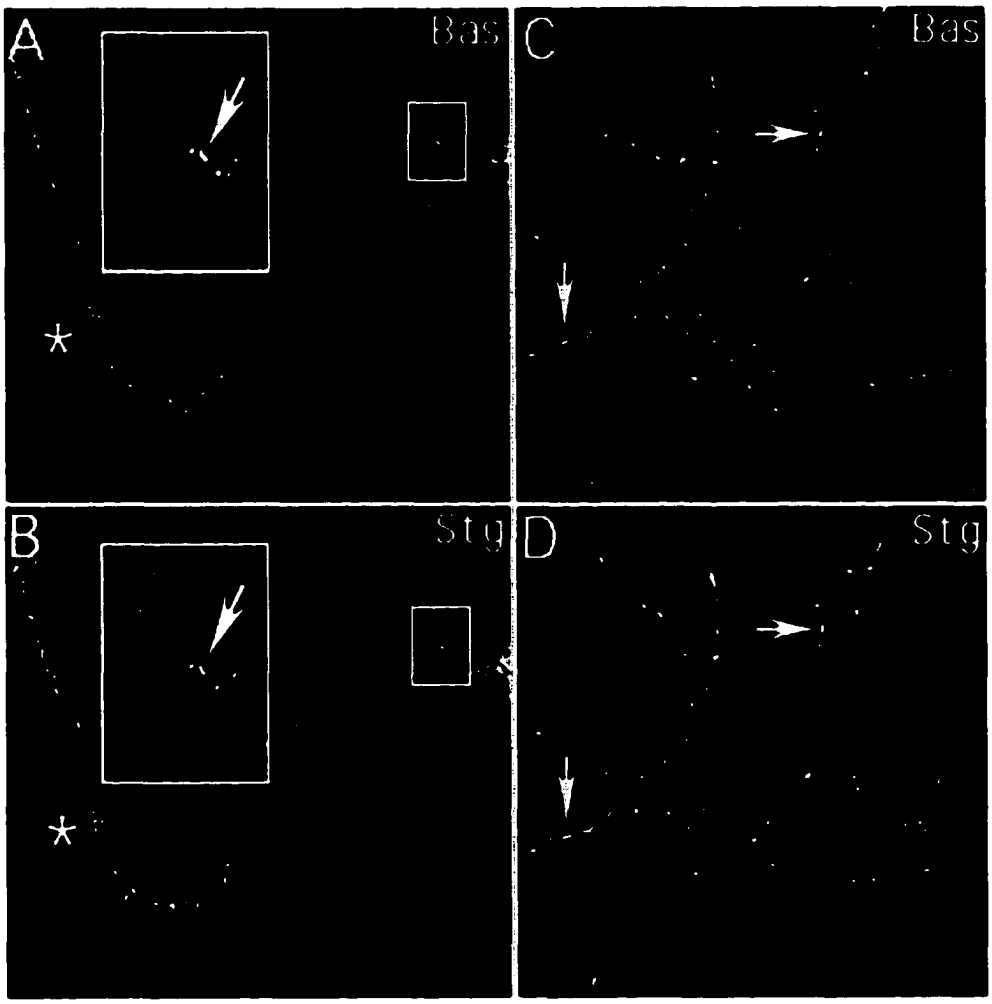

Figure 17. Bassoon Clusters Colocalize With Synapotagmin I Clusters Along Dendritic Profiles

At 4 div and 14 div, neurons were fixed and double labeled for Bassoon (A and C) and Synaptotagmin I (B and D). The axon in each neuron is indicated by star (*). In 4 div neurons (A and B), Bassoon is concentrated primarily in axons and their growth cones (A). Newly formed Bassoon clusters along dendritic profiles colocalized with Synaptotagmin I clusters (arrows in A and B). In 14 div neurons (C and D), a large number Bassoon and Synaptotagmin I clusters can be seen to colocalize (arrows in C and D). Scale bars: 20 μm.

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synaptic vesicle recycling, antibodies against the luminal domain of Synaptotagmin I (Stg-lu Ab) were used to label synaptic vesicles that were recycling from the plasma membrane (Kraszewski et al., 1995). When neurons cultured for 4 div were fixed after a 15 min labeling step, internalized Stg-lu Ab were primarily seen as distinct large clusters along somato-dendritic regions (arrows in Figure 18B and 18E). In neurons double stained with Bassoon antibodies, all recycled synaptic vesicle clusters colocalized with the larger Bassoon positive clusters (Figure 18A-18C). Similar results were obtained when fixed neurons were stained with Synaptophysin antibodies. However, we observed a pool of synaptophysin immunoreactivity that did not label by Stg-lu Ab (arrowheads in Figure 18D-18F). These sites presumably represent clusters of synaptic vesicles that were not actively recycling at the time of the uptake experiment or sites of vesicle clustering that have not yet acquired the capability of active recycling. These data indicate that newly formed Bassoon clusters coincide with active sites of synaptic vesicle recycling.

Early Recruitment of Bassoon at Newly Forming Excitatory and Inhibitory Synapses

The above data show that both functional inhibitory and excitatory synapses are present in 4 div hippocampal cultures. To examine whether Bassoon can be found at both types of synapses as they are assembled, we compared the distribution of Bassoon to several synaptic marker proteins. For excitatory synapse, neurons cultured for 4, 10, and 21 div were labeled with antibodies against GKAP, a structural component of the PSD (Naisbitt et al., 1997), GluR1 subunits of the AMPA receptor, or the dendritic spine protein α-actinin (Wyszynski et al., 1997). In stage 3 neurons (3-4 div), GKAP and

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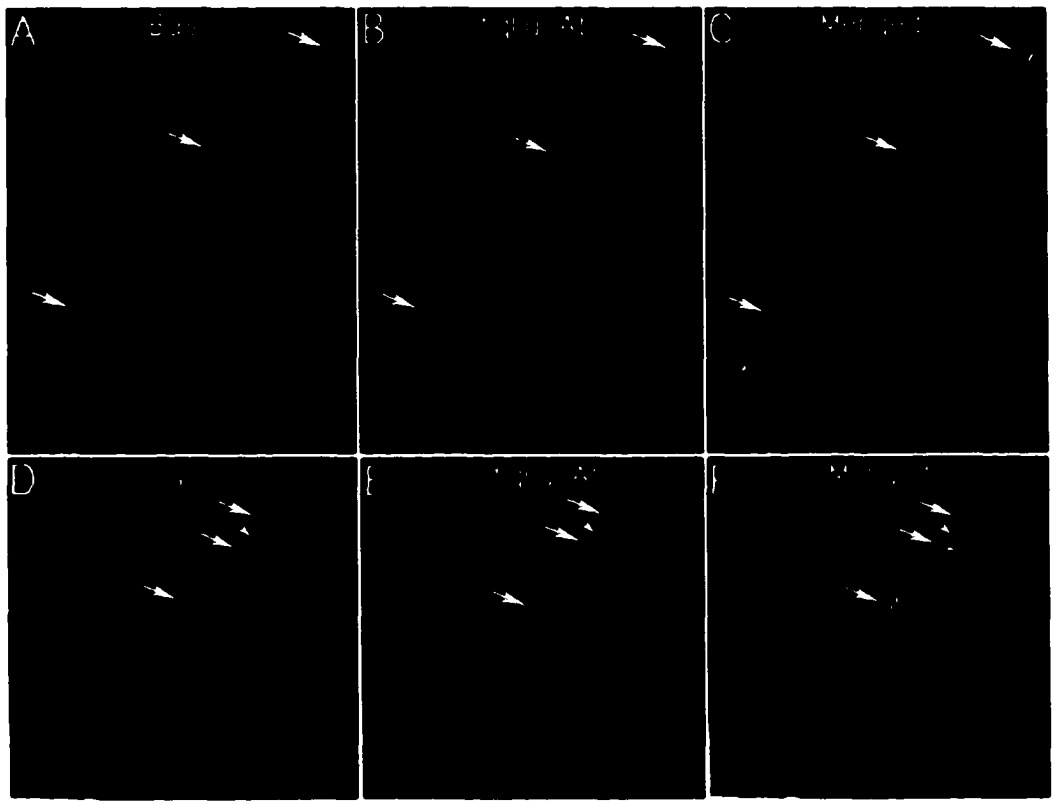

Figure 18. Synaptic Vesicles Recycle at Bassoon Clustering Sites

Neurons cultured for 4 div were fixed after a 15 min incubation with antibodies against the luminal domain of Synaptotagmin I (Stg-lu) at 37°C. Neurons were then stained for Bassoon (A) or Synaptophysin (D). Internalized Stg-lu Ab are found at large clusters (arrows in A-F) present along cell somata and dendritic profiles (D and E), indicating that Stg-lu Ab has labeled the sites of SV clustering and recycling. At least one large synaptophysin cluster remained unlabeled by Stg-lu Ab (arrowheads in D-F). Large Stg-lu positive clusters are also seen to colocalize with Bassoon clusters (arrows in A, B, and C). Scale bars: 10 μm.

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GluR1 staining were diffuse and restricted to somato-dendritic areas, as compared to the somato-axonal distribution of Bassoon (stars in Figure 19A and 19B and Figure 20A and 20B). As observed with Synaptotagmin I antibodies, the first small GKAP and GluR1 clusters present along cell somata and proximal dendrites colocalized with a subset of Bassoon clusters (Figure 19A and 19B). Bassoon clusters that do not colocalize appear to represent inhibitory synapses (see below). In stage 4 and 5 neurons (10 and 21 div, respectively), the number of GKAP and GluR1 clusters along dendritic profiles has increased, while the diffuse somato-dendritic staining is decreased. At all three stages, 100% of the GKAP and GluR1 clusters contain Bassoon (arrows in Figure 19C-19F). In total, this represents about 55% of all Bassoon clusters. In contrast, α-actinin immuno-reactivity remained diffuse in the somato-dendritic compartment of neurons cultured for 4 and 10 div (Figure 21). The α-actinin clusters, found in association with dendritic spines, were found to cocluster with Bassoon clusters after 14 div (Figure 21). These data indicate that α-actinin localizes to excitatory synapses later than Bassoon, GKAP, and GluR1.

To examine the appearance of Bassoon in inhibitory synapses, neurons were double labeled for Bassoon and $GABA_A$ receptor α subunits ($GABA_AR$). At 4 div, most of the $GABA_AR$ immunoreactivity was diffusely distributed in the somato-dendritic compartment (Figure 22B). However, a few clusters of $GABA_AR$, colocalizing with a subset of Bassoon clusters, were observed on cell somata and along dendritic profiles (arrow in Figure 22B). As neurons matured, the number of $GABA_AR$ clusters per cell colocalizing with Bassoon clusters were found to increase (Figure 22C-22F). However, after quantification the percentage of $GABA_AR$/Bassoon clusters per cell was found to

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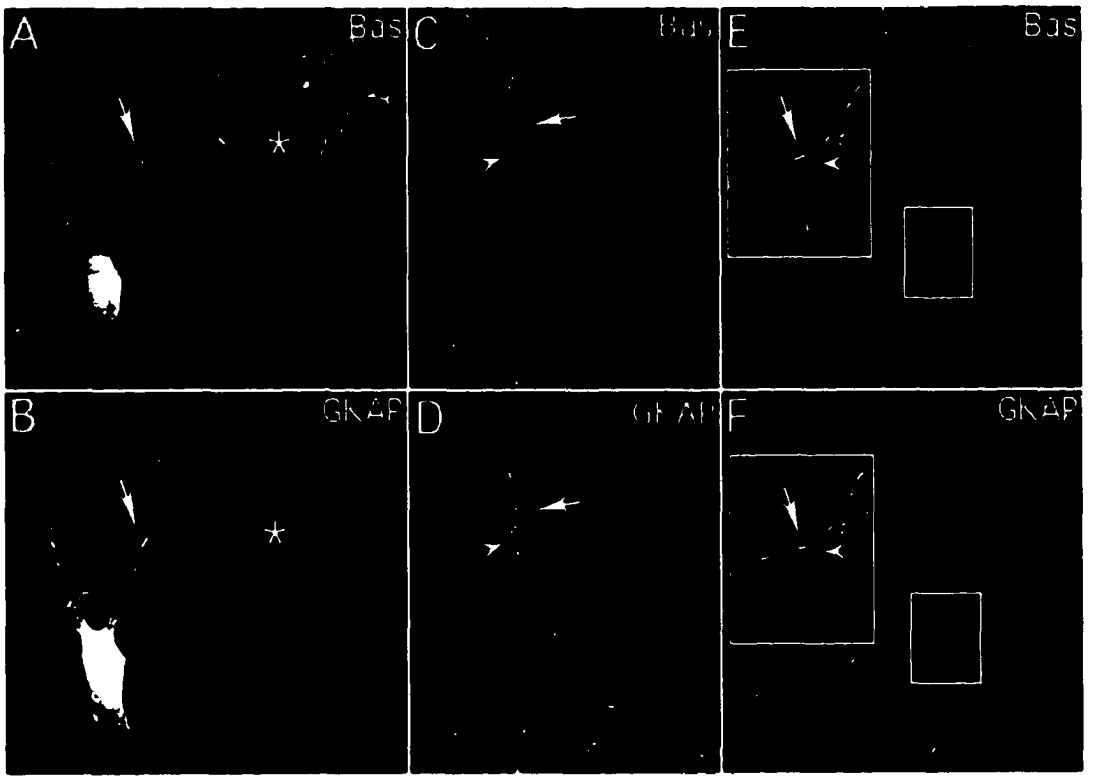

Figure 19. Co-Clustering of Bassoon With GKAP in Differentiating Neurons

At 4, 10, and 14 div, neurons were double labeled for Bassoon (Bas; A, C, and E) and GKAP (B, D, and F). At 4 div, most of the GKAP immuno-reactivity was found diffuse in the somato-dendritic compartment (B), with a few GKAP clusters observed to co-localize with Bassoon clusters (arrows in A and B). At 10 and 14 div, as the level of diffuse GKAP staining decreased, the numbers of Bassoon and GKAP clusters were observed to increase. A subpopulation of the Bassoon clusters lacking GKAP immuno-reactivity is indicated by arrowheads in C-F. Scale bars, 20 μm.

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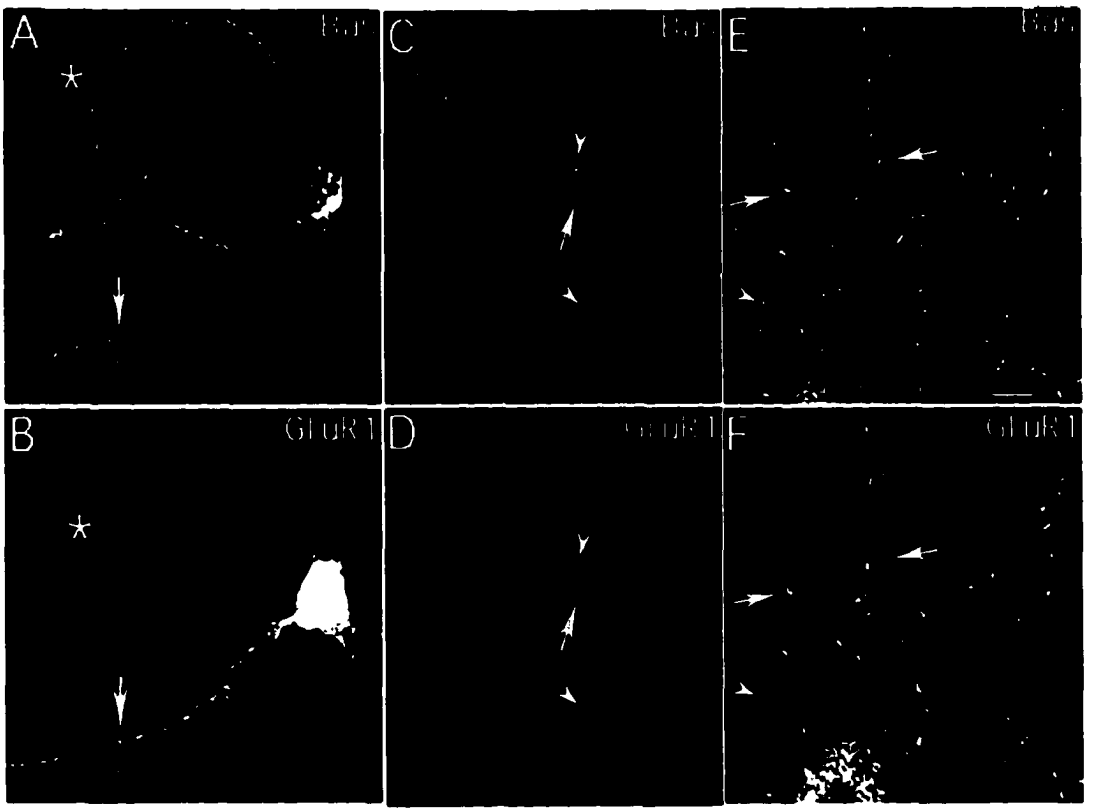

Figure 20. Coclustering of Bassoon With GluR1 in Differentiating Neurons

At 4, 10, and 14 div, neurons were double labeled for Bassoon (Bas; A, C, and E) and GluR1 (B, D, and F). At 4 div, most of the GluR1 immuno-reactivity was found diffuse in the somato-dendritic compartment (B) with a few GluR1 clusters observed to co-localize with Bassoon clusters (arrows in A and B). At 10 and 14 div, as the level of diffuse GluR1 staining decreased, the numbers of Bassoon and GluR1 clusters were observed to increase. A subpopulation of the Bassoon clusters lacking GluR1 immuno-reactivity are indicated by arrowheads in C-F. Scale bars, 20 μm.

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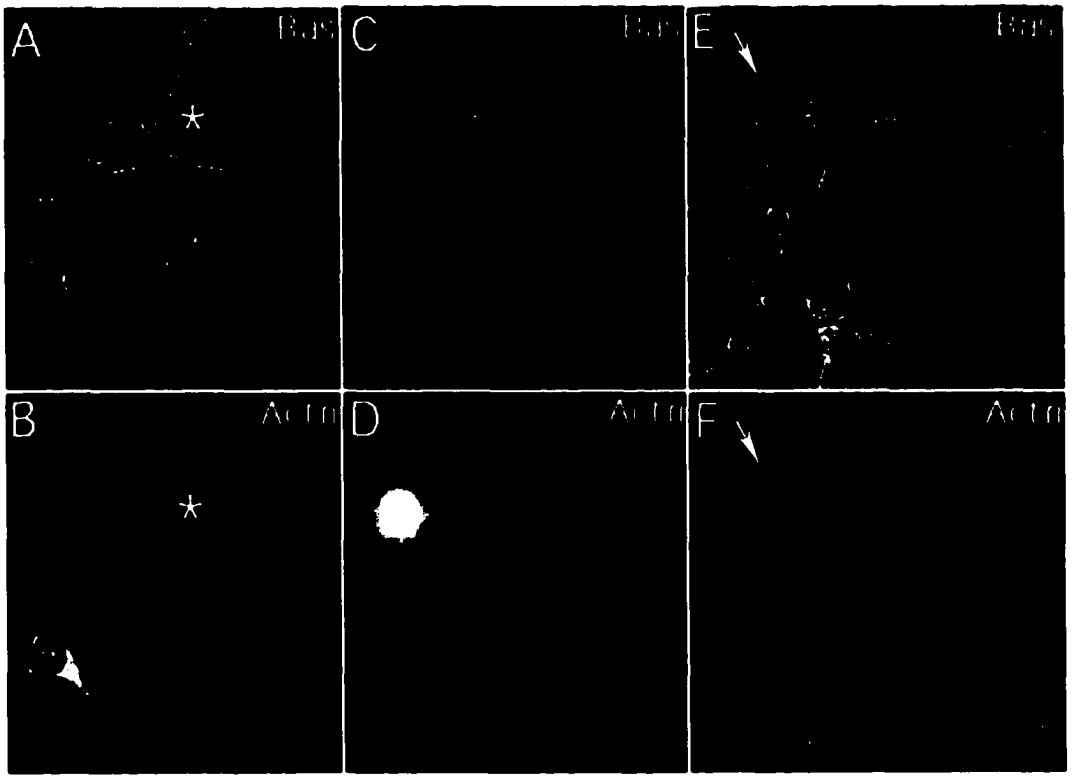

Figure 21. Coclustering of Bassoon with α-Actinin in Differentiating Neurons

At 4, 9, and 21 div neurons were double labeled for Bassoon (Bas; A, C, and E) and α-actinin (Actn; B, D, and F). At 4 div, Bassoon was concentrated in axon (asterisk in A) and at 9 div, Bassoon was mostly clustered on the surface of soma and dendrites (C), while α-actinin was diffusely distributed in soma and dendrites (B and D). At 21 div, α-actinin became clustered in spines (F) and colocalized with a subpopulation of Bassoon clusters (E). Arrows point to examples of colocalization. Scale bars, 20 μm.

