Neuron, Vol. 29, 131–143, January, 2001, Copyright ©2001 by Cell Press

Assembling the Presynaptic Active Zone: A Characterization of an Active Zone Precursor Vesicle

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Summary

The active zone is a specialized region of the presynaptic plasma membrane where synaptic vesicles dock and fuse. In this study, we have investigated the cellular mechanism underlying the transport and recruitment of the active zone protein Piccolo into nascent synapses. Our results show that Piccolo is transported to nascent synapses on an ~80 nm dense core granulated vesicle together with other constituents of the active zone, including Bassoon, Syntaxin, SNAP-25, and N-cadherin, as well as chromogranin B. Components of synaptic vesicles, such as VAMP 2/synaptobrevin II, synaptophysin, synaptotagmin, or proteins of the perisynaptic plasma membrane such as GABA transporter 1 (GAT1), were not present. These studies demonstrate that the presynaptic active zone is formed in part by the fusion of an active zone precursor vesicle with the presynaptic plasma membrane.

Introduction

Synapses of the central nervous system (CNS) are highly specialized asymmetric sites of cell–cell contact designed for the rapid and repetitive signaling between neurons and their targets (Burns and Augustine, 1995). The plasma membrane of the presynaptic compartment contains a specialized region, known as the active zone, where synaptic vesicles (SVs) are thought to dock and fuse. The active zone is characterized ultrastructurally as an electron-dense meshwork of cytoskeletal filaments intimately associated with the plasma membrane, embedded with clusters of SVs, and juxtaposed to the electron-dense postsynaptic density (PSD) (Landis et al., 1988; Hirokawa et al., 1989; Gotow et al., 1991). The cytoskeletal matrix associated with the active zone (CAZ) is thought to play a fundamental role in defining neurotransmitter release sites, keeping the active zone in register with the postsynaptic reception apparatus and regulating the mobilization of SVs and the refilling of release sites (Garner et al., 2000a).

The assembly of functional active zones is a key event during the formation of synapses in CNS. The formation of synapses presumably begins with the protrusive activity of one or both of the future synaptic partners and the establishment of a new axodendritic physical contact. Subsequent steps include the assembly of a specialized junctional cytoskeletal matrix that serves to stabilize the adhesion site as well as to promote the recruitment and clustering of neurotransmitter receptors, ion channels, and SVs (Rao et al., 1998; Zhai et al., 2000). Real-time imaging studies in both slice and hippocampal cultures have revealed that individual synaptic connections may form relatively quickly (Dailey and Smith, 1996, Ziv and Smith, 1996; Baranes et al., 1998; Engert and Bonhoeffer, 1999; Horch et al., 1999; Maletic-Savatic et al., 1999; Okabe et al., 1999; Ahmari et al., 2000; Jontes et al., 2000). For example, activity-induced recycling of SV has been observed as soon as 30 min after the initial axodendritic contact (Vardinon-Friedman et al., 2000; see also Ahmari et al., 2000). In contrast, the recruitment of glutamate receptors, presumably to the postsynaptic compartment, seems to be delayed on average by another 40 min (Vardinon-Friedman et al., 2000). These studies suggest that first, synaptogenesis involves a sequential series of events, and second, the presynaptic active zone may become functionally active prior to the maturation of the postsynaptic reception apparatus.

Currently, the cellular mechanisms that underlie the establishment of active zones and the PSD remain unclear. Open questions include the following: In what form are synaptic proteins sorted and transported to nascent synapses? Does the assembly of synaptic junctions involve the sequential addition of synaptic cytoskeletal proteins to cell–cell contact sites or involve the transport and recruitment of synaptic junctional proteins as preformed complexes? Clearly, for integral membrane proteins such as neurotransmitter receptors, ion channels, adhesion molecules, and SV proteins, vesicular intermediates are required for their sorting and transport. Cytoskeletal proteins such as actin, tubulin, and clathrin, in contrast, are generally supposed to be transported by slow-transport mechanisms (Hirokawa, 1998; Goldstein and Philp, 1999; Hannah et al., 1999).