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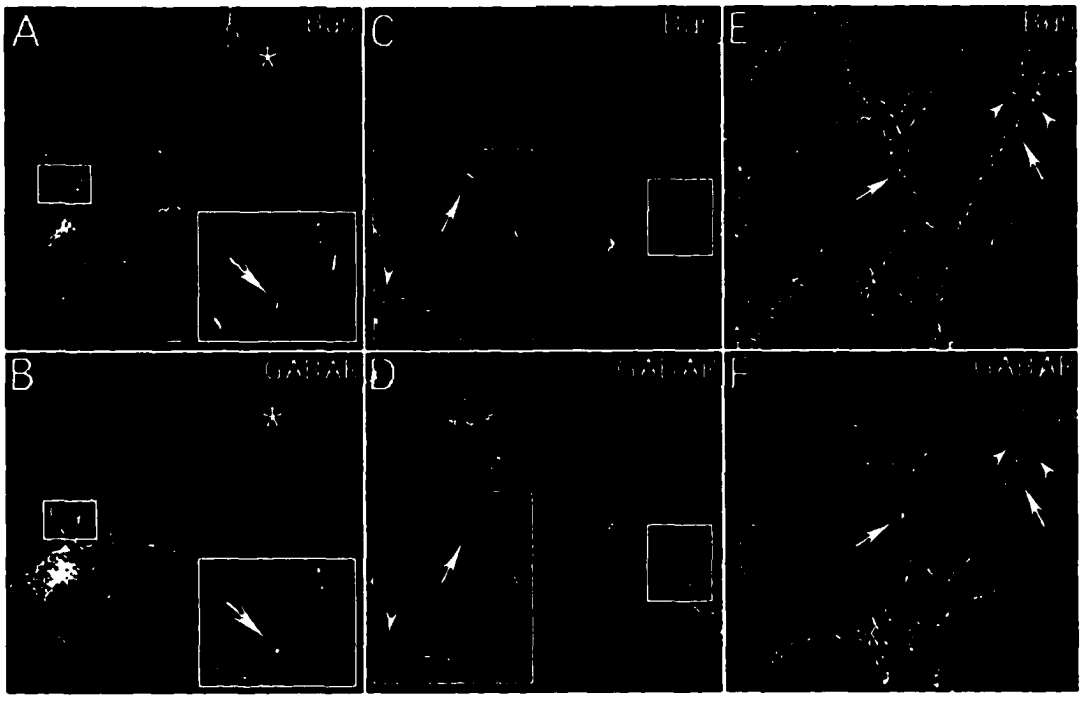

Figure 22. Early Coclustering of Bassoon with $GABA_AR$

At 4, 10, and 14 div, neurons were double labeled for Bassoon (Bas; A, C, and E) and $GABA_AR$ (GABAR; B, D, and F). At 4 div, $GABA_AR$ immuno-reactivity was mostly found diffuse in somato-dendritic compartment. However, numerous $GABA_AR$ clusters were observed co-localizing with Bassoon at this time (arrows in A and B). At 10 and 14 div, the numbers of Bassoon $GABA_AR$ clusters were seen to increase, while the diffuse cytoplasmic staining of $GABA_AR$ was observed to decrease (D). $GABA_AR$ clusters colocalize with a subpopulation of the Bassoon clusters (arrows in C-F), and the Bassoon clusters lacking $GABA_A$ clusters are indicated by arrowheads in C-F. Scale bars, 20 μm.

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decrease as the cultures mature (Figure 23; 68 ± 11%, 42 ± 2% and 38 ± 7% at 4, 10, and 21 div, respectively). The decrease in percentage of $GABA_AR$/Bassoon clusters was associated with a reciprocal increase in the percentage of GluR1/Bassoon clusters (26 ± 3%, 57 ± 11%, and 54 ± 2% at 4, 10, and 21 div, respectively; Figure 23). The ratio of GluR1/Bassoon (26 ± 3%), to $GABA_AR$/Bassoon (68 ± 11%) receptor clusters (~1/3) at 4 div corresponds very well to the ratio of excitatory to inhibitory events (~1/3) at this developmental stage (Figure 24). These observations are consistent with previous studies showing that, in the CNS, GABA synapses form earlier than glutamatergic synapses (Chen et al., 1995). To correlate the initial appearance of Bassoon clusters at 4 div with the acquisition of synaptic activity of neurons at this stage, spontaneous miniature synaptic events were recorded from cells cultured for 4 div. Among the seven cells recorded, six showed excitatory activity, and five showed inhibitory activity (Figure 24A). The inhibitory events happened at a higher frequency than excitatory events. Totally, 293 inhibitory events and 92 excitatory events were recorded (Figure 24B). These data show that functional inhibitory and excitatory synapses are present at this developmental stage and are consistent with the hypothesis that the sites of colocalization of Bassoon and cycling vesicles represent young synapses.

Taken together these data show that the formation of Bassoon clusters in differentiating neurons correlates well with the formation of physiologically functional excitatory and inhibitory synapses.

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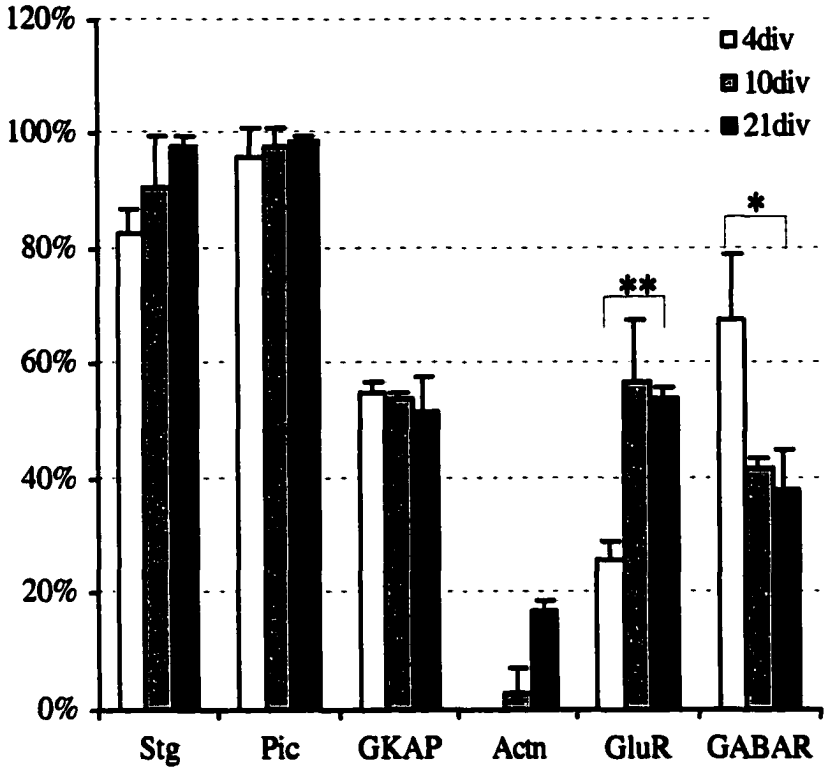

Figure 23. Quantitative Analysis of the Colocalization Between Bassoon and Various Synaptic Proteins

At 4, 10, and 21 div, neurons were double stained with anitbodies against Bassoon and one of the six synaptic proteins. Stained clusters were evaluated and counted (see Methods). At least 10 neurons were counted for each pair at each stage. The number of Bassoon clusters was normalized to 100% in each pair, and the percentage of the colocalizing clusters for each synaptic protein at each stage was displayed. Pic, Piccolo; Stg, synaptotagmin; Actn: α-actinin; GluR, AMPA receptor GluR1 subunit; GABAR, $GABA_A$ receptor subunit.
* $p < 0.05$, ** $p < 0.01$

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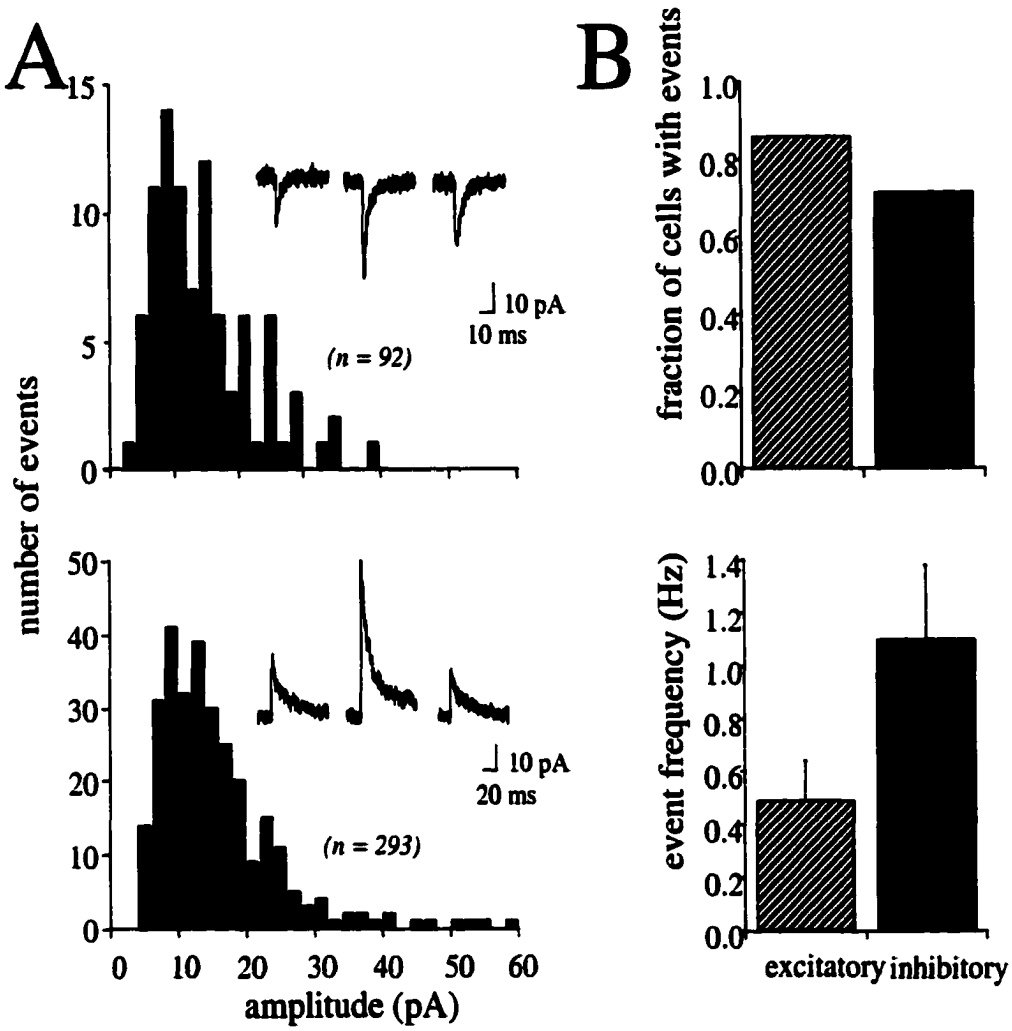

Figure 24. Miniature Synaptic Events in Hippocampal Neurons Cultured for 4 div

(A) Miniature synaptic current amplitude histograms for excitatory (upper panel) and inhibitory (lower panel) events recorded from two different hippocampal neurons. Bin size = 2 pA. Representative examples of synaptic events are shown (insets). Recording duration was 313 s and 213 s for the excitatory and inhibitory events, respectively.

(B) Histograms of the fraction of cells with detectable events (upper panel) and mean frequency of events for those cells with detectable events (lower panel). Total cells (n = 7): excitatory (6/7); inhibitory (5/7).

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Discussion

In situ, Bassoon was recently identified as a component of the CAZ that is assembled at active zones. Based on its large multidomain structure, it was suggested that Bassoon plays a role in defining synaptic vesicle release sites and regulating the vesicle cycle at existing synaptic contacts (Dieck et al., 1998). When Bassoon is first recruited to newly differentiating presynaptic boutons and whether it may be involved in neurotransmitter release from axonal growth cones and developing synapses were not examined. In this study, we investigated whether temporally the arrival of Bassoon at newly forming synapses coincides with the clustering and recycling of synaptic vesicles. Bassoon and its transcripts were present at early stages of neuronal differentiation. Moreover, Bassoon was first recruited to both excitatory and inhibitory synapses concomitant with an axonal/presynaptic accumulation and the cycling of synaptic vesicles. The early arrival of Bassoon in these compartments suggests it may be involved in structurally defining neurotransmitter release sites and the regulation of the synaptic vesicle cycle.

The early expression of Bassoon as well as Piccolo in differentiating neurons is supported by developmental Northern blots, in situ hybridization on rat brain sections and immuno-fluorescent studies of primary hippocampal neurons (Dieck et al., 1998; Zhai et al., 2000). Specifically, by Northern analysis, Bassoon transcripts were found to be present in rat brain as early as embryonic day 19, which is a stage of rapid proliferation, differentiation, and migration of neuronal cells (Zhai et al., 2000). Both Bassoon and Piccolo transcript levels reach a peak around postnatal day 10-20, corresponding to the period of robust synaptogenesis in the developing rat brain (Gaarskjaer, 1981; Melloni

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and DeGennaro, 1994; Zhai et al., 2000; 2001). A comparable temporal expression pattern was also observed in individual brain subregions by in situ hybridization. For example, in the hippocampus peak levels of Bassoon transcripts are observed around postnatal day 21, which is a time that corresponds to the major period of neuronal differentiation and synaptogenesis in the hippocampus (Gaarskjaer, 1981; Melloni and DeGennaro, 1994). Immunocytochemically, Bassoon was detected in early differentiating cultured hippocampal neurons. For example, similar to the synaptic vesicle proteins Synaptotagmin I (Figure 17) and Synapsin I (Fletcher et al., 1991; Mundigl et al., 1993; Matteoli et al., 1995), Bassoon immuno-reactivity was initially found in cell soma and all minor neurites in stage 2 neurons. Subsequently, it became asymmetrically sorted into axonal profiles and their growth cones in stage 3 neurons and clustered along dendritic profiles in stage 4 neurons. Interestingly, the punctate pattern observed for Bassoon and Piccolo in immature axons is atypical for most cytoskeletal proteins such as MAP2, tau, α-actinin, or the growth cone-associated protein GAP-43 (Goslin et al., 1990) but similar to synaptic vesicle proteins. They may represent a transport particle or hot spots of synaptic vesicle recycling at the plasmalemma. The nature of the Bassoon and Piccolo positive puncta in axons will be addressed in the following chapters.

An important issue raised by the localization of Bassoon and Piccolo to the cytomatrix assembled at the active zone of mature synapses in situ (Cases-Langhoff, 1996; Dieck et al., 1998; Brandstatter et al., 1999; Richter et al., 1999) is whether they play a general role in defining sites of synaptic vesicle cycling. Our data show that Bassoon and Synaptotagmin I coclusters are present along dendritic profiles at early stages of neuronal differentiation. Furthermore, we found that early Bassoon clusters are

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sites of synaptic vesicle recycling, as assessed by the uptake of antibodies against the luminal domain of Synaptotagmin I (Matteoli et al., 1992; Kraszewski et al., 1995). This suggests that Bassoon is recruited to the presynaptic bouton at the initial phase of synaptogenesis. This conclusion is supported by the colocalization of Bassoon clusters on immature neurons with aggregation sites of postsynaptic proteins such as GKAP, GluR1, or $GABA_AR$. The existence of functional synapses in early hippocampal cultures is further confirmed by the electrophysiological detection of spontaneous miniature excitatory and inhibitory events at this developmental stage. These data are consistent with previous studies showing that synapses appear at sites of cell-cell contact between hippocampal neurons around 4 div (Fletcher et al., 1994; Matteoli et al., 1995; Rao et al., 1998). Taken together, these observations indicate that Bassoon clusters along the dendrites of immature hippocampal neurons are found at functional synaptic junctions. Moreover, this early recruitment of Bassoon and Piccolo to developing synapses, as well as its localization to the cytoskeleton associated with active zones of mature synapses (Dieck et al., 1998; Brandstatter et al., 1999; Richter et al., 1999), suggests that they may participate in structurally defining neurotransmitter release sites of newly forming presynaptic boutons.

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PICCOLO IS TRANSPORTED TO NERVE TERMINALS IN ASSOCIATION WITH 80 NM DENSE CORE GRANULATED VESICLES DURING SYNAPTOGENESIS

Summary

The active zone is a specialized region of the presynaptic plasma membrane where synaptic vesicles dock and fuse. The assembly of the cytomatrix at the active zone is an important step in synaptogenesis. To understand the process of the assembly of the CAZ, we investigated the cellular mechanism that underlies the sorting and transport of Piccolo to the active zone. In immature neurons, Piccolo exhibited a punctate pattern that was similar yet distinct to synaptic vesicles. Our characterization of the Piccolo puncta revealed that it was associated with ~80 nm dense core vesicles that were concentrated in axonal growth cones and in association with nascent synapses. These vesicles were highly reminiscent of the granulated vesicles initially identified by Vaughn (1989), suggesting that CAZ protein Piccolo is transported to nascent synapses on an active zone precursor vesicle. The fusion of such vesicle with the plasma membrane at sites of axonal-dendritic cell-cell adhesion may deposit Piccolo and initiate the establishment of the presynaptic active zone. We refer to the Piccolo-associated vesicle as a granulated vesicle to avoid confusion with and to draw a distinction from the classical neuropeptide-containing dense core vesicles that are typically 100 nm in diameter.

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Introduction

To establish and maintain the polarity of neurons, newly synthesized proteins and organelles are sorted and transported to proper locations in neurons in order to fulfill their specific functions. Proteins are produced mainly in the cell soma and sorted either to axon or dendrites. In axons, proteins move from the cell body toward the terminal at different rates. The fastest moving group of proteins (with velocity ~200 mm per day), named the fast component (FC), is the group of proteins associated with membrane organelles. The slowest moving group (with a velocity ~0.5 mm per day), the slow component a (SCa), contains tubulin and neurofilament subunit proteins. A second slow moving group (velocity ~2 mm per day), called the slow component b (SCb), contains actin, spectrin, clathrin, metabolic enzymes, and other axonal proteins (Lasek et al., 1984). During neurite outgrowth and axon elongation, membrane flow towards the growing axon is one mechanism for the incorporation of new proteins and for membrane expansion into the growing ends (Bradke and Dotti, 1997). This flow contains transgolgi network (TGN)-derived vesicles, components involved in membrane transport and insertion, mitochondria, peroxisomes, and a cytosolic iron regulatory protein (Bradke and Dotti, 1997).

Studies on the synaptogenesis of the developing spinal cord provide the strongest evidence to date that these precursor vesicles may exist (Vaughn, 1989). Here, granulated vesicles (60-80 nm in diameter) with spicules (electron dense projections) were observed at sites of synapse formation where the small patches of active zone like structure were found to be opposed to a modest PSD-like membrane specialization (Figure 25A). These vesicles were not observed in adult synapses (Vaughn, 1989). The

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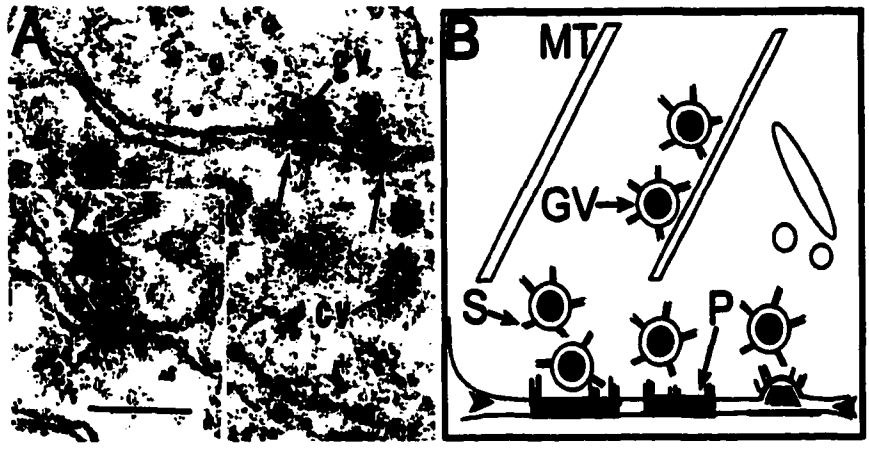

Figure 25. Dense Core Vesicles Were Observed in E15 Spinal Cord Neurons

(A) EM micrographs of E15 spinal cord (Vaughn, 1989). Granulated vesicles with dense cores are observed in close association with the cytoplasmic faces of developing synaptic membranes. Some of them have dense spicules projecting from their perimeters (arrows in insert). When vesicles are located near the presynaptic membranes, these spicules appear to blend with the material, forming paramembranous densities
(B) Schematic diagram of a nascent synapse based on (A).

GV, granulate vesicle; CV, coated vesicle; S, spicule; P, patches of newly forming active zone; MT, microtubule.

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morphological similarities between the spicules and the CAZ present at mature synapses raised the possibility that these granulated vesicles may carry structural/cytoskeletal components of the active zone. These observations provide evidence for the involvement of transport vesicles in the delivery of materials necessary for synaptic differentiation. The cytoskeleton, especially microtubules, provides tracks for the trafficking of membrane organelles. If the microtubular network is depolymerized by nocodazole, both axonal and dendritic transport of integral membrane glycoproteins are inhibited (Cid-Arregui et al., 1995). This indicates that presynaptic membrane proteins are transported to nerve terminals on vesicles that move along microtubules during axonal differentiation. However, how cytomatrix proteins like Piccolo are sorted and transported to the nerve terminal is not known.

In this chapter, I have investigated the cellular mechanism underlying the sorting, transport, and recruitment of Piccolo into nascent synapses to gain clues to the mechanisms that direct the assembly of presynaptic active zones.

Results

Piccolo is Associated With Vesicle-Like Light Membrane at Early Stage of Development

The punctate pattern exhibited by Piccolo in axonal growth cones (Figure 16A and 16C) could represent the association of Piccolo with vesicular membranes or a proteinecious particle. To understand the nature of the Piccolo puncta, I first examined the biochemical properties of Piccolo in E18 rat brain using a floatation assay. This stage is chosen because it represents an early stage of neuronal differentiation prior to the time of robust synaptogenesis (Altman and Das, 1965). E18 brain homogenates were

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hypotonically lysed and subjected to 100,000 × g centrifugation to yield the supernatant (S100) and pellet (P100) fraction. Although a small amount of Piccolo is present in S100, more than 90% of Piccolo was found in P100 (Figure 26A). P100 or S100 fraction was then adjusted to 2 M sucrose and loaded as a layer of a discontinuous sucrose gradient underneath layers of 1.2 M, 0.8 M, and 0.3 M sucrose. After centrifugation, fractions were assayed for the presence of Piccolo or synaptophysin, a synaptic vesicle protein, by Western blotting. As shown in Figure 26B, Piccolo in the P100 fraction was found in 0.3 M and 0.8 M layers together with synaptophysin, indicating that Piccolo is associated with light membranes similar to synaptic vesicles. In contrast, Piccolo in S100 fraction remained at the bottom of the gradient, indicating that Piccolo in the supernatant is not associated with membranes (Figure 26B). As a control, when P100 fraction was treated with nonionic detergent Triton X100 before the floatation assay, Piccolo and synaptophysin remained at the bottom of the gradient indicating that the ability of both Piccolo and synaptophysin to float is depended on membrane integrity. Interestingly, when adult rat brain (P30) was used in the experiment, Piccolo stayed at the bottom of the gradient while synaptophysin was found in the 0.3 M layer, suggesting that in mature brain Piccolo is primarily associated with synaptic junctions but not light membranes. This latter result is consistent with the restricted synaptic junctional localization of Piccolo in mature brain (Figure 13; Cases-Langhoff et al., 1996). These data indicate that at early stages of development Piccolo may be transported to nerve terminals in association with a vesicle.

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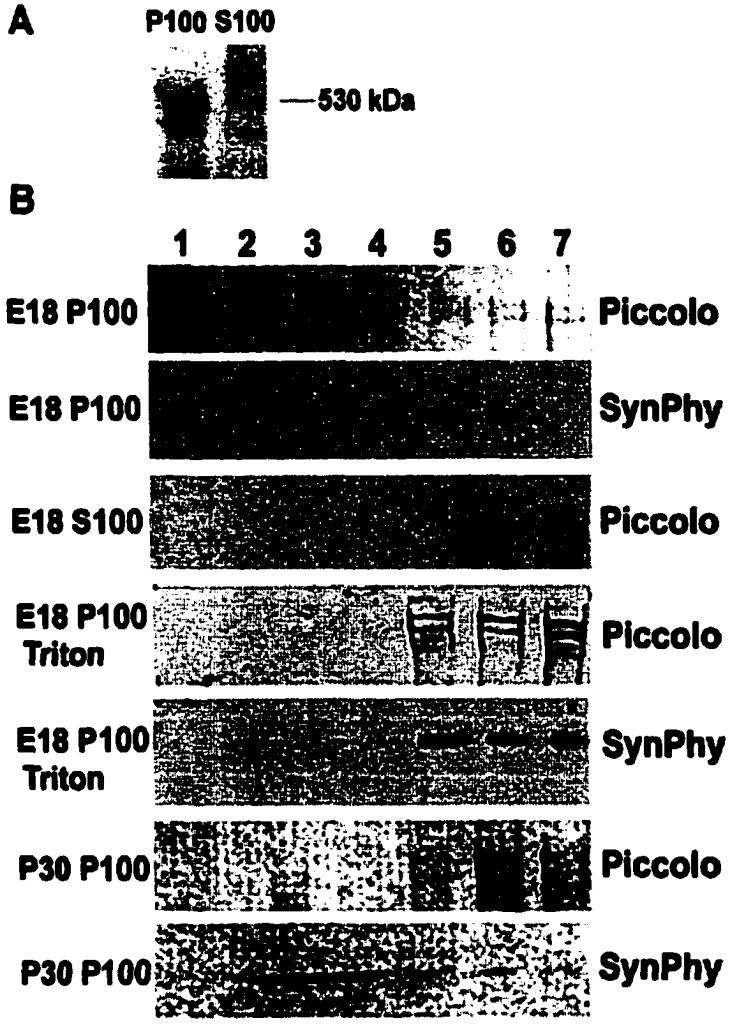

Figure 26. Piccolo is Associated With Light Membranes in Embryonic Rat Brain

(A) Western blot of Pellet (P100) and supernatant (S100) fractions from hypotonically lysed E18 brains homogenate, stained with rabbit Piccolo antibodies.
(B) Western blots of P100 or S100 fractions from either E18 or P30 rat brain homogenates after running on a flotation gradient and stained with antibodies against Piccolo or synaptophysin. Gradients were prepared by adjusting P100 or S100 fractions with 2 M sucrose and loading them at the bottom of a sucrose gradient of 0.3 M, 0.8 M, and 1.2 M. After centrifugation, fractions were taken from the top (fraction 1) of the gradient to the bottom (fraction 7). In membrane disruption experiment, P100 was also treated with 2% Triton X100 for 30 min before being adjusted to 2 M sucrose. SynPhy, synaptophysin.

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Piccolo is Transported to Nerve Terminals in Association With an 80 nm Dense Core Granulated Vesicle

To further examine whether in immature neurons Piccolo is associated with a vesicle, EM was used to study the ultra-structure of the putative Piccolo transport vesicle. An immuno-isolation approach was used to purify the Piccolo complex. The light membrane fractions containing Piccolo from E18 were incubated with Piccolo rabbit polyclonal antibody coated beads (Piccolo-rAb beads). The isolated bound fractions were processed for EM. Samples processed with 3% paraformaldehyde and 1% glutaradehyde (Fix 1) revealed spherical structures with electron dense cores surrounded by clouds of less dense material (Figure 27A1). Using 4% glutaraldeyde and 0.8% tannic acid in 0.1 M cacodylate buffer (Fix 2) to enhance the fixation, closed spherical vesicles with dense cores were found attached to Piccolo-rAb beads with an average outer diameter of 80.2 ± 8.1 nm (n = 88; Figure 27A2 and A3). Similar results were obtained using Bassoon-mAb beads (Figure 27B). Vesicles of 50 to 60 nm with clear center were found on synaptophysin-mAb beads (n = 25; Figure 27C). No vesicular elements were attached to the control immunoglobulin G (IgG) beads (Figure 27D). As described below, this immuno-isolation procedure is highly specific, leading to the selective isolation of Piccolo or synaptophysin, respectively, as assessed by Western blotting. These data support the hypothesis that Piccolo in immature neurons is associated with a transport vesicle, referred to as the PTV.

To examine whether granulated vesicles of approximately 80 nm are actually present in immature axons, 3 div hippocampal cultures were processed for EM. Interestingly, granulated vesicles with similar size (80 nm) and morphology as the granulated vesicle isolated with Piccolo antibodies were found in axons in the vicinity of

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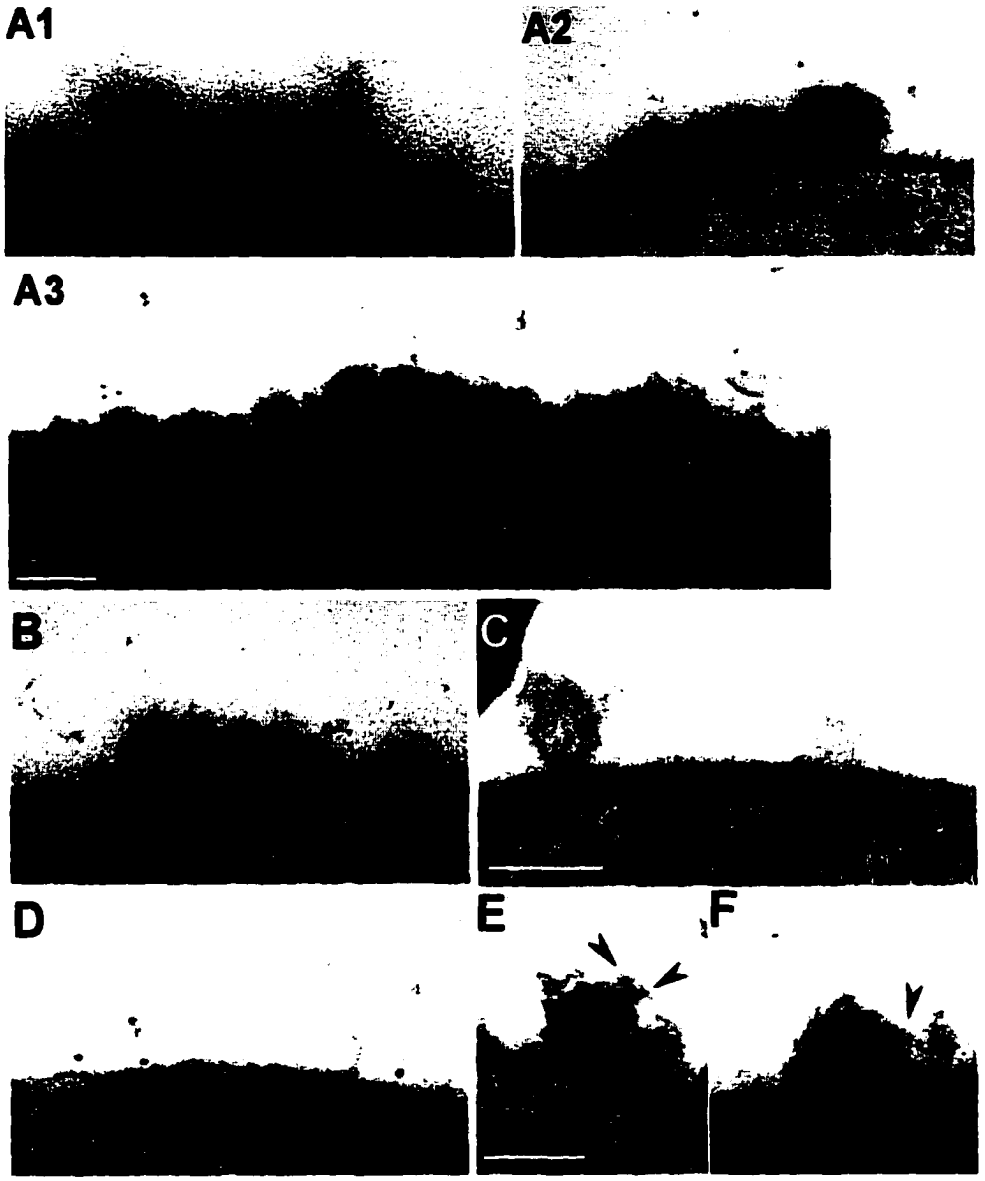

Figure 27. The PTV is a Dense Core Granulated Vesicle With ~80 nm Diameter

The light membrane fractions collected from the flotation assay (Figure 24B, fractions 2 through 4) were incubated with Piccolo-rAb beads (A1-A3), Bassoon-mAb beads (B), synaptophysin-mAb beads (C), or the irrelevant IgG beads (D). The beads were then collected and extensively washed, fixed, and processed for EM. In A1, the beads were fixed by Fix 1 (see Methods), and in A2-D the beads were fixed by Fix 2 to enhance the membrane. (E) The Bassoon-mAb beads isolated fractions were incubated with Piccolo rAb, followed by 6 nm gold conjugated anti-rabbit secondary antibody. Piccolo is present on the Bassoon-mAb isolated dense core vesicles (arrowheads in D). (F) Piccolo-mAb beads isolated fractions were incubated with Bassoon-mAb, followed by 5 nm gold conjugated anti-mouse secondary antibody. Bassoon is present on the Piccolo-rAb coated beads isolated dense core vesicle (arrowhead in F). Scale bars, 100 nm in A3 for A-B, in C for C-D, in E for E-F.

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nascent synapses (Figure 28A). Here, an axon, which is characterized by parallel microtubules, make contact with a cell soma, which is characterizaed by the presence of polyribosomes (Figure 28A, small arrowheads). These granulated vesicles are distinct from the clear-centered vesicles, presumably synaptic vesicles, and clathrin-coated vesicles (Figure 28A and 28B). Interestingly, granulated vesicles were found along microtubules, suggesting a microtubule-based transport mechanism. Furthermore, in cell soma ~80 nm dense core granulated vesicles are present close to the initial axon segment (Figure 28D), as well as in a perinuclear region in association with Golgi stacks (Figure 28C), suggesting that this vesicle is derived from the Golgi apparatus. These data, together with the perinuclear and axonal localization of Piccolo puncta in immature neurons revealed by immunofluorescent staining (Figure 16), indicate that, shortly after synthesis, Piccolo becomes associated with Golgi derived dense core granulated vesicles.

The association of Piccolo and Bassoon with these 80 nm granulated vesicles in vivo was confirmed by silver enhanced immno-gold EM with Piccolo antibodies (Figure 29A-29C) and Bassoon antibodies (Figure 29D). The quantitative analysis of this experiment was summarized in Table 1. When Piccolo antibodies were used, 117 dense core vesicles were found decorated with one or more gold particles (1.4 gold particles per dense core vesicle). Of the 184 gold particles, 88.9% of the gold particles were found in association with dense core granulated vesicles, 2.6% were found to labele other types of vesicular organelle, and 8.5% were found associated with nonvesicular material. Similarly, when Bassoon antibodies were used, 72 dense core vesicles were found decorated with gold particles (1.33 gold particles per dense core vesicle). Of the 112 gold particles, 91.5% of the gold particles were found in association with dense core granulated vesicles, 2.1% found labeled other types of vesicular organelle, and 6.4% were

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Figure 28. Dense Core Granulated Vesicles are Present in Axons and Cell Soma of 3 div Hippocampal Neurons

EM micrographs of hippocampal neurons cultured for 3 div. In (A), an axon comes into contact with the soma of a neighboring neuron, and dense core granulated vesicles are observed (arrows). A high magnification micrograph of the selected region (dashed line box) is shown in (B). 80 nm dense core granulated vesicles are observed along microtubules (MT; arrows). Clear center vesicles were also present (small arrow). A coated vesicle can be observed invaginating from the plasma membrane (asterisk). An electron dense region most likely represents a nascent synapse (arrowheads) and can be observed between the axon and an adjacent cell soma. Dense core granulated vesicles were also observed in close association with Golgi stacks (arrow in C) and in cell soma close to the initial segment of axon (arrow in D). Scale bars, 100 nm in all panels.

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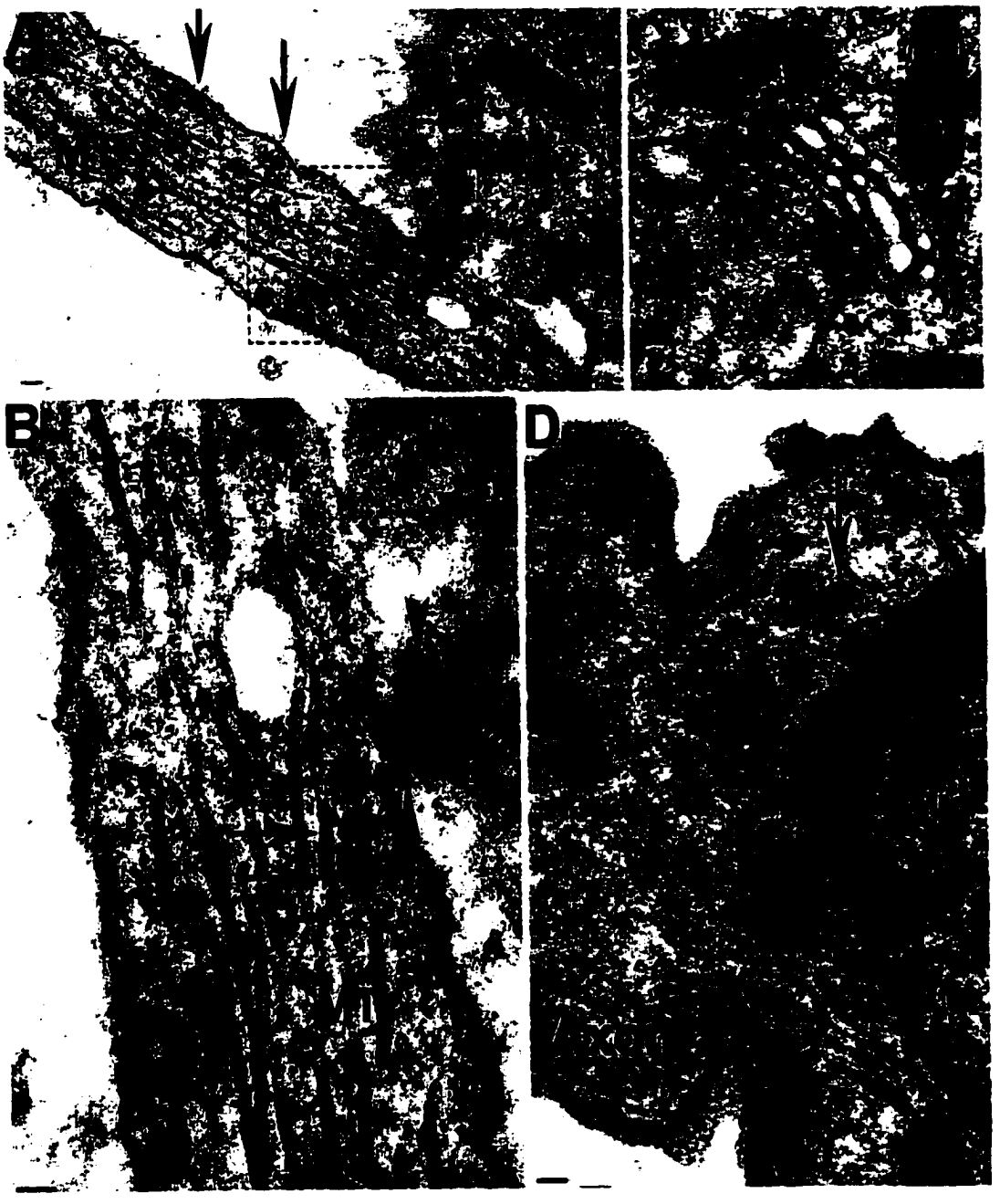

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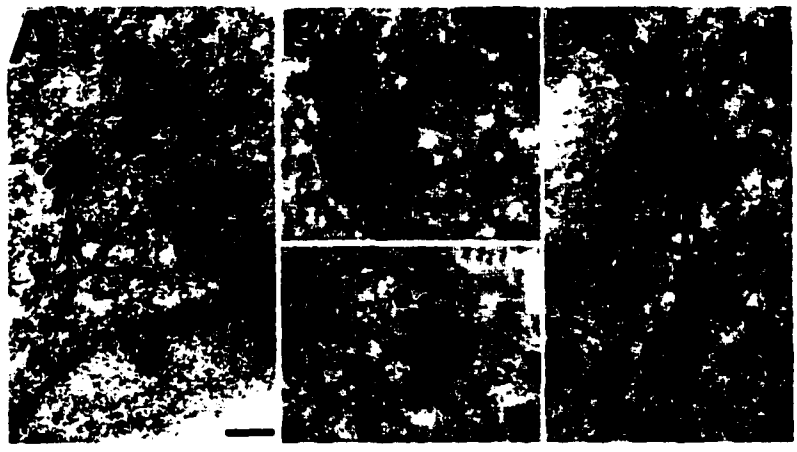

Figure 29. Piccolo and Bassoon are Present on Dense Core Vesicles

The presence of Piccolo (A-C) and Bassoon (D) on granulated vesicles was visualized by silver enhanced immunogold EM on cultured hippocampal neurons. Gold particles labeled dense core vesicles (arrows in A and D), but not other type of vesicles (arrowheads in A and D). Scale bars, 100 nm in all panels.

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Table 1. Quantitative Analysis of the Immunogold EM on Cultured Hippocampal Neurons

Antibody used	Piccolo Ab	Bassoon Ab	Control Ab
Total number of labeled DCVs	117	72	6
Total number of gold particles	184	112	38
Number of gold particles per DCV	1.40	1.33	N/A
Percentage of gold particles decorated DCVs	88.9%	91.5%	15.8%
Percentage of gold particles decorated other types of membrane structure	2.6%	2.1%	N/A
Percentage of gold particles decorated non-vesicular material	8.5%	6.4%	N/A

Piccolo polyclonal antibody 44aII and Bassoon monoclonal antibody were used. In control experiment, primary antibody was replaced by rat serum (see Methods). DCV, dense core vesicles.

found associated with nonvesicular material. In the control experiment, 78% of the gold particles were not found associated with any specific type of organelle, and only 15.8% of the particles (6 out of 38) were seen in close proximity to the 80 nm dense core granulated vesicles. These vesicles, once sorted and transported into axons and their growth cones, can readily participate in the assembly of active zones, as suggested by Vaughn (1989) and diagrammed in Figure 25B.