One clue to the mechanisms underlying synaptogenesis of CNS synapses came from studies by Ahmari et al showing that clusters of vesicles containing synaptic proteins are found at sites of newly forming synapses (Ahmari et al., 2000). 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 perisynaptic plasma membrane is unclear. Studies on the synaptogenesis of the developing spinal cord provide the strongest evidence to date that these precursor vesicles may exist

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(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 are apposed to a modest PSD-like membrane specialization (Vaughn, 1989). The 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. To date, however, a molecular characterization of these granulated vesicles has not been performed.

Our molecular characterization of CNS synapses has recently led to the identification of two structurally related CAZ proteins called Piccolo and Bassoon (Cases-Langhoff et al., 1996; tom Dieck et al., 1998; Fenster et al., 2000). They are multidomain zinc finger proteins of 530 and 420 kDa, respectively (tom Dieck et al., 1998; Wang et al., 1999; Fenster et al., 2000), that are restricted within nerve terminals to the active zone of both excitatory and inhibitory synapses (Cases-Langhoff et al., 1996; tom Dieck et al., 1998; Brandstatter et al., 1999; Richter et al., 1999; Wang et al., 1999; Zhai et al., 2000). Biochemical, molecular, and ultrastructural studies indicate that they are structural/cytoskeletal components of the active zone, where they are thought to perform a scaffold function (Garner et al., 2000a). Analysis of synaptogenesis in cultured hippocampal neurons has revealed that Bassoon is expressed at the early stages of neuronal differentiation, arriving at newly forming synapses prior to or simultaneously with the acquisition of evoked SV recycling (Vardinon-Friedman et al., 2000; Zhai et al., 2000). However, a similar analysis for Piccolo has not been yet performed.

In this study, we have investigated the cellular mechanisms 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. We found that Piccolo was selectively sorted into axonal growth cones at early stages of neuronal differentiation and was recruited into nascent synapses coincidentally with the acquisition of activity-induced recycling of SVs. In immature neurons, Piccolo exhibited a punctate pattern that was similar yet distinct to SVs. Our characterization of the Piccolo puncta revealed that it was associated with ~80 nm dense core granulated vesicles that were concentrated in axonal growth cones and was associated with nascent synapses. These vesicles were highly reminiscent of the granulated vesicles initially identified by Vaughn (1989), suggesting that components of active zone are transported to nascent synapses on an active zone precursor vesicle in which the fusion with the plasma membrane at sites of axonal–dendritic cell–cell adhesion may initiate the establishment of the presynaptic active zone. We refer to the Piccolo-associated vesicle as a granulated vesicle to avoid confusion with and draw a distinction from the classic neuropeptide-containing dense core vesicles.

Results

Piccolo Is Expressed at Early Stages of Synaptogenesis in Developing Rat Brain

The localization of Piccolo at the CAZ in mature synapses (Cases-Langhoff et al., 1996) suggests a fundamental role for Piccolo in defining the active zone as the site of SV docking and fusion. If true, one would expect Piccolo to be expressed at early stages of neuronal differentiation and prior to synaptogenesis. Developmental Northern blots were initially used to examine the temporal expression of Piccolo in the developing rat brain. Total RNA from postnatal day 1 (P1) to P75 rat brain was hybridized with radiolabeled Piccolo cDNA (clone sap44a) (Cases-Langhoff et al., 1996). Two transcripts of 16 and 17 kb were detected, indicating that Piccolo is encoded by at least two different transcripts, presumably because of alternative splicing (Figure 1A). At P1, the 16 kb band is the most prominent and increases steadily throughout the first 2 weeks of postnatal development. In contrast, expression of the 17 kb transcript, while initially low, increases during the next 2 weeks of development, with some drop in the adult brain. These data demonstrate that Piccolo transcripts are expressed at early stages of neuronal differentiation and throughout the peak period of synaptogenesis, which occurs between days P10–P30 (Altman and Das, 1965; Melloni and DeGennaro, 1994).