Piccolo Transport Vesicles are Distinct From Synaptic Vesicles

Although the migration pattern of Piccolo in floatation assay is very similar to synaptophysin, the EM analysis of PTV revealed a morphologic distinction from typical

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synaptic vesicles. To further confirm this observation, biochemical approaches and double-labeling immunofluorescent microscopy were used to distinguish PTV from synaptic vesicles. First, the immuno-isolation assay was used to compare the components present in each type of vesicle. Beads coated with Piccolo antibodies or synaptophysin antibodies were used to isolate PTV or synaptic vesicles respectively. The isolated fractions were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and evaluated for the presence of Piccolo and synaptic vesicle proteins by Western blotting. As shown in Figure 30, Piccolo Ab coated beads isolated Piccolo but none of the synaptic vesicle proteins (VAMP2/synaptobrevin, synaptophysin, and synaptotagmin) were found. In reciprocal experiments with synaptophysin antibody coated beads, Piccolo was not isolated, but all of the above synaptic vesicle proteins were present. None of these proteins were isolated with beads coated with an irrelevant control IgG. A peri-synaptic protein GABA transporter 1 (GAT1) was probed as a negative control. GAT1 is not present in either Piccolo antibody or synaptophysin antibody isolated fraction. These results suggest that Piccolo is not associated with synaptic vesicles but rather a distinct vesicle complex. Double label immuno-fluorescent microscopy on the axons and growth cones of 3 div neurons was used to confirm these results. Cultures were double stained for Piccolo and synaptotagmin or VAMP2/synapto-brevinII. As shown in Figure 31, Piccolo puncta did not colocalize with either VAMP2/synaptobrevinII or synaptotagmin, even though the general staining patterns are very similar. The immuno-isolation and the double-labeling experiments clearly demonstrated that the PTVs are distinct from synaptic vesicles.

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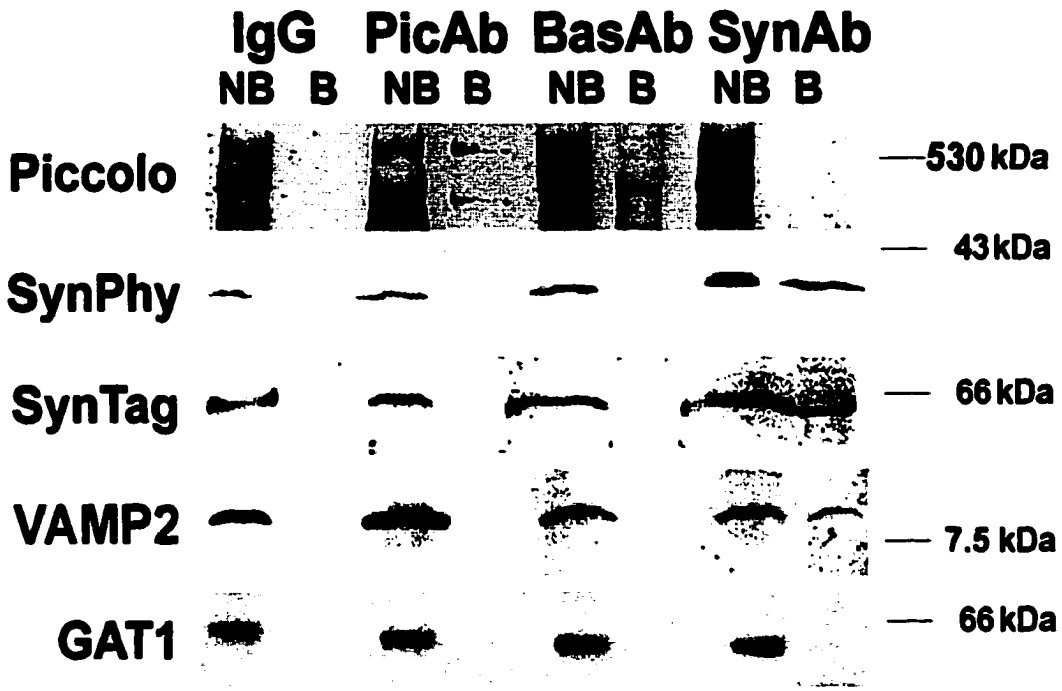

Figure 30. Piccolo Ttransport Vesicles Do Not Contain Components of Synaptic Vesicles

Light membrane fractions immuno-isolated with beads coated with Piccolo, Bassoon, synaptophysin, or the irrelevant IgG antibodies were tested by Western blotting. The supernatant fractions were saved as the nonbound subfraction (NB), and the beads were extensively washed. The beads bound subfraction (B), and NB fractions were resolved by SDS-PAGE and subjected to Western blotting to detect the presence of Piccolo, synaptophysin (SynPhy), synaptotagmin (SynTag), VAMP2/synaptobrevin2 (VAMP2), and GABA transport 1 (GAT1).

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Discussion

In this study, I examined how Piccolo is recruited into nascent synapses to gain insights into the mechanisms underlying presynaptic active zone assembly. Piccolo was found associated with 80 nm Golgi derived granulated vesicles that are asymmetrically sorted into axons and their growth cones and recruited into nascent synapses at a time that corresponds to the acquisition of activity-induced synaptic vesicle recycling. One prerequisite of synapse assembly is that synaptic proteins need to be correctly sorted and transported. Numerous studies have shown that microtubule-dependent vesicular trafficking plays a fundamental role in the differential transport of proteins to their distinct membrane specializations (see Bradke and Dotti, 1998; Hirokawa, 1998; Goldstein and Philp, 1999). Real time imaging studies on cultured neurons have shown that vesicles of different types and shapes accumulate at nascent synapses (Kraszewski et al., 1995; Ahmari et al., 2000) and thus may participate in the formation of nerve terminals. However, the relationship between these vesicles, their cargoes, and the assembly of presynaptic subdomains such as the active zone is unclear. In the previous chapter, I followed the changing spatial distribution of Piccolo during neuronal differentiation and found that it is sorted into axons at early stages and displayed a vesicle-like punctate pattern. In this chapter, I demonstrated that Piccolo is transported to nerve terminals in association with 80 nm vesicles that are morphologically distinct from synaptic vesicles. This conclusion is supported by biochemical analysis, double-labeling immunofluorescent microscopy, and EM studies. These observations suggest that cytoskeletal components of the active zone like Piccolo are not necessarily recruited to nascent synapses from cytosolic pools but rather from a vesicular intermediate (see the following chapter).

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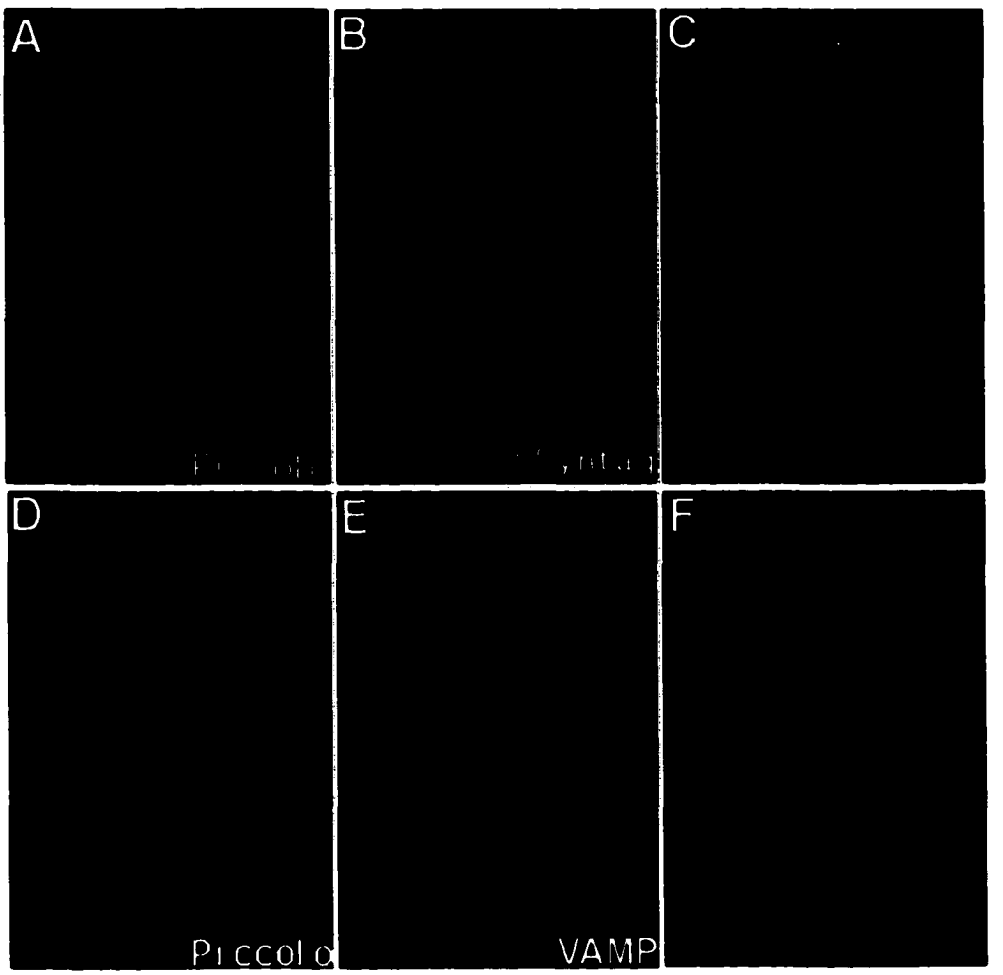

Figure 31. The Piccolo Transport Vesicle is Distinct From Synaptic Vesicles

Neurons cultured for 4 div were double labeled for Piccolo (A and D) in green and synaptotagmin (SynTag; B) or VAMP2/synaptobrevin2 (E) in red. The distal portions of axons and their growth cones were displayed in a vertical orientation with their growth cones at the top. Piccolo and synaptotagmin (B) or VAMP2 (E) showed no colocalization. Scale bars: 10 μm in all panels.

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The EM micrographs revealed a close association of PTV with microtubules in axons of cultured hippocampal neurons (Figure 28). This suggests that PTV may use a microtubules-based transport mechanism. Although more direct evidence is needed, this is predicted based on the studies on axonal transport of synaptic vesicles and other types of vesicles (Goldstein and Yang, 2000). It is likely that Piccolo is associated with the outside of the PTVs, and upon fusion, Piccolo will be deposited at the nascent synaptic junction where it can participate in the subsequent assembly of the cytomatrix underlying the active zone. Figure 25B depicts a possible mechanism utilized by neurons to assembly active zones. In this model, Piccolo is associated with a PTV that is specifically sorted into axons and their growth cones. These vesicles can readily participate in the assembly of active zones by fusion with the plasma membrane at sites of cell-cell adhesion. The fusion will lead to the localization of all membrane-associated proteins present on or in the PTV to active zones, as well as the deposition of the dense core material into the nascent synaptic cleft. It is likely that the localization and clustering of membrane-associated proteins at a confined space in active zones will result in the formation of electron-dense patches observed in mature active zones by EM (Landis et al., 1988).

An interesting issue raised by our studies concerns the biogenesis of the PTV. Recent studies by Ahmari et al. (2000) have observed the presence of vesicular tubules as well as dense core granulated vesicles at nascent synapses. The latter is likely to be a PTV. This has lead to the suggestion that presynaptic junctional proteins are recruited to nascent synapses from vesicles budding from these vesicular tubules (Roos and Kelly, 2000). At present it seems unlikely that the PTV buds from these vesicular tubules but rather is derived from the Golgi. This conclusion is supported by both the punctate peri-

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nuclear appearance of Piccolo and Bassoon in immature neurons and the presence of 80 nm granulated vesicles in close association with Golgi stacks in the soma of differentiating neurons. This situation is rather analogous to the biogenesis of both synaptic vesicles and large dense core vesicles (LDCVs) (Huttner et al., 1995; Hannah et al., 1999). It is also unclear whether the vesicular tubules associated with nascent synapses represent endosomal membranes or a repository for at least some component of mature synapse. The presence of the synaptic vesicle proteins VAMPII/synaptobrevin, synapsin, and synaptotagmin, as well as calcium channels within this cluster of vesicles (Ahmari et al., 2000), suggests that perhaps both scenarios are true. With regard to the utility of green fluorescent protein (GFP)-VAMP2/synaptobrevinII to tag the cluster of vesicles associated with nascent synapses as observed by Ahmari and colleagues, it should be noted that we did not find VAMP2/synaptobrevinII associated with the PTV. Finally, the close association of the granulated vesicles with axonal microtubules suggests an active microtubule-based transport of the PTV from the soma to nascent synapses.

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THE PICCOLO TRANSPORT VESICLE IS A PRECURSOR OF THE ACTIVE ZONE

Summary

To ensure precise neurotransmitter release, the machinery of synaptic vesicle exocytosis has to be assembled properly at the active zone. The correct localization depends on accurate transport and targeting. One mechanism of transport and targeting is the transport of structurally and/or functionally related proteins as a multimeric complex. It is not clear if presynaptic proteins are transported in a preformed complex. Previous chapter has shown that the CAZ protein Piccolo is transported to nascent synapses on a dense core granulated vesicle. To understand the mechanism of presynaptic assembly, we dissected the components of the PTV and found that Piccolo transport vesicles carry the components of synaptic vesicle exocytosis machinery. These components have been genetically proven to be involved in either SNARE complex formation, such as syntaxin and SNAP-25, or SNARE complex regulation, such as Munc18, Munc13, complexin, Ca^{2+} channel, and Rab3. In addition, PTV carries all four high molecular weight CAZ proteins: Piccolo, Bassoon, RIM, and Munc13. Furthermore, PTV also contains key adhesion molecules of synaptic junctions, such as N-cadherin, N-CAM, and syndecan2. Interestingly, in searching for the proteins making up the dense core, we identified a neuropeptide precursor Chromogranin B, suggesting PTV may contain peptides or growth factors that act on postsynapse formation. These results suggest that the PTV is a preformed complex that serves as an active zone precursor.

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Introduction

Given the complexity of synaptic functions, it is not surprising that a large number of proteins are present at synapses. Proteins localized at synaptic junctions can be divided into several groups: proteins involved in exo- and endocytosis; neurotransmitter receptors, transporters, and ion channels; protein kinases, phosphatases, and other cellular enzymes that closely interact with proteins at the junction; and cytoskeleton proteins, adhesion molecules, and cytomatrix proteins.

One of the major functions of presynaptic nerve terminals is the activity-dependent release of neurotransmitter. This process is accomplished by calcium-dependent synaptic vesicle exocytosis followed by endocytosis. Synaptic vesicle exocytosis only occurs at the active zone, while clathrin-mediated endocytosis is largely observed at peri-active zonal areas (Bartlett and Banker, 1984; Brodin et al., 2000). Synaptic vesicle exocytosis is a multistep, highly regulated process. Many proteins have been identified to be involved in this process, and, interestingly, most of them do not localize on synaptic vesicles.

One class of ion channels present in the presynaptic plasma membrane is calcium channels. Neurotransmitter release is triggered by elevation in intracellular Ca^{2+}, which is controlled by multiple types of Ca^{2+} channels synergistically (Mintz et al., 1995). Two plasma membrane proteins, syntaxin and SNAP-25, are directly involved in vesicle fusion by forming SNARE complex with synaptic vesicle protein VAMP/synaptobrevin. The SNARE complex interacts with N-type and P/Q-type Ca^{2+} channels (Sheng et al., 1994; Rettig et al., 1996). Specifically, syntaxin interacts with the $\alpha 1$ subunit of N-type or P/Q-type channel, and SNAP-25 interacts with P/Q-type channel. The interaction

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between SNARE and N-type channel is calcium dependent, and disruption of this interaction by a peptide sequence of the syntaxin-binding site of α1A subunit alters calcium-dependence of neurotransmitter release (Sheng et al., 1994; Rettig et al., 1997). Therefore, syntaxin and SNAP-25 not only are components of fusion complex but also regulate the vesicle fusion. A large protein family that participate in membrane trafficking is Rab proteins. Rab proteins are members of the ras superfamily of small GTPases. Approximately 40 Rab proteins have been identified in mammals, all with specific subcellular localizations (Pfeffer 1994; Novick and Zerial, 1997). Among them, Rab3a has been strongly implicated in synaptic vesicle dynamics (Fischer von Mollard et al., 1994). In *C. elegans* Rab3 knockout mutants display a decrease in the number of vesicles at active zones and a concomitant increase along the length of axons, indicating that Rab3 may facilitate the movement of vesicles to target sites (Nonet et al., 1997). Although murine Rab3a knockout displays only very minor phenotypes, potential Rab effectors, including Rabphilin, RIM, and PRA-1 (prenylated Rab acceptor), suggest possible roles for Rabs. Rab3 may attach or detach vesicles from the cytoskeleton through its interaction with Rabphilin3a, which interacts with α-actinin (Kato et al., 1996). Rab3 may regulate SNARE complex assembly through its interaction with PRA-1, which can interact with SNARE protein VAMP/synaptobrevin (Bucci et al., 1999). Munc18, the neuronal homolog of yeast Sec1, is another gene required for synaptic transmission. Interestingly, genetic analyses suggested that Munc18 has both positive and negative effects. Mutation of the Munc18 homolog UNC18 in *C. elegans* caused a severe paralysis that can be rescued by murine Munc18, and heterozygous knockout of the *Drosophila* homologue Rop caused loss of normal synaptic activity (Gengyo-Ando et al.,

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1996; Wu et al., 1998). On the other hand, overexpression of *Drosophila* homologue Rop can also decrease neurotransmitter release (Schulze et al., 1994). Recent structural studies suggest that Munc18 regulates synaptic vesicle fusion by its direct interaction with syntaxin (Misura et al., 2000). Munc18 binds a closed conformation of syntaxin, and this binding prevents the formation of SNARE complex (Yang et al., 2000). A possible model of the SNARE core complex nucleation has been proposed based on studies of Rab3 and Munc18 (Lin and Scheller, 2000). In this model, Syntaxin starts in the closed conformation bound to Munc18. Rab3 and its effector then cause a conformational change in Munc18 through direct or indirect interaction, leading to destabilization of syntaxin's linker region between helices, which open up syntaxin and allow the nucleation of SNARE core complex.

As discussed earlier, among four known large CAZ proteins, Piccolo, Bassoon, Munc13, and, RIM, Munc13 is the only protein for which a defined function in the synaptic vesicle cycle was assigned. Munc13, a mammalian homologue of *C. elegans* UNC13, is a novel target of the diacylglycerol second messenger pathway and is an essential component of the synaptic vesicle priming apparatus (Betz et al., 1998). Munc13 deficient synapses are morphologically normal but do not contain fusion competent vesicles (Augustin et al., 1999). Recently, the mechanism of synaptic vesicle priming has been partially unveiled. Munc13 may mediate the priming process through its interaction with RIM and syntaxin (Betz et al., 1997; Brose et al., 2000; Betz et al., 2001). Disruption of the RIM and Munc13 interaction in wild-type neurons results in a deficiency in synaptic vesicle priming that is reminiscent of the Munc13 knockout phenotype, suggesting this interaction is likely to be the key in vesicle priming (Betz et

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al., 2001). It is interesting to note that RIM is a Rab3 effector. Therefore, RIM can nicely fit in the above SNARE core complex nucleation model proposed by Lin and Scheller (2000), although the details of the mechanism still need to be worked out. Many proteins regulate the synaptic vesicle cycle through their interactions with syntaxin. In addition, to the syntaxin binding proteins discussed previously, there is yet another protein, Complexin, that also binds to the SNARE complex. Complexin is a cytosolic protein that regulates the functions of SNAREs by competing with α-SNAP (McMahon et al., 1995). Injection of recombinant complexin II into presynaptic neurons in *Aplysia* buccal ganglia caused a decrease of neurotransmitter release, and complexin II-deficient mice had impaired long-term potentiation although ordinary synaptic transmission and short-term plasticity were normal (Ono et al., 1998; Takahashi et al., 1999).

Before the vesicle fusion event, synaptic vesicles have to be targeted to active zones. In yeast, the Sec6/8 complex, or excoyst, acts to target vesicles to sites of exocytosis but does not actually participate in the fusion event itself (Aalto et al., 1993; TerBush and Novick, 1995; Guo et al., 1999). The mammalian Sec6/8 complex is a 743 kDa, eight-subunit complex homologous to the yeast Sec6/8 complex (Hsu et al., 1998). In rat brain, Sec6/8 complex appears to localize to regions of active synaptogenesis. However, the immunoreactivity almost disappears in adult brains, and mature synapses apparently do not require the presence of the complex (Hazuka et al., 1999). Although the exact function of Sec6/8 in brain is not clear, it is strongly suggested that the complex plays an important role in vesicle targeting, especially during the establishment of synapses (Lin and Scheller, 2000).

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To establish synaptic connections and hold the synaptic junction in place, structural components have to be precisely localized to synaptic junctions early enough to enable the acquisition of synaptic function. The cortical cytoskeleton at the presynaptic terminal is thought to consist of fodrin/spectrin, ankyrin and protein 4.1-like proteins, actin filaments, SAPs, and Piccolo (Burns and Augustine, 1995; Garner and Kindler, 1996). To keep the pre- and postsynaptic membranes in register, cell adhesion molecules (NCAM) and cadherins are involved. NCAM-180 binds to spectrin and fodrin (Kramer et al., 1997). The cadherin/catenin complex is widely expressed in brain. In the mouse cerebellum and hippocampus, N-cadherin and E-cadherin are differentially expressed in early and late synapses where they serve a primary adhesive function. N- and E-cadherin are distributed in a mutually exclusive pattern because the distribution does not colocalize or overlap at the same synaptic complexes (Fannon and Colman, 1996).

The protein composition of presynaptic terminal has been under intensive scrutiny, and, as a result, our knowledge about the molecular mechanism underlying synaptic functions has expanded greatly. However, very little is known about how proteins are localized to synaptic junctions. The localization of proteins is pivotal to synaptic function. The correct localization depends on accurate transport and targeting. One mechanism of transport and targeting is the transport of structurally and/or functionally related proteins as a mutimeric complex. For example, in axonal transport, clathrin, clathrin uncoating ATPase (HSC70), and the 30-36 kDa clathrin-associated proteins (CAPs), which regulate assembly of clathrin into coated vesicles, are transported as a complex in SCb (Gower and Tytell, 1987; Black et al., 1991). Similarly, actin filaments and proteins bound to actin are transported together in SCb (Sheetz et al.,

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1998). It is not clear if presynaptic proteins are transported in a preformed complex. Recently, studies by Ahmari et al. (2000) have shown that clusters of vesicles containing synaptic proteins are found at sites of newly forming synapses. These data suggest that nerve terminals may be assembled in part from preformed complexes. However, whether these vesicular clusters represent a general reservoir for presynaptic proteins or whether they represent precursor vesicles each involved in the assembly of specific presynaptic subdomains such as the active zone or peri-synaptic plasma membrane is unclear. This chapter will address this question by dissecting the components of PTV and thus unraveling the mechanism of presynaptic assembly.

Results

Piccolo Transport Vesicles Contain Other CAZ Proteins

The presence of a presynaptic cytomatrix protein Piccolo on the outside of a dense core granulated vesicle suggests that this vesicle may also carry other cytomatrix proteins and serve as a precursor complex to the active zone. To test this hypothesis, immuno-isolated PTV preparations were resolved by SDS-PAGE and evaluated for the presence of other CAZ proteins by Western blotting. As shown in Figure 32, when Piccolo antibody-coated beads were used, the other three large CAZ proteins, Bassoon, RIM, and Munc13, as well as CASK, were also copurified with Piccolo, suggesting that the PTV carries all four large CAZ proteins known so far. These results were confirmed by double-label immunofluorescent microscopy on the axons and growth cones of 3 div neurons. Cultures were double stained for Piccolo and Bassoon or RIM. As shown in Figure 33, Piccolo puncta revealed a high degree of colocalization with Bassoon puncta

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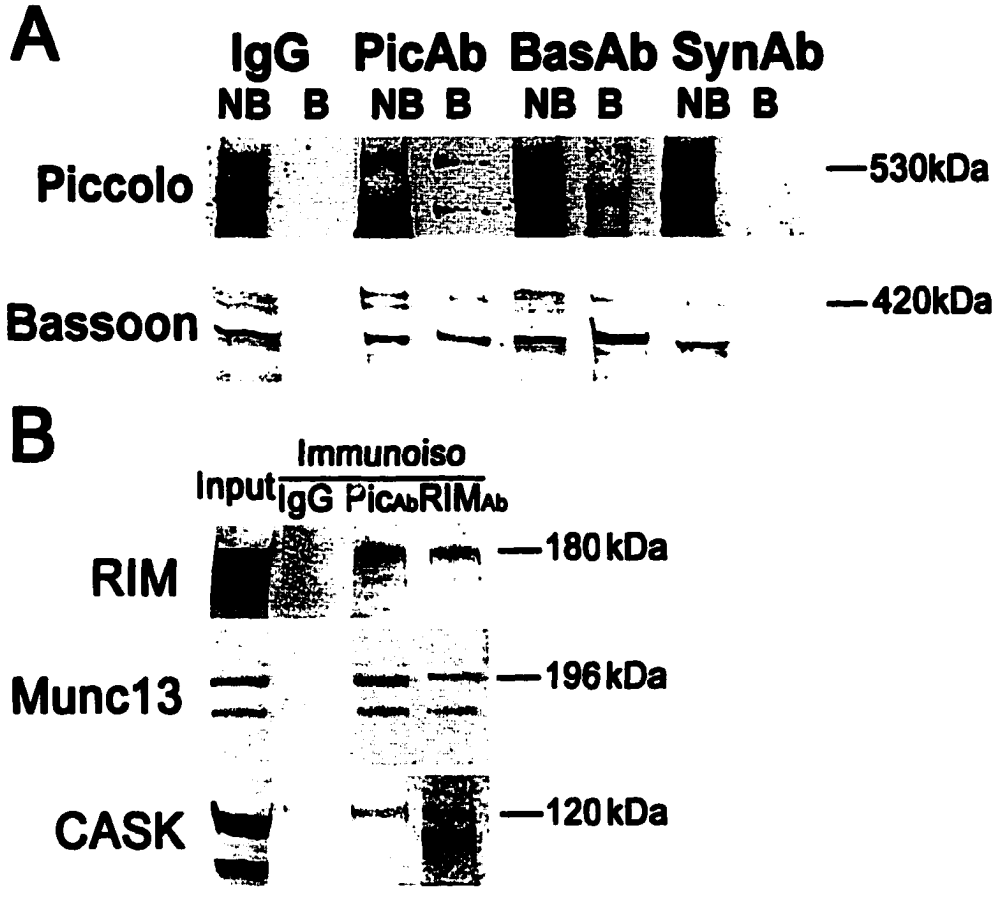

Figure 32. Piccolo Transport Vesicles Contain CAZ Proteins

(A) Western blots of light membrane fractions immuno-isolated with beads coated with Piccolo, Bassoon, synaptophysin, or the irrelevant IgG antibodies. The supernatant fractions were saved as the nonbound subfraction (NB), and the beads were extensively washed. The beads bound subfraction (B), and NB fractions were resolved by SDS-PAGE and subjected to Western blotting to detect the presence of Piccolo and Bassoon. (B) Western blots of light membrane fractions immuno-isolated with beads coated with Piccolo, RIM, or the irrelevant IgG antibodies. The beads bound subfraction (B), and the input were resolved by SDS-PAGE and subjected to Western blotting to detect the presence of RIM, Munc13-1, or CASK.

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(>95%; Figure 33A-33C) and a moderate level of colocalization with RIM punta (Figure 32D-32F).

Piccolo and Bassoon seem to have identical distribution in cultured neurons; therefore, it is likely that they are transported on identical vesicles. The association of Bassoon with dense core granulated vesicles was further confirmed both by EM analysis immuno-isolated fractions (Figure 27B) and by immuno-EM of cultured neurons (Figure 29D). EM analysis of magnetic bead coated with our Bassoon antibodies revealed the presence of 80 nm dense core vesicles (Figure 27B) similar to those observed when the beads were coated with Piccolo antibodies (Figure 27A). The association of Bassoon with 80 nm dense core granulated vesicles was confirmed by immuno-gold EM analysis of cultured hippocampal neurons (Figure 29D). Additionally, immuno-gold EM analysis of isolated PTVs also confirmed that Piccolo and Bassoon were both present on the PTV (Figure 27E-27F). When the PTV fractions isolated by Piccolo-rAb coated beads were incubated with Bassoon-mAb, followed by 5 nm gold conjugated anti-mouse secondary antibody, gold particles were seen decorating the tufts of material on the membrane surface, suggesting Bassoon is present on the dense core vesicle isolated by Piccolo-rAb coated beads (arrowhead in Figure 27F). Similarly, when Bassoon-mAb bead isolated fractions were incubated with Piccolo rAb, followed by 6 nm gold conjugated anti-rabbit secondary antibody, Piccolo is present on the dense core vesicle isolated by Bassoon-mAb coated beads (arrowheads in Figure 27E).

Taken together, these results strongly suggest that the CAZ proteins, Piccolo, Bassoon, RIM, Munc13, and CASK, are associated with dense core granulated vesicles in

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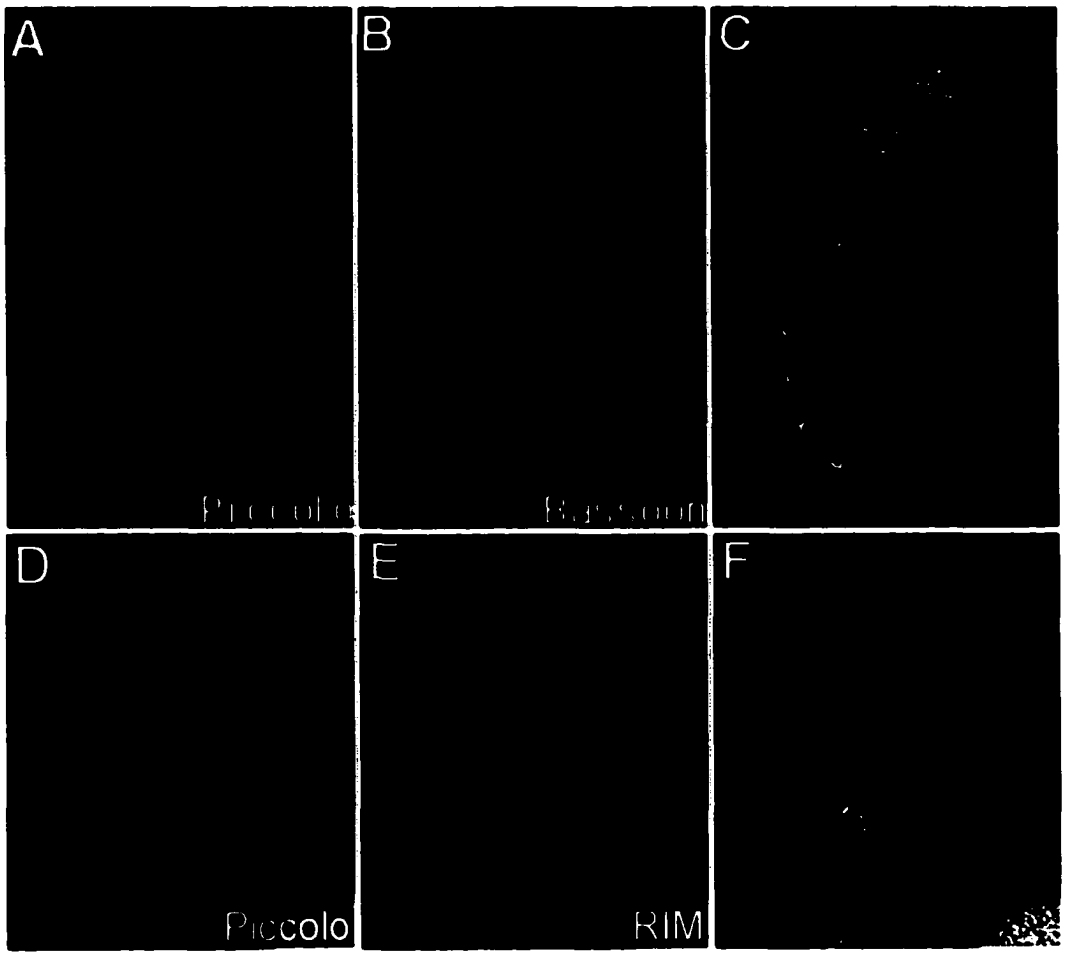

Figure 33. Piccolo Co-Localizes With CAZ Protein Bassoon or RIM

Neurons cultured for 4 div were double labeled for Piccolo (A and D) in green and Bassoon (B) or RIM (E) in red. The distal portions of axons and their growth cones were displayed in a vertical orientation with their growth cones at the top. A high degree of colocalization can be observed between Piccolo and Bassoon (C), and Piccolo partially colocalized with RIM (F). Scale bars: 10 μm in all panels.

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vivo and that the PTV is a preformed complex for the cytomatrix at the active zone. Upon fusion, PTVs can deliver these proteins to the newly forming active zone and initiate the assembly of the cytomatrix.

Piccolo Transport Vesicles Contain Components of Exocytosis Machinery

The main function of the active zone is the docking, fusion, and recycling of the synaptic vesicles. Although a number of components of the exocytosis machinery have been identified, it is not known how these proteins are assembled. They can either be individually recruited to the active zone or they can be assembled from a preformed complex. PTVs carried not only Piccolo but also other CAZ proteins, suggesting it may be a precursor for the active zone. To further test this hypothesis, we evaluated the presence of the following components of exocytosis machinery. The functions of these proteins in exocytosis pathway are well studied, especially by genetic approaches. As shown in Figure 34, these proteins have been identified associated with PTV: the plasma membrane SNARE proteins syntaxin and SNAP25 (Figure 34A); the vesicle trafficking regulatory protein Rab3a (Figure 34A); the SNARE binding proteins Munc18, Munc13, and Complexin (Figure 34B and 34C); the α1 and β subunits of calcium channel (Figure 34B); and the component of Sec6/8 complex Sec6 (Figure 34B). The Western blot analysis of Complexin 2 revealed multiple bands. Adult rat brain total membranes were used as a control for the banding pattern. The predicted molecular weight for Complexin 2 is 19 kDa. The 38 kDa, 76 kDa, 114 kDa, 152 kDa, and 190 kDa bands were likely to represent dimer, tetramer, hexamer, octomer, and decamer, respectively (Figure 34C). The immuno-isolation results were confirmed by double-label immunofluorescent

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Figure 34. Piccolo Transport Vesicles Contain Components of Exocytosis Machinery

(A)Western blots of light membrane fractions immuno-isolated with beads coated with Piccolo, Bassoon, synaptophysin, or the irrelevant IgG antibodies. The beads bound subfraction (B), and nonbound (NB) fractions were resolved by SDS-PAGE and subjected to Western blotting to detect the presence of Piccolo, syntaxin, SNAP25, and Rab3a.
(B)Western blots of light membrane fractions immuno-isolated with beads coated with Piccolo or the irrelevant IgG antibodies. The beads bound subfraction (B), and the input fraction were resolved by SDS-PAGE and subjected to western blotting to detect the presence of Munc18, Calcium channel $\alpha 1$ subunit (alpha1), $\beta 1$ subunit (beta1), or Sec6.
(C)Western blots of light membrane fractions immuno-isolated with beads coated with Piccolo or the irrelevant IgG antibodies. The beads bound subfraction (B) and the input fraction were resolved by SDS-PAGE and subjected to western blotting to detect the presence of Complexin2. Brain total membrane was used as a control. BM, brain membranes.

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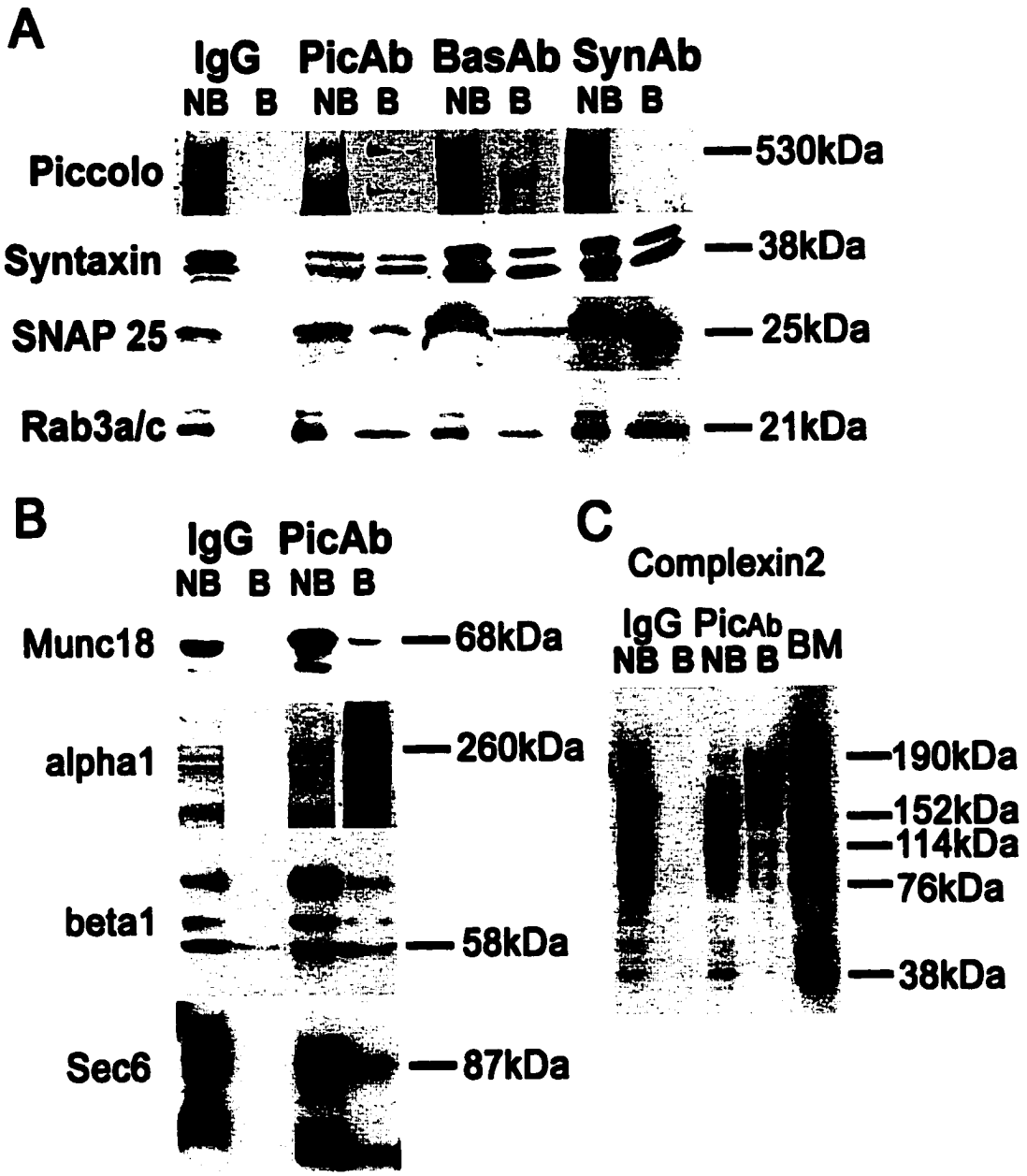

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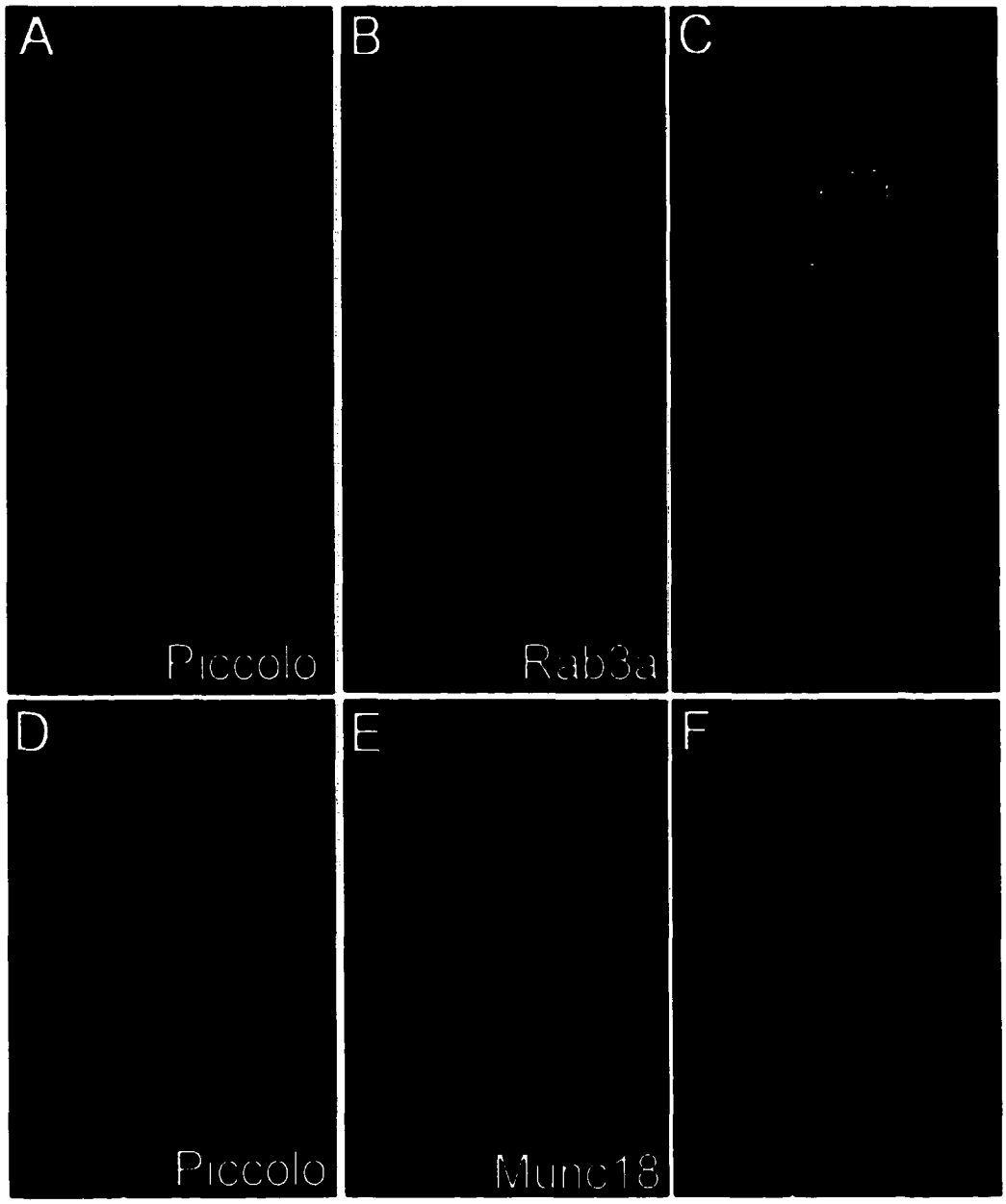

Figure 35. Piccolo Colocalizes With Components of Exocytosis Machinery

Neurons cultured for 4 div were double labeled for Piccolo (A and D) in green and Rab3a (B) or Munc18 (E) in red. The distal portions of axons and their growth cones were displayed in a vertical orientation with their growth cones at the top. Piccolo partially colocalized with Rab3a (C) or Munc18 (F). Scale bars: 10 μm in all panels.

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mircoscopy on the axons and growth cones of 3 div neurons. As shown in Figure 35, Piccolo puncta revealed a moderate level of colocalization with Rab3a (Figure 35A-35C) or Munc18 punta (Figure 35D-35F). Although some colocalization was observed between Piccolo puncta and syntaxin1A, SNAP25, or calcium channel (data not shown), a predominant plasma membrane staining pattern of these proteins, as observed by others (Garcia et al., 1995), precluded a clear conformation of their presence on the PTV.

Piccolo Transport Vesicles Contain Synaptic Adhesion Molecules

Adhesion molecules play an important role in the process of synaptogenesis. If the active zone is partially assembled from the PTV, it is possible that the PTV also carries synapse specific adhesion molecules. We tested the presence of N-cadherin, N-CAM, and Syndecan2 in immuno-isolated PTV preparations. As shown in Figure 36A, these three proteins were all present. In double-label immunofluorescent experiments, a moderate level of colocalization was observed between Piccolo and N-cadherin (~50%, Figure 37A-37C).

Piccolo Transport Vesicles Contain Classical Dense Core Vesicle Protein Chromogranin B and Neuregulin

The association of active zone proteins with dense core granulated vesicles is unexpected and suggests a novel potential role for classical peptide containing dense core vesicles during synaptogenesis. To begin to address this important issue, we examined whether the classical dense core vesicle protein chromogranin B is present in the PTV. Chromogranin B immuno-reactivity was selectively found on Piccolo or Bassoon antibody-coated beads but not on synaptophysin or IgG coated beads (Figure 36B).

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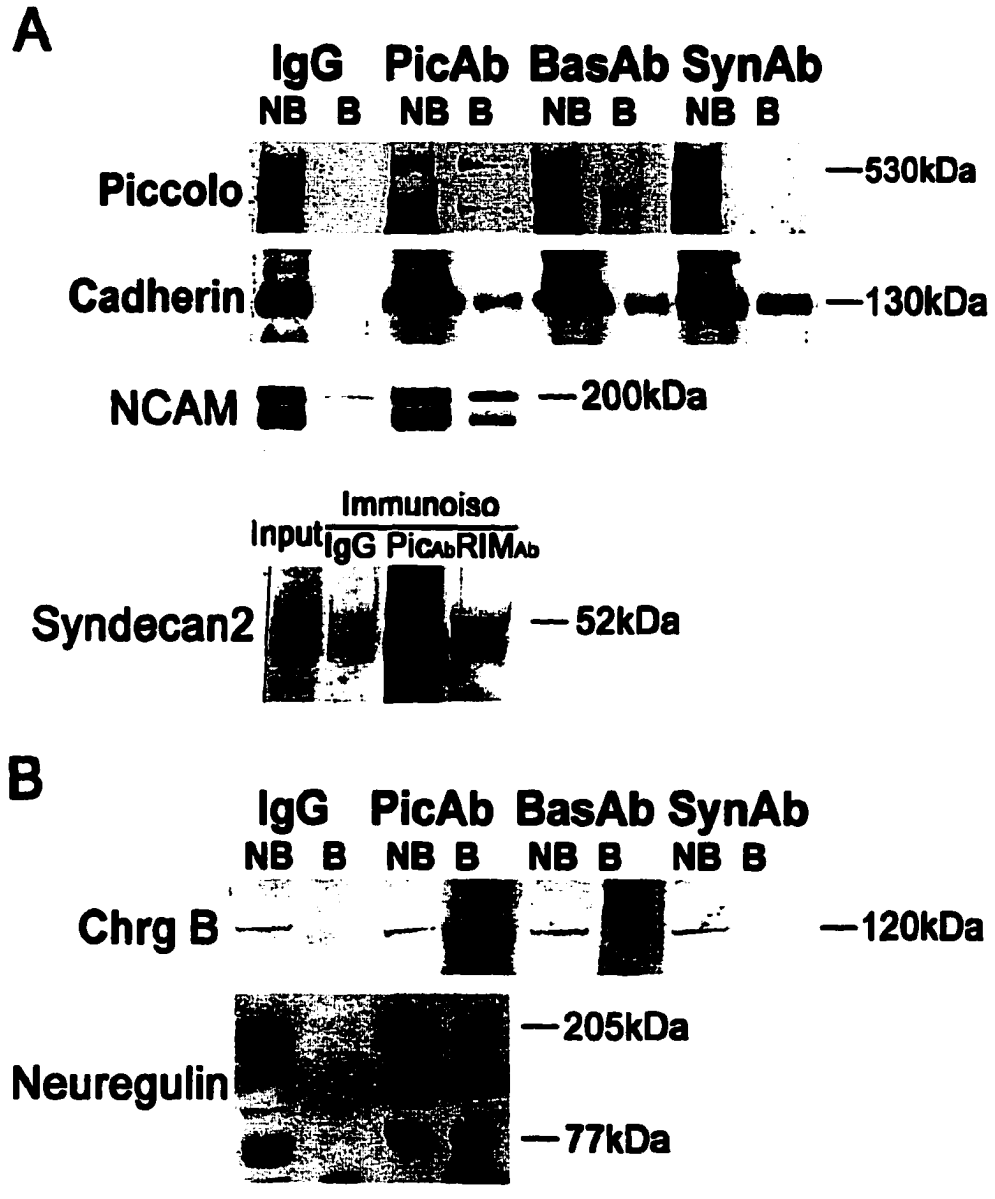

Figure 36. Piccolo Transport Vesicles Contain Synaptic Adhesion Molecules, Chromogranin B, and Neuregulin

(A) Western blots of light membrane fractions immuno-isolated with beads coated with Piccolo, Bassoon, synaptophysin, or the irrelevant IgG antibodies. The beads bound subfraction (B), and nonbound (NB) fractions were resolved by SDS-PAGE and subjected to Western blotting to detect the presence of Piccolo and synaptic adhesion molecules N-cadherin and NCAM. For detecting Syndecan2, RIM antibody-coated beads were also used.
(B) The beads bound subfraction (B), and the NB fractions were resolved by SDS-PAGE and subjected to Western blotting to detect the presence of dense core vesicle protein Chromogranin B (ChrgB) and neuregulin.

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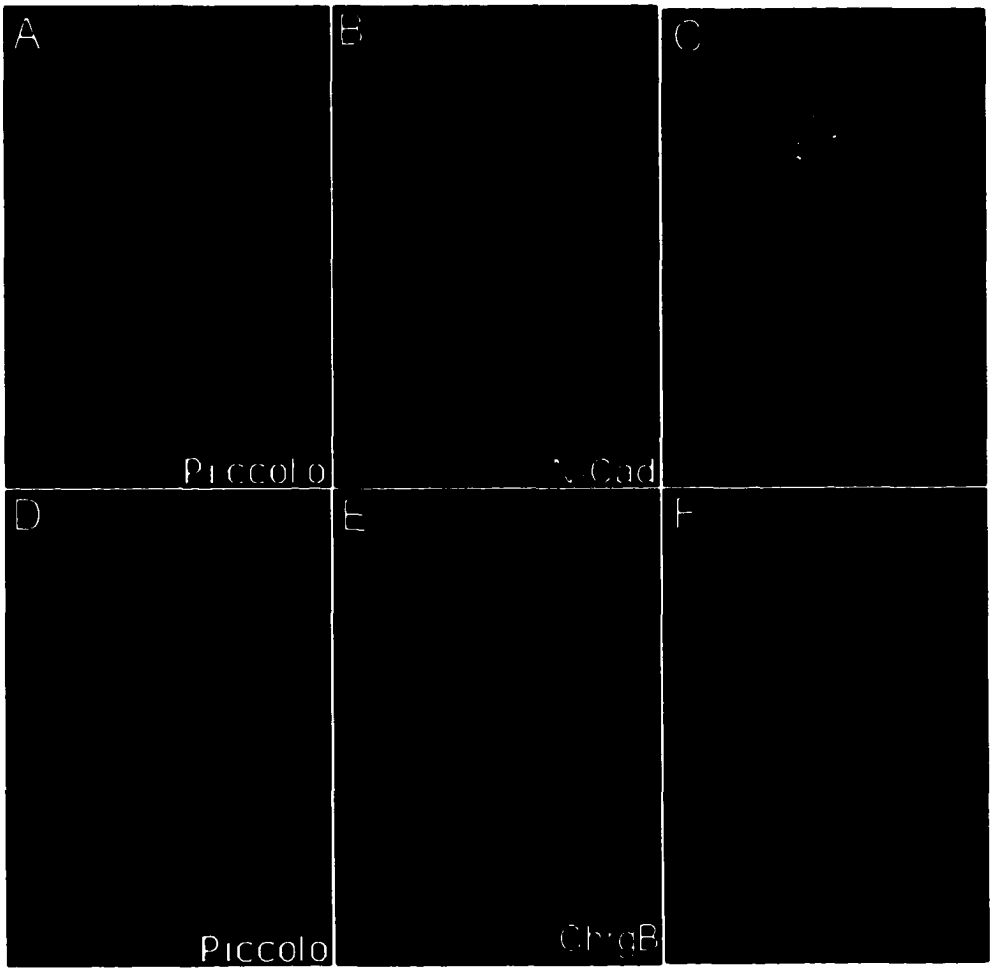

Figure 37. Piccolo Co-Localizes With N-Cadherin or Chromogranin B

Neurons cultured for 4 div were double labeled for Piccolo (A and D) in green and N-cadherin (B) or Chromogranin (E) in red. The distal portions of axons and their growth cones were displayed in a vertical orientation with their growth cones at the top. Piccolo partially colocalized with N-cadherin (N-Cad) (C) or Chromogranin B (ChrgB) (F). Scale bars: 10 μm in all panels.

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Furthermore, chromogranin B and Piccolo immuno-reactive puncta were found to colocalize in immature axonal growth cones (Figure 37D-37F).

When the PTV fuses with the plasma membrane, the content of the dense core including Chromogranin B will be released into the cleft and easily accessed or even will act on the postsynaptic membrane. The process of postsynapse differentiation is not clear. Although agrin and neuregulin are found in the CNS, whether they are involved in synaptogenesis is not known. As a first attempt to uncover the postsynapse inducing molecules, we tested the presence of neuregulin in the PTV. Interestingly, we did detect neuregulin in the PTV complex (Figure 36B), suggesting that neuregulin can be transported in PTV and released upon fusion.

Some Components of the PTV are in Direct Association With Piccolo

The biochemical and EM ultrastructural studies revealed that the PTV is an ~80 nm dense core vesicle complex carrying more than a dozen proteins. Some of these proteins are transmembrane proteins, such as syntaxin, calcium channel $\alpha 1$ subunit, N-Cadherin, N-CAM, and Syndecan2. Some of these proteins associate with membrane through lipid modification, such as Rab3a and SNAP25. However, some of them do not have a membrane association domain, such as CAZ proteins (Piccolo, Bassoon, RIM, and Munc13) and cytosolic proteins (Complexin, Munc18, and calcium channel β subunit). Understanding how these proteins associate with vesicles and knowing the biochemical composition of the PTV will be very important to reveal the mechanisms of transport and active zone assembly. As a first attempt, a detergent extraction analysis was used to dissect out the protein components that directly interact with Piccolo from those that do

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not interact with Piccolo. In this experiment, immuno-isolated PTV preparation was treated and washed with Restricted interruption of protein association (RIPA) buffer to disrupt the membranes. The Piccolo antibody coated beads purified Piccolo and the proteins that bind Piccolo, while the resulting supernatant contained proteins that were in the PTV complex but not in direct association with Piccolo. As shown in Figure 38A, Bassoon and calcium channel β1 subunit bind Piccolo directly, but NCAM and syntaxin mainly do not interact with Piccolo. As a control for the experiment, synaptophysin antibody coated beads were used to immuno-isolate synaptic vesicles, and were subsequently treated with RIPA buffer. As prediected, syntaxin did not bind synaptophysin directly (Figure 38B).

Discussion

The biochemical analyses of the PTV complex demonstrated that major components of exocytosis machinery, as well as the CAZ proteins and synaptic adhesion molecules, are transported in association with 80 nm granulated vesicles. These components are all required for the assembly of active zones, and, therefore, the Piccolo transport vesicle is likely to serve as a precursor for the presynaptic active zone. Previous chapters have shown that the PTV is a dense core vesicle distinct from synaptic vesicles, as the PTV does not contain any of the synaptic vesicle signature proteins we have tested. In this chapter, I have shown that the PTV carries a protein complex comprised of components of the mature active zone. The proteins found thus far fall into four different catagories: CAZ proteins, components of synaptic vesicle exocytosis machinery, synaptic adhesion molecules, and neuropeptide precursor protein.

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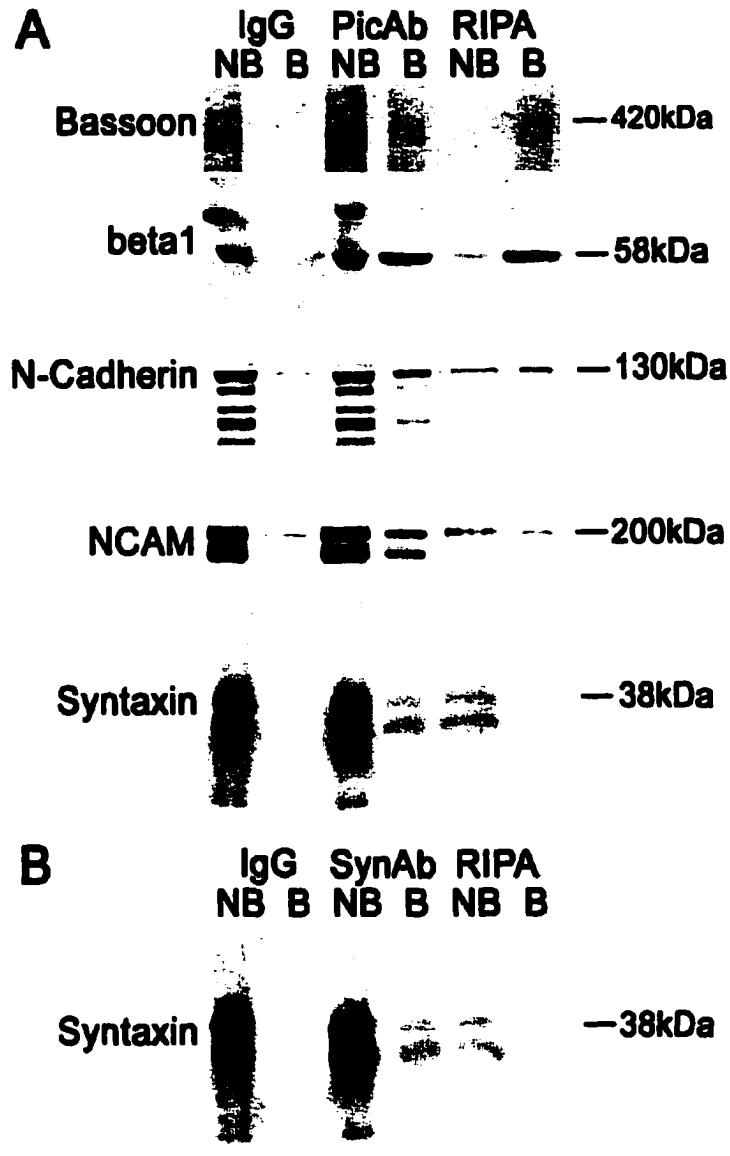

Figure 38. Bassoon and Calcium Channel β1 Subunit Interact Directly With Piccolo

(A) Light membranes were incubated with Piccolo antibody-coated beads or IgG control beads. Immuno-isolated PTV fractions were treated with RIPA buffer to disrupt the membrane. The beads bound subfraction (B), and the NB fraction and the RIPA buffer-treated supernatant and bound fraction were resolved by SDS-PAGE and subjected to Western blotting to detect the presence of Bassoon, Calcium channel β1 subunit, N-cadherin, NCAM, and syntaxin.
(B) As a control, synaptophysin antibody was used to immuno-isolate synaptic vesicles and also was subjected to RIPA buffer treatment. Fractions were resolved and Western blotted for syntaxin.

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Among the CAZ proteins, Piccolo and Bassoon seem to be present on identical vesicles, suggesting they may use the same transport mechanism. CAZ proteins RIM and Munc13 have also been shown to have regulatory effects on neurotransmission through their interactions with Rab3 or SNARE proteins. The components of exocytosis machinery that have been identified in association with the PTV either are directly involved in SNARE formation, such as syntaxin and SNAP25, or are closely regulate SNARE formation, such as Munc18 and Complexin, or required for the neurotransmission process, such as Ca^{2+} channel subunits and Rab3a. These proteins together provide the building blocks for functional synaptic vesicle release sites. The fusion of the PTV with the presynaptic plasma membrane is expected to deposit these proteins all at once, which will allow the insertion of the exocytosis machinery in a fast and efficient manner. The adhesion molecules N-cadherin and Syndecan2 have high specificity and are important for synapse recognition. When carried by PTVs and deposited upon fusion, they can quickly initiate the recognition between the pre- and postsynaptic elements and promote the establishment of new synaptic junctions.

An interesting and surprising finding about the PTV is its dense core. Like other dense core vesicles, the core is expected to be released into the extracellular space. In the case of the PTV, this should be the nascent synaptic cleft. The identification of Chromogranin B and neuregulin in the PTV provided the first clues to the potential role of this dense core. It is possible that the dense core also contains other neuronpeptides and trophic factors that can induce the differentiation of the postsynaptic and perhaps also the presynaptic sides of synaptic junctions after they have been released into the cleft of newly forming synapses. It is mostly unclear whether neuregulin plays a direct role in the

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differentiation of CNS glutamatergic or GABAergic synapses (Sanes and Scheller, 1997), although a recent study suggested that neuregulin may have bidirectional signaling effects in the process of synaptogenesis between mossy fibers and cerebellar granule cells in cerebellum (Ozaki et al., 2000). Our studies suggested a possible mechanism for their transport and deposition.

Based on these findings, we would like to propose a model for synaptogenesis and the assembly of active zones. As shown in Figure 39, during neuronal differentiation and neurite outgrowth, Piccolo and other active zone proteins are transported on PTVs along axon to growth cones. The fusion of the PTV with the newly forming presynaptic plasma membrane may promote the rapid establishment of new active zones as well as postsynaptic differentiation. Specifically, (a) cell adhesion molecules such as N-cadherin and Syndecan2 in the PTV could help establish stable trans-synaptic cell-cell adhesion and facilitate the subsequent recruitment and localization of postsynaptic structural proteins and neurotransmitter receptors (Tanaka et al., 2000), (b) The insertion of CAZ proteins such as Piccolo and Bassoon as well as components of the synaptic vesicle exocytotic machinery and its regulatory proteins at the nascent active zone site would facilitate the rapid acquisition of activity-induced synaptic vesicle recycling as well as confine their fusion to this site, (c) The fusion of the PTV at nascent synapses is expected to lead to the deposition of its dense core into the newly forming synaptic cleft. The content of this core is potentially composed of neuropeptides, neurotrophic factors, and ECM proteins that are expected to play direct roles in the differentiation of the PSD, such as promoting the synapse specific recruitment of subclasses of neurotransmitter receptors and their associated PSD proteins.

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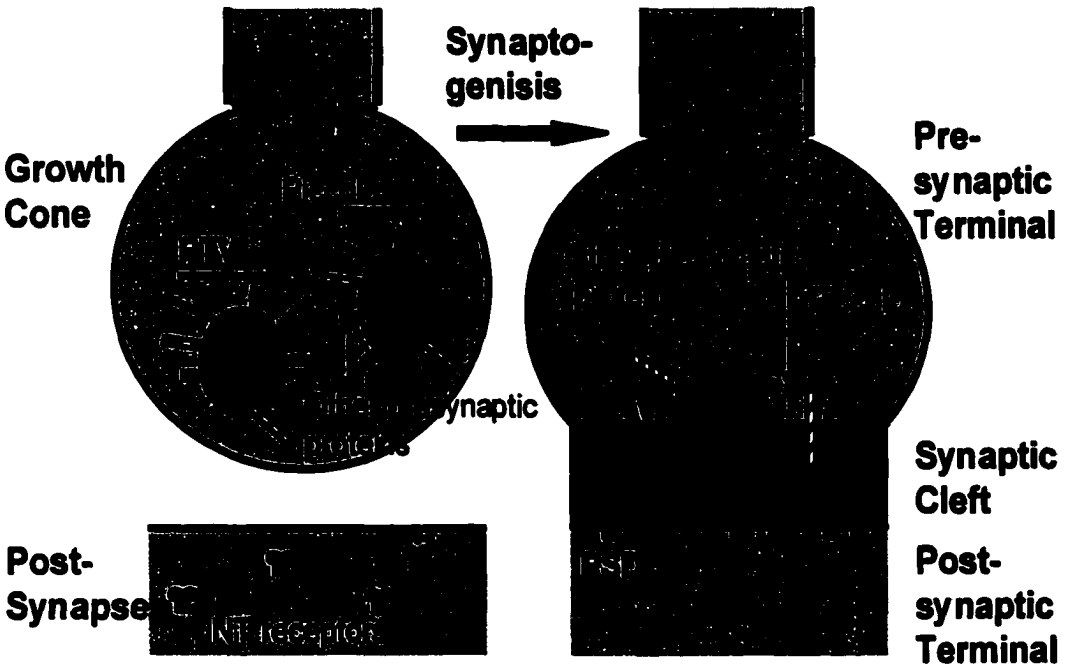

Figure 39. Model of Synaptogenesis

PTVs are transported along the axon to the growth cone during neuronal differentiation. When growth cone is in contact with postsynaptic target membrane, the PTV is expected to fuse with the plasma membrane and deposit all the active zone proteins into a relatively confined place and facilitate the assembly of active zone. The content of the core is also released to the cleft, which may form the ECM and may send signals to the postsynapse and induce the formation of the PSD.

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SUMMARY AND CONCLUSIONS

Summary of Results

The excitable cells of the nervous system are joined into a network by connections called synapses. The structure of synapses is highly ordered and precisely tuned to ensure the fidelity of neurotransmission. Uncovering the mechanism underlies the assembly of the presynaptic active zone and is central to the understanding of the mechanisms underlying neural development, synaptogenesis, synaptic plasticity, and learning. The series of studies presented in this dissertation demonstrated that a novel mechanism utilized by the presynaptic neuron initiated the assembly of the active zones in an efficient and accurate manner and also suggested that it may represent a common mechanism for synaptogenesis in the CNS.

The first study, Piccolo is Specifically Localized to the Presynaptic Nerve Terminal of Both Excitatory and Inhibitory Synapses but not to the NMJ, laid down the foundation for the entire series of studies. This study demonstrated the following. First, Piccolo is specifically localized to the presynaptic terminals of both excitatory glutamatergic synapses and inhibitory GABAergic or glycinergic synapses, but not to the cholinergic neuromuscular junctions. Second, Piccolo is very tightly associated with the synaptic junction, and it is highly enriched in the synaptic junctional preparations. This study suggested that Piccolo is a general component of the active zone cytomatrix at different types of synapses in the CNS. Therefore, it is likely that different types of

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synapses utilize a similar mechanism for the assembly of Piccolo into the presynaptic cytomatrix despite the differences in size, morphology, and physiological properties.

The second study, Temporal Appearance of the CAZ Proteins Bassoon and Piccolo During Neuronal Development, examined the developmental distribution of Piccolo and its structural homologue Bassoon using cultured hippocampal neurons as a model system. This study demonstrated the following. First, both Piccolo and Bassoon are expressed early in development and are selectively sorted into axons during the early stage of neuronal differentiation. Second, as synaptogenesis begins, the clustering of Bassoon and Piccolo along dendritic profiles occurs simultaneously with or precedes the localization of other synaptic marker proteins. Third, the clustering of Bassoon coincides with the acquisition of the recycling of the synaptic vesicles. This study suggested that the localization of Piccolo and Bassoon to the presynaptic active zone is an early event during the process of synaptogenesis, indicating an active role Piccolo and Bassoon are likely to play in the assembly of synaptic junctions.

The first two studies have provided evidence for the involvement of Piccolo and Bassoon in synapse assembly and a rationale for using Piccolo and Bassoon as target proteins to study the assembly of the presynaptic active zone. The third study, Piccolo is Transported to Nerve Terminals in Association With an 80 nm Dense Core Granulated Vesicles During Synaptogenesis, unveiled one facet of the cellular mechanism of assembly by revealing how Piccolo is transported and sorted to the active zone. This study demonstrated the following. First, in immature neurons, Piccolo is associated with an ~80 nm dense core vesicle, termed PTV. Second, the PTV is likely to be derived from the Golgi in soma. Third, the PTV is structurally and molecularly distinct from synaptic

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vesicles. This study is the first to illustrate the cellular mechanism for the sorting and transport of a presynaptic cytomatrix protein. It also suggested a novel function for classical dense core vesicles.

The final study, The Piccolo Transport Vesicle is a Precursor of the Active Zone, uncovered the mechanism of active zone assembly by dissecting the components of Piccolo transport vesicle. This study demonstrated the following. First, the PTV carries five CAZ proteins: Piccolo, Bassoon, RIM, Munc13, and CASK. Second, the PTV contains all the major components of synaptic vesicle exocytosis machinery. Third, the PTV contains synaptic adhesion molecules such as N-cadherin, N-CAM, and Syndecan2. Finally, Chromogranin B is one of the components that make up the dense core of the PTV. By revealing the molecular composition of the Piccolo transport vesicle, this study suggested a novel mechanism that the presynaptic neuron uses to assemble the active zone in a fast and efficient manner.

This series of studies has used presynaptic cytomatrix proteins Piccolo and Bassoon as the target proteins to uncover a novel dense core granulated vesicle that appears to serve as a precursor vesicle for the active zone. The existence of such vesicle suggests that components of the presynaptic nerve terminal are unlikely to be recruited individually but rather assembled from a preformed complex.

Discussion

The Presynaptic Function and the Cytomatrix of the Active Zone

The presynaptic axon terminal is highly specialized to convert an electrical signal into an intercellular chemical signal via calcium-dependent exocytosis of neurotransmit-

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ter. Molecular elements comprising the presynaptic axon terminal (bouton) include voltage-gated calcium channels; the protein complexes involved in vesicle docking, fusion, and recycling; and the cytoskeletal components that clustered synaptic vesicles and define the active zone as the site of neurotransmitter release and synaptic vesicle membrane recycling. It has been well known for many years that the plasma membrane associated with the presynaptic active zone is composed of an electron-dense meshwork of cytoskeletal filament (Landis et al., 1988), although the nature of its molecular composition is unclear. The recent molecular characterizations of the proteins highly enriched in the presynaptic terminal in different organisms have provided valuable insights into the molecular organizations of the CAZ and the regulation of active zone functions. In mammalian synapses, CAZ proteins include several high molecular weight, multidomain structure proteins such as Piccolo, Bassoon, Munc13, RIM1, and Oboe/RIM2 (Garner et al., 2000a). In *Drosophila* and *C. elegans*, homologues have been identified for Munc13, RIM1, and Oboe/RIM2 (Brose et al., 2000). Although the homologues for Piccolo and Bassoon in lower organisms have not yet been identified, recently, two presynaptic high molecular weight proteins, Highwire in *Drosophila* and RPM-1 in *C. elegans*, have been discovered (Scheafer et al., 2000; Wan et al., 2000; Zhen et al., 2000). It is very plausible that using large scaffolding molecules to build synaptic structures is a conserved mechanism throughout evolution.

The characterization of these multidomain proteins and the protein-protein interactions via individual domains has been a useful avenue to understand the functions of active zones. Among mammalian CAZ proteins, RIM1 is the best-characterized protein so far. RIM1 was originally identified as a Rab3a interacting molecule (Wang et

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al., 1997). It contains a zinc finger domain, a PDZ domain, and two C2 domains (Figure 4). The zinc finger domain binds specifically to Rab3 in a GTP-dependent manner, and this interaction regulates exocytosis in PC12 cells (Wang et al., 1997). Interestingly, recent studies by Südhof and colleagues revealed that RIM1 also interacts with Munc13-1, synaptotagmin, and liprin through yeast two-hybrid assays (Südhof and Schoch, personal communication). Munc13-1 and synaptotagmin are both involved in the regulation of synaptic vesicle exocytosis. Liprin is a multivalent protein that functions to localize Leukocyte common antigen-related receptor (LAR) family tyrosine phosphatases at focal adhesion sites on the plasma membrane, regulating their interaction with the extracellular environment and their association with substrates (Serra-Pages et al., 1998). It has also been suggested that, in *C. elegans*, liprin regulates the differentiation of presynaptic termini, in particular the formation of the active zone, by acting as an intracellular anchor for tyrosine phosphatase signaling at synaptic junctions. Liprin (SYD-2) protein is localized at presynaptic termini independently of the presence of vesicles, and active zones of liprin (syd-2) mutants were significantly lengthened, and the synaptic transmission is partially impaired (Zhen and Jin, 1999). These studies provide compelling evidence that CAZ proteins RIM1 and Munc13 form a protein scaffold in presynaptic nerve terminal, and the fact that they interact with each other adds another level of complexity.

Piccolo and Bassoon each contain more than 10 protein homology domains that are potentially involved in protein-protein interactions (Fenster et al., 2000). Of particular interest are the two double-zinc finger motifs located in the N-terminal half of both proteins, and they are highly homologous with the zinc finger domain in RIM1 and

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Rabphilin (Dieck et al., 1998; Fenster et al., 2000). Although interacting proteins for the zinc fingers of Bassoon have not yet been identified, studies from our laboratory have demonstrated that Piccolo zinc fingers do not bind Rab3a like the RIM1 zinc finger does but instead interact with PRA1, a 21 kDa molecule originally identified based on its ability to bind Rab3a (Martincic et al., 1997; Bucci et al., 1999; Fenster et al., 2000). The exact function of PRA1 is not very clear, but its interaction with two fundamentally diverse protein families involved in vesicle trafficking (rabs and VAMP2/Synaptobrevin II) suggested that PRA1 may play a role in regulating the interaction of VAMP2/Synaptobrevin II with t-SNARE components (Martincic et. al., 1997; Bucci et al., 1999). Alternatively, PRA1 may also affect the ability of rabs to interact with their effectors. If this is the case, a transient association of PRA1 with Piccolo in the active zone could serve to unmask rab3A and/or VAMP2/Synaptobrevin II on synaptic vesicles. This would allow rab3A to interact with RIM1 in a GTP-dependent manner and VAMP2 to interact with syntaxin and SNAP-25 to initiate the formation of the SNARE docking complex at the active zone.

CAZ proteins RIM1, Munc13, and Piccolo have been suggested to directly or indirectly regulate synaptic vesicle recycling. Their restricted distribution at the active zone and their multidomain structure strongly suggested that CAZ proteins play a role as scaffolding molecules tethering and localizing other (relatively small) proteins to a confined region, ensuring the proper functioning of presynaptic nerve terminals. Figure 40 summarizes what we know about the players in presynaptic active zones and their interactions.

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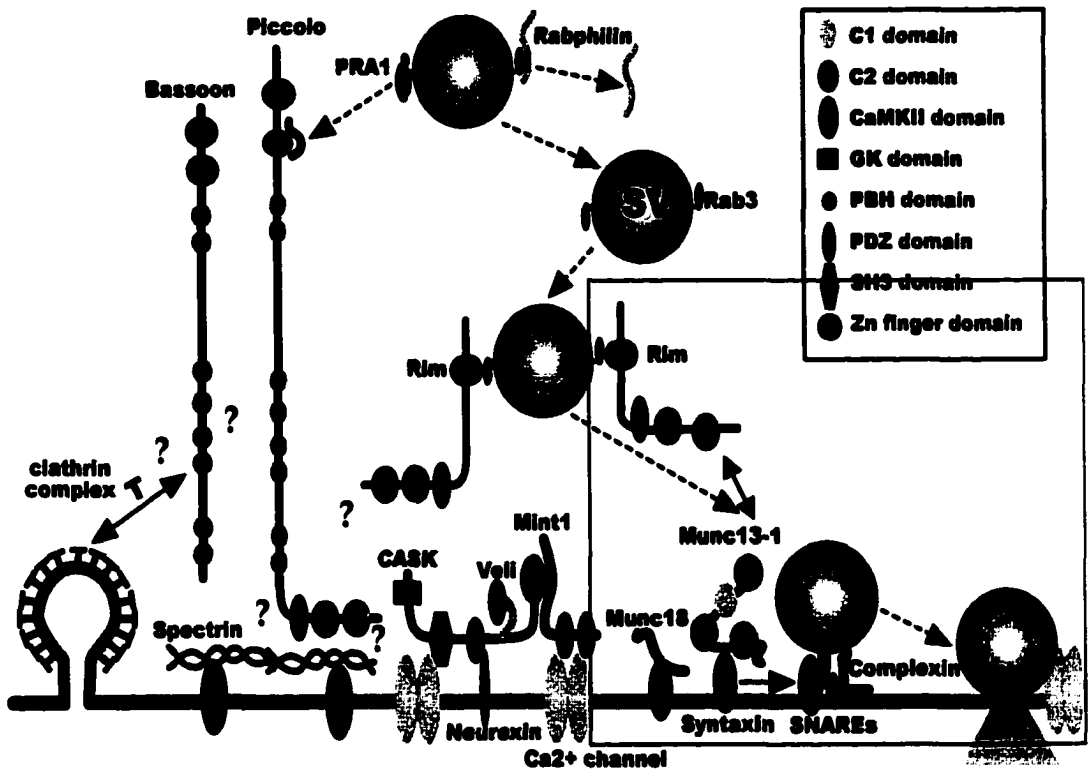

Figure 40. Molecular Model of the Structure and Interactions of the Active Zone Proteins

The cytomatrix at the active zone is thought to provide a scaffold for the protein machinery involved in synaptic vesicle recycling. Piccolo and Bassoon are the largest members of the CAZ protein family, and they share 10 homology domains. Piccolo zinc fingers interact with the prenylated Rab3 acceptor protein PRA1, suggesting a role in synaptic vesicle exocytosis. RIM interacts with Rab3a and may regulate vesicle trafficking. RIM also interacts with Munc13 and SNARE protein syntaxin. Munc13 regulates the SNARE complex assembly via its interaction with syntaxin. Munc13 also contains domains that interact with spectrin, phorbol esters, and calcium/phospholipids. CASK contains a SH3 domain that binds to N-type calcium channel, a PDZ domain that binds neurexin and syndycan2, a CaMKII-like domain that binds Mint1. A box is drawn to include all the known components of exocytosis machinery. These proteins have been shown to directly participate in or regulate synaptic vesicle exocytosis. These proteins are all found present in PTV.

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The CNS Synapses May Use a Vesicular Transport Mechanism for Assembly

Synaptogenesis is a remarkable cellular event. At the correct time and place, a motile growth cone touches a potential target membrane, and both parties transform into a stable synaptic contact. The most noticeable change is the morphological transformation. The presynaptic growth cone develops into a bouton filled with synaptic vesicles, and a PSD will be formed at the opposing postsynaptic plasma membrane. What is not very obvious is how the recruitment of the large amount (hundreds) of synaptic proteins lead to the formation of the synapse and how the machinery for neurotransmission is assembled at the synaptic junction. The large amount of effort by many groups on dissecting the components of PSDs has resulted in a detailed but rather static map of the assembly of PSDs. Studies using cultured hippocampal neurons have suggested that components are sequentially recruited to synaptic sites during a period of several days (for review, see Lee and Sheng, 2000). In contrast, studies using novel imaging approaches suggest that functional synapses may form within a few hours (Dailey and Smith, 1996; Ziv and Smith, 1996; Maletic-Savatic and Malinow, 1998; Toni et al., 1999, Jontes et al., 2000; Vardinon-Friedman et al., 2000). Especially, at the presynaptic terminal the capacity of synaptic vesicle to recycle in an activity-dependent manner can be acquired around 20 to 40 min after the initial axon-dendrite contact (Vardinon-Friedman et al., 2000). These observations suggest that synapses, at least the presynaptic terminal, uses a fast "touch and go" mechanism for assembly. It is unlikely that the components of the active zone and the machinery of synaptic vesicle recycling are recruited individually. The studies presented in this dissertation have suggested a vesicle-based model for the partial assembly of the presynaptic active zone. In this

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model, neurons bulk pack all the proteins that go to the same destination into a small number of vesicular complexes and then transport and deposit them all at once. More importantly, the vesicular transport mechanism allows for a fast and efficient assembly of synapses from preformed complexes, which is consistent with the short time required for the synapse assembly observed by real-time imaging.

It has been known for many years that there is a large number of vesicular membrane structure present in differentiating axons in cultures (Bradke and Dotti, 1997, 1998; Ruthel and Banker, 1998) or in developing spinal cord neurons in situ (Vaughn, 1989). Although the functions of these vesicles are not known, it is a generally accepted that they are likely to be transport vesicles carrying (membrane) proteins. In particular, Vaughn (1989) revealed by EM the ultrastructure of a dense core vesicle in close proximity to a developing synapse in spinal cord neurons. A recent study by Ahmari et al. (2000) suggested that the presynaptic active zone is assembled from "cytoplasmic transport packets." They used GFP-VAMP as a labeling tool and observed a cloud of vesicular membrane structures with different size and morphology moving together along the axon to the site of synapse formation. This study implies that different types of vesicles such as synaptic vesicles, dense core vesicle, and tubular membranes are in one "packet," using similar transport mechanisms (Ahmari et al., 2000; Roos and Kelly, 2000). Our studies presented here have focused on one type of vesicle, specifically a dense core vesicle, and have shown that this dense core vesicle (PTV) is morphologically and biochemically distinct from synaptic vesicles. Interestingly, when examined by EM, dense core vesicles and clear-center synaptic vesicles can be seen in close association with microtubules in axons (Figure 28). However, these vesicles do not form aggregates

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nor are they present in close proximity with each other but rather exhibit discrete patterns (Figure 28). It is possible that different types of vesicles move at a similar rate along microtubules and arrive at the newly forming bouton around the same time, but they do not have to be attached with each other. Our studies suggest that individual vesicles can be transported independently. Furthermore, the ultrastructure of the PTV highly resembles what Vaughn has observed in embryonic spinal cord neurons, suggesting that this may be a common mechanism of transport and assembly used by different types of neurons.

The PTV is the Precursor for the Active Zone and May Represent Functional Units of Active Zones

The biochemical characterization uncovered a partial molecular composition of the PTV. Interestingly, the PTV contains five CAZ proteins and the major components of the exocytosis machinery. The final destination of all of these proteins is the active zone. The PTV does not contain proteins that eventually localize to peri-synaptic regions, such as the GAT1, or proteins that only present on synaptic vesicles. The PTV is likely to be the precursor complex dedicated to active zone assembly. The advantage of packing all active zone proteins into such a vesicle is that it allows efficient assembly. First, forming a vesicular complex will ensure a fast microtubule-based transport (Sheetz et al., 1998). Second, packing all the components for exocytosis machinery and the regulatory proteins in one vesicle will allow the deposition of these proteins in a spatially confined region soon after the initial contact is made with a postsynaptic target. The model of assembling from active zone precursor vesicular complex provided a logical explanation for the

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"touch and go" phenomenon observed by many groups, with regard to both timing and molecular aspects.

Assuming that each PTV has all or most of the components needed to establish a synaptic vesicle release site, in the correct stoichiometry, it is interesting to speculate how many SV fusion sites a single PTV may contain. Quantitative analysis of hippocampal excitatory synapses has revealed that the area of a single synaptic vesicle docking site at active zones is 3844 nm^2 (62 × 62 nm) in brain and 5929 nm^2 (77 × 77 nm) in hippocampal culture (Schikorski and Stevens, 1997). The surface area of an 80 nm dense core granulated vesicle is around 20160 nm^2. Assuming that the surface area of this vesicle translates into an active zone of equal size, the fusion of one 80 nm dense core vesicle would yield about four vesicle docking sites (5.2 in brain or 3.4 in culture). This said, EM and electrophysiological studies have shown that there are active zones with three or less vesicle docking sites (Schikorski and Stevens, 1997). This suggests that the number of synaptic vesicle release sites formed by the fusion of the PTV may be less than four. Although theoretically this is an attractive model for the assembly of SV release sites, it remains elusive whether this granulated vesicle contains a complete complement of active zone proteins or whether the fusion of addition precursor vesicles is required for active zone assembly.

The Assembly of the Presynaptic Nerve Terminal

The presynaptic nerve terminal is a structurally defined region, containing three functionally and structurally distinct subdomains: the active zone, synaptic vesicle pools, and the peri-synaptic plasma membrane (Landis et al., 1988). There are proteins

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specifically localized to each domain. The biogenesis of synaptic vesicles has been well characterized (Huttner et al., 1995; Hannah et al., 1999). Our studies presented here suggested a possible mechanism for the biogenesis of the active zone. Interestingly, we did not detect the peri-synaptic protein GAT1 in either synaptic vesicle preparations or in PTV preparations. Given the fact that GAT1 is a transmembrane protein (Jursky et al., 1994), it is likely that GAT1 is transported and inserted to the plasma membrane via a vesicular intermediate. This vesicle may also carry other peri-synaptic proteins. If that is the case, assembling from a vesicular precursor complex would be a common mechanism used by presynaptic neuron, in which distinct types of vesicles may be assigned for individual subdomains.

Although our biochemical studies have shown that CAZ proteins Piccolo, Bassoon, and RIM1 are present in PTVs, only Piccolo and Bassoon shared a high degree of co-localization, while Piccolo and RIM1 were only partially colocalized (Figure 33). This result indicates that there may be more than one type of active zone precursor vesicle present. Piccolo and RIM are both restricted to the active zone, but their protein structures are quite different (Figure 4), suggesting that Piccolo and RIM have distinct functions at the active zone. It is likely that Piccolo and RIM1 each have their own set of interacting molecules and specialized localization within the active zone. The presence of an "RIM transport vesicle" would allow an even more defined regulation of the assembly.

The Cross Talk Between the Pre- and Postsynaptic Elements During Synaptogenesis

The formation of synapse requires precise coordination of pre- and postsynaptic neurons. Axons are guided to their target by the combinatorial actions of signaling

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molecules such as netrins, semaphorins, and IgCAMs (Tessier-Lavigne and Goodman, 1996; Winberg et al., 1998; Mueller, 1999). Upon encountering their proper target, axons cease to extend and develop into presynaptic bouton. It is known that target-derived trophic factors are involved in activity-dependent synapse formation (Levi-Montalcini, 1996). In particular, it has been shown in cultured hippocampal neurons that brain derived neurotrophic factor (BDNF) induced formation of both excitatory and inhibitory synapses, whereas neurotrophin-3 (NT-3) induced formation of only excitatory synapses (Vicario-Abejon et al., 1998). However, until recently the exact signaling pathway underlying the morphological transformation of a growth cone to a synaptic bouton has remained a mystery. Excitingly, Hall and colleagues have recently utilized a pontine explant/granule cell coculture system to demonstrate that the secreted signaling molecule WNT7a functions during synaptogenesis at the mossy fiber-granule cell synapse (Hall et al., 2000). WNT7a is a member of a large family of secreted WNT molecules that have previously been demonstrated to participate in a variety of developmental events such as cell fate decisions, cell polarity, and patterning events (Cadigan and Nusse, 1997). Hall et al. (2000) presented a model for presynaptic morphogenesis. In this model, WNT is secreted from target cells (granule cells) and binds to the frizzled receptors (fz) on the growth cone membrane and activates an intracellular signaling cascade that represses the activity of glycogen synthase 3β kinase (GSK3β). GSK3β regulates the stability of microtubules via phosphorylation of several microtubule-associated proteins such as Tau, MAP-1B, and MAP-2 (Goold et al., 1999). Target-secreted WNT7a therefore induces the cytoskeletal rearrangement and the growth motility through GSK3β pathway. When axon growth cone encounters its target, initial contact will be established. Recent studies by

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Scheiffele et al. (2000) suggest that the initial contact leads to the formation of the neuroligin-neurexin complex and that intercellular signaling via the neuroligin-neurexin complex is sufficient to drive the synaptic vesicle aggregation at the presynaptic terminal. These studies provide compelling evidence that WNT and neuroligin function as target (postsynaptic) derived signals that act on presynaptic elements and drive their differentiation.

For a long time the NMJ has been the best model synapse to study synaptogenesis, and we have gained remarkable insight into the role of neuron (presynaptic) derived signals for postsynaptic differentiation. Agrin and neuregulin are released from the presynaptic motoneuron and act through MuSK and erbB receptors, respectively, to induce the clustering and synthesis of postsynaptic acetylcholine receptors (Sanes and Lichtman, 1999). In searching for the postsynaptic receptor clustering factor, O'Brien et al. (1999) identified a secreted immediate-early gene product Narp (neuronal activity-regulated pentraxin). Overexpression of recombinant Narp increases the number of excitatory but not inhibitory synapses in cultured spinal neurons, and Narp-expressing HEK293T cells can induce the aggregation of neuronal AMPA receptors. Narp may function like Agrin specifically to induce the aggregation of receptors at excitatory synapses (O'Brien et al., 1999). A recent study suggested a possible role for neuregulin in synaptogenesis between mossy fibers and cerebellar granule cells (Ozaki et al., 2000). This study demonstrated that a soluble form of neuregulin is generated from the membrane-anchored form via proteolytic cleavage. The cleaved form of neuregulin has been found to act trans-synaptically to regulate the expression of the NMDA receptor subunit NR2C, whereas the membrane-anchored form

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of neuregulin showed a homophilic-binding activity between neuregulinα1s from pre- and postsynaptic elements (Ozaki et al., 2000). This study indicated that neuregulin induces postsynaptic differentiation by regulating the expression of NMDA receptors. Interestingly, our preliminary study has shown that neuregulin is likely to be present in PTV (Figure 36). The PTV may provide a transport and packaging mechanism for neuregulin and other postsynapse differentiation factors. They can be released from the presynaptic terminal when the PTV fuses with the plasma membrane and act on the postsynaptic signaling pathway to induce the postsynaptic differentiation.

The cross talk between the developing pre- and postsynaptic elements not only conducts the information exchange between two sides but also coordinates the developmental processes occurring simultaneously at pre- and postsynaptic sides and ensures the precision and accuracy of synaptogenesis.

Conclusion

The studies presented here have revealed that components of the active zone are packaged together on a dense core transport vesicle. The fusion of such an active zone precursor vesicle at nascent synapse is likely to constitute a fundamental mechanism used by neurons to initiate the assembly of synapses. Moreover, such a mechanism could also explain prior observations, suggesting that synaptic vesicles acquire the capacity to recycle at new axo-dendritic sites less than 1 hr of axo-dendritic contact (Ahmari et al., 2000; Vardinon-Friedman et al., 2000).

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Future Directions

Dissect the Components of Active Zone Precursor Vesicles (PTVs and RTVs)

PTVs, as well as RIM transport vesicles, are multi protein complexes. The collection of the protein identified in this dissertation probably just represents the tip of the iceberg. Other known and unknown proteins carried by these vesicles can very well be important for our understanding of presynaptic function and synaptogenesis. The conventional "guess and test" approach used in this dissertation is very useful for testing the presence of individual protein of interest; however, a high-through put analysis on the vesicle by proteomics will be much more efficient in dissecting the complete molecular composition.

Uncover the Mechanisms Underlying the Fusion of Active Zone Precursor Vesicles

Since a majority of the membrane fusion events within a cell use SNARE complex-mediated membrane fusion mechanism (Südhof and Scheller, 2000), it is likely that a SNARE complex is also involved in the fusion of Piccolo transport vesicle with the plasma membrane. A distinct set of SNARE components may be involved in the fusion of this vesicle. A more interesting question is to understand the signal that triggers the fusion and the potential regulators of the fusion process. A combination of molecular and real-time imaging approach will be very beneficial.

Identify the Postsynaptic Differentiation Factors

Any biological event in vivo never happens in a solitary manner. The development of the presynaptic terminal progresses in precise coordination with the

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differentiation of the postsynapse. Identifying the postsynapse differentiation factors carried in PTV is central to the understanding of the CNS synaptogenesis. These factors will link the pre- and postsynaptic terminals together and tie up the seemingly solitary events.

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MATERIALS AND METHODS

Materials

The rabbit and mouse Bassoon antisera were raised against a 75 kDa GST-Bassoon fusion protein, and polyclonal rabbit Piccolo antibody were against the GST-44a2 fusion protein as previously described (Cases-Langhoff et al., 1996; Dieck et al., 1998). They were affinity purified using fusion protein coupled actigel (Sterogene). All three antibodies were used at a dilution of 1:200. The other antibodies used were as follows: mouse monoclonal anti-α-actinin antibody (clone EA-53; Sigma Chemical Co.; 1:200); monoclonal anti-calcium channel β1 subunit antibodies (Transduction Laboratories); polyclonal anti-calcium channel α1, β1 subunit antibodies (gift from A. Maximov, University of Texas, Southwestern Medical Center); polyclonal anti-ChromograninB antibodies (Santa Cruz Biotechnologies); monoclonal anti-Complexin2 antibody (Transduction Laboratories); rabbit polyclonal anti-GKAP antibody (Naisbitt et al., 1997; 1:200); rabbit polyclonal anti-GluRI antibody (Chemicon International Inc.; 1:50); mouse monoclonal anti-$GABA_A$ receptor, β chain antibody (clone bd 17; Roche Diagnostics GmbH; 1:30); rabbit anti-MAP2b antibody (Kindler et al., 1990) at a dilution of 1:15,000; monoclonal anti-Munc13 antibodies (Transduction Laboratories); monoclonal anti-Munc18 antibodies (Transduction Laboratories); mouse monoclonal anti-Rab3a antibody (Transduction Laboratories); monoclonal anti-RIM1 antibodies (Transduction Laboratories); polyclonal anti-syndecan2 antibodies (gift from J. Couchman, University of Alabama at Birmingham); mouse monoclonal anti-

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Synaptotagmin I antibody (Cl 41.1; gift from R. Jahn, Max Planck Inst.;1:250); mouse monoclonal anti-synaptophysin antibody (Roche Diagnostics GmbH, 1:250); rabbit polyclonal anti-Synaptotagmin I luminal domain antibody (gift from P. DeCamilli, Yale University, New Haven, CT); and rabbit polyclonal anti-syntaxin antibody (gift from M. Quick, University of Alabama at Birmingham).

Methods

Hippocampal Cultures

Primary cultures of hippocampal neurons were prepared from Sprague-Dawley rat embryos at age E19. Hippocampi were dissected out, stored in HBSS (HEPES buffered Hank's balanced salt solution without Ca^{2+} and Mg^{2+} pH 7.3), and dissociated with the Papain dissociation system (Worthington). In brief, tissue was digested in papain solution (oxygen saturated Earle's balanced salt solution, pH 7.3, containing 20 units/ml papain, 1 mM L-cysteine, 0.5 mM Ethylenediaminetetraacetic acid (EDTA) and 0.1 units/ml DNase I), shaking very gently at 37°C for 15 min. After trituration, cells were centrifuged and resuspended in Earle's balanced salt solution, pH 7.3, containing bovine serum albumin (BSA)-ovomucoid protease inhibitor (1 mg/ml) and 0.1 units/ml DNase I. Cells were plated onto poly-D-lysine coated 18 mm Ø glass coverslips (Assistent) at densities from 2 to 5 x 10^4 cells/coverslip. Conditioned culture medium was used in plating and maintaining the neurons. This medium was prepared as described (Ye and Sontheimer, 1998). Briefly, confluent astrocytes (8 to 14 days old) were incubated in Earle's minimum essential medium without glutamine (Gibco BRL) containing 10% fetal bovine serum (Hyclone) and 20 mM glucose for 6 to 10 hr at 37°C in a 5% CO_2/95% air

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humidified atmosphere. The conditioned medium was filtered through a 0.2 μm bottom top filter and used within 14 days. The neurons were grown for up to 45 days. To inhibit the proliferation of glial cells, Ara-C (1-2 μM, Sigma) was added 3 days after plating.

Immunocytochemistry

Cultures were fixed in 3.7% formaldehyde in phosphate buffered saline (PBS), pH 7.4 for 20-30 min, washed twice with PBS, and permeabelized for 5 min with 0.25% Triton X 100. Nonspecific binding was blocked for 4 hr to overnight at 4°C with PBS containing 2% BSA, 5% fetal bovine serum (FBS), 2% glycine, 50 mM NH_4Cl, and 0.05% NaN_3. Cells were incubated with primary antibodies diluted in 3% FBS in PBS overnight at 4 °C. After washing in PBS, cells were incubated with either fluorescein-isothiocyanate (FITC) or Texas Red conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Sigma). Cultures were washed with PBS, rinsed with distilled water, dried, and mounted using Vectashield mounting medium (Vector). Fluorescent images were taken with a Nikon Diaphot 300 microscope equipped with a Photometrics CH250 CCD-camera. Digital images were processed and displayed with IP lab Spectrum and Adobe Photoshop.

Synaptotagmin Luminal Domain Antibody (Stg-lu Ab) Uptake

For Stg-lu Ab uptake (Kraszewski et al., 1995), cultures were incubated with 2.0 μg Stg-lu Ab/ml for 15 min at 37°C. Cells were fixed after two washes and processed for immunofluorescent labeling for Stg-lu Ab and Bassoon (using mouse Bassoon antibody), or Synaptophysin.

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Quantitation of Fluorescent Labeling

To quantify the data from the immunocytochemistry, neurons were randomly chosen for image acquisition. At each stage (4, 10, and 21 div), five to seven cells were chosen, and images were taken in both green and red channels. The area selected for analysis was greater than 5,000 μm^2. For all the images, haze was removed, and the intensity of the staining was monitored using IP lab Spectrum software. Clusters were defined as fluorescence intensity of a puncta above a certain threshold. For the described experiments, the threshold intensity was set at a value that was 2-fold or greater in intensity than the diffuse fluorescence present along unstained dendritic segments. In each pair of images, the percentage of clusters that colocalize with the total number of Bassoon clusters was calculated. Except for Piccolo clusters, the number of Bassoon clusters outnumbered other synaptic proteins at all three stages. The data were analyzed using SPSS software (SPSS Inc.).

Immunohistochemistry of Rat Diaphragm

P7 rats were perfused with 4% (wt/vol) paraformaldehyde in 100 mM phosphate buffer (PB, pH 7.4). The diaphragm was removed, postfixed overnight in 2% (wt/vol) paraformaldehyde-phosphate buffer (PB) and cryoprotected in 30% (wt/vol) sucrose in PB (until tissue sinks in the solution or for a maximum of 48 hr). Tissue was embedded in Tissue-Tek (Miles Inc.) and frozen at -70°C. Diaphragm sections (6 μm) were blocked with 5% (vol/vol) fetal calf serum (FCS) in PBS for 2 hr., and incubated overnight with FITC-conjugated α-bungarotoxin (Sigma) and rabbit anti-Piccolo antibodies (1:100 dilution), mouse anti-Bassoon antibodies (1:100 dilution), or a mouse antibody against

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synaptotagmin (1:100 dilution) at 4°C in 5% FBS/PBS. Fluorescent images were collected and processed as described above.

Synaptosome Preparation

The isolation of synaptosome, synaptic plasma membrane, and synaptic junctional preparations was performed as described (Cases-Langhoff et al., 1996). Equal amounts of proteins from each fraction were separated by a 3-15% SDS-PAGE; transferred to nitrocellulose membranes (MS Inc.) and immunoblotted with anti-Piccolo, rab3A, PRA1, and VAMP2/Synaptobrevin II antibodies followed by alkaline phosphatase (AP) conjugated secondary antibodies.

Brain Membrane Floatation Assay

The flotation assay was modified from Balch et al. (1984). Briefly, E18 brains were dissected out and homogenized in homogenization buffer (5 mM HEPES, pH 7.4, 0.5 mM EDTA, 0.3 M sucrose, protease inhibitor cocktail). Homogenate was centrifuged at 800 g for 20 min, and the crude membrane in the supernatant was hypotonically lysed by adding 9 volumes of H_2O. The crude membrane was then centrifuged at 100,000 g for 1 hr. The pellet was P100 and the supernatant is S100. P100 or S100 fraction was then adjusted to 2 M sucrose and loaded as a layer of a discontinuous sucrose gradient underneath layers of 1.2 M, 0.8 M, and 0.3 M sucrose. The sucrose gradient was centrifuged at 350,000 g for 3 hr. Fractions were taken from the top of the gradient to the bottom.

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Immuno-isolation

The immuno-isolation assay was modified from the method described by Henley and McNiven (1996). Briefly, tosylated super-paramagnetic beads (Dynabeads M-500 Subcellular; Dynal Inc.) were incubated overnight with a goat anti-rabbit or anti-mouse linker IgG (Jackson Immunology) at 10 μg/mg beads in borate buffer (100 mM H_3BO_3, pH 9.5). For this and all subsequent steps, beads were collected with a magnetic device (MPC, Dynal, Inc.). Beads were washed with PBS/0.1% BSA and blocked with Tris blocking buffer (0.2 M Tris, pH 8.5, 0.1% BSA) for 4 hr at 37°C. Linker IgG-coated beads were then incubated overnight at 4°C with rabbit polyclonal anti-Piccolo antibody, monoclonal anti-Bassoon antibody, or monoclonal anti-synaptophysin antibody at a concentration of 10 μg/mg beads in incubation buffer (PBS, pH 7.4, 2 mM EDTA, 5% FBS). The primary antibody-coated beads and the control linker IgG-coated beads were then incubated overnight at 4°C with the light membrane fractions (0.3 and 0.8 M sucrose gradient fractions). Beads were then collected and washed five times with incubation buffer and three times with PBS at 10 min each and saved as bound fractions (B). Supernatants were saved as nonbound fractions (NB). The B and NB fractions were subsequently analyzed by EM and Western blotting techniques. To access proteins that are in direct association with Piccolo, an RIPA buffer treatment was used. The immuno-isolated fractions were further treated with RIPA buffer for 15 min on ice, and the supernatant was saved as unbound fraction, and the beads were further washed five times with RIPA buffer and three times with PBS and saved as bound fraction.

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Electron Microscopy

Beads bound fractions were fixed by either 3% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer (Fix 1) or 4% glutaraldehyde, 0.8% tannic acid in 0.1 M Cacodylate buffer (Fix 2) to enhance the fixation. After fixation, the bead fractions were rinsed, postfixed by 1% OsO_4, dehydrated, and embedded. Cultured hippocampal neurons at 3 to 5 div were fixed by 0.1% glutaldehyde, 4% paraformaldhyde, and 1% tannic acid in PBS and processed for EM. Immuno-EM on cultured neurons was done as described by Gross et al. (2000). Briefly, after fixation, neurons were rinsed with 10% methanol and 0.03% H_2O_2 in PBS for 5 min, washed with PBS, and permeabilized with 0.05% saponin in HBSS for 10 min. After washing with PBS, cells were blocked with 2% normal goat serum, 0.05% saponin, and 0.1% NaN_3 for 1 hr and incubated overnight with anti-Piccolo antibody diluted in blocking buffer or blocking buffer without primary antibody. After blocking with 0.2% BSA in PBS, neurons were incubated with ultrasmall gold coupled secondary antibody (EMS). After washing, neurons were postfixed with 2% glutaradehyde for 5 min. The gold labeling was silver enhanced for 20 min using GP-US kit (EMS). Membranes were marked by 1% OsO_4 for 30 min in dark. Then the cells were washed, dehydrated, and processed for EM. Sections of 2 × 1.6 μm were imaged, and gold particles were counted and catogorized based on their location. If the gold particle was within 20 nm of an organelle, the gold particle was scored as associated with this organelle. Twenty five Piccolo antibody-labeled sections, 20 Bassoon antibody labeled sections, and 20 control sections were counted. The counting and scoring were performed double blinded.

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Electrophysiological Recording From Hippocampal Neurons in Culture

After 4 days in culture, hippocampal neurons grown on cover-slips were placed in a recording chamber and mounted on the stage of an inverted microscope (Diaphot 200, Nikon). Neurons were continuously superfused with extracellular media containing (in mM) NaCl, 155; $CaCl_2$, 2; $MgCl_2$, 2; HEPES, 10, glucose, 10; pH 7.4. Standard whole-cell patch clamp recordings were obtained under visual control with Sylgard-coated pipettes filled with intracellular medium containing (in mM) Cs-methansulphonate, 130 140; CsCl, 10; HEPES, 10; EGTA, 10. Miniature synaptic currents were recorded in the presence of Tetanus toxin (TTX) (100 nM). Miniature inhibitory postsynaptic currents (mIPSPs) were isolated in the presence of 6,7-Dinitroguinoxaline (DNQX) (10 μM) at a holding potential of 0 mV. Miniature excitatory postsynaptic currents (mEPSPs) were isolated in the presence of bicuculline (100 μM) at a holding potential of minus 40 mV. Events were recorded to videotape using a Digital Data Recorder (Instrutec). Off-line video tape recordings were digitized at 10 kHz and low-pass filtered at 2 Hz using a Digidata 1200 A/D board (Axon Instruments) on an 80586-based computer using AxoScope software (Axon Instruments). Miniature synaptic events were detected automatically as negative (excitatory) or positive (inhibitory) deflections that were > 3 fold above noise using a Mini Analysis program (Synaptosoft). All records were visually inspected, and artificial events were excluded from final analysis.

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APPENDIX

IACUC APPROVAL NOTICE

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THE UNIVERSITY OF
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Office of the Provost

MEMORANDUM

DATE: December 4, 2000

TO: Craig Garner, Ph.D.
CIRC-663 0021
FAX: 975-5097

FROM: Clinton J. Grubbs, PhD, Chairman
Institutional Animal Care and Use Committee

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on December 3, 2000.

Title of Application: Assembling the Presynaptic Junction

Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office for Protection from Research Risks (Assurance Number A3285-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

CJG/mlw

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GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Rong Zhai

Graduate Program Neurobiology

Title of Dissertation Assembling the Presynaptic Active Zone: The Characterization of an Active Zone Precursor Vesicle

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name		Signature
Craig C. Garner	**, Chair**	Craig Garner
Kent T. Keyser		Kent T. Keyser
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Date 7/18/01

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