In a previous study, Piccolo transcripts were found to be present in most regions of the adult rat brain and were the most abundant in the hippocampus and cerebellum (Cases-Langhoff et al., 1996). To test further the potential involvement of Piccolo during neuronal differentiation, we analyzed the spatial and temporal expression pattern of Piccolo and its transcripts in developing rat cerebellum. In situ hybridization of P5, P10, and P75 cryosections revealed the presence of a strong hybridization signal initially in the premigratory undifferentiated granule cells situated in the external granule cell layer of P5 and P10 cerebella (Figure 1B). As development proceeds and granule cells begin to accumulate in the inner granule cells layer, the hybridization signal is also observed in the differentiating neurons. No signal was observed in white matter tracks (data not shown) and in the molecular layer of either P10 or P75 cerebellar sections (Figure 1B). Furthermore, no specific hybridization signals were detected when sections were either incubated with a 100-fold excess of unlabeled oligonucleotide, treated with RNase before hybridization, or hybridized with a sense probe (see Cases-Langhoff et al., 1996).

Examination of the spatial appearance of Piccolo in adjacent cerebellar sections by immunoperoxidase staining revealed a robust and specific staining of the emerging molecular layer in P5 brains. As the cerebellum develops, a strong staining was seen not only in the inner region of the molecular layer but also in the newly forming outer regions of the molecular layer (Figure 1C). Moreover, at P5, Piccolo was found to exhibit a punctate pattern, similar to synaptophysin, on the surface of Purkinje cell dendrites, which were visualized with antibodies against MAP2, a dendritic microtubule-associated protein (Matus et al., 1986), by double-labeled immunofluorescence microscopy (Figures 1D and 1E). These data demonstrate that in situ Piccolo is expressed at early stages of neuronal differentiation and may become localized at newly formed synapses.

Expression of Piccolo in Differentiated Hippocampal Neurons

Using hippocampal cultures as a model system to study synaptogenesis (Goslin and Banker, 1991; Matteoli et

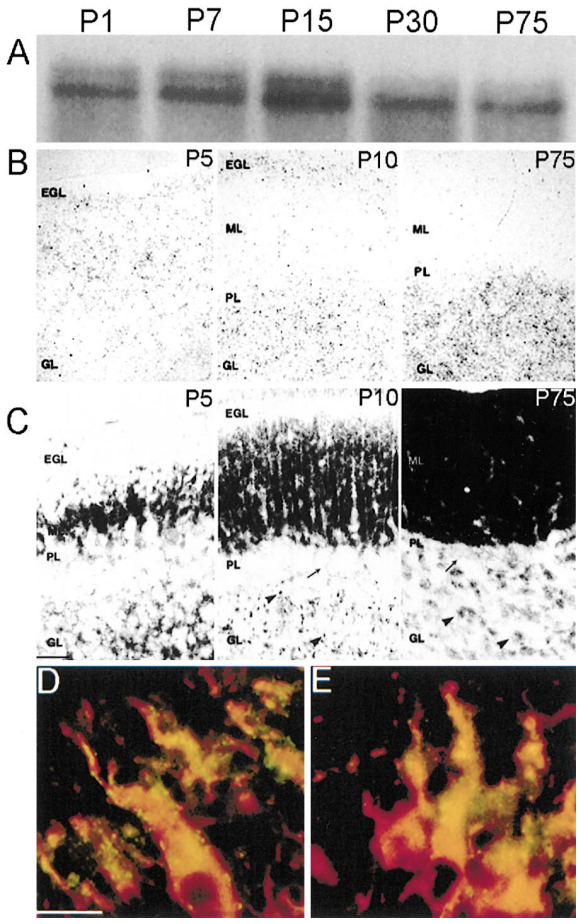

Figure 1. Expression of Piccolo during Rat Brain Development

(A) Total RNA was isolated from rat brain at the age of postnatal days 1, 7, 15, 30, and 75 (P1, P7, P15, P30, and P75). Total RNA was separated on 1.2% formaldehyde agarose gels and blotted onto Qiabrane Nylon (Qiagene, Chatsworth, CA). Nylon membrane was hybridized with α-^{32}P-labeled cDNA probes. Two bands at 16 and 17 kb were visible at P1. The intensity of both bands rose and then declined from P1 to P75, but the highest intensity of both 16 and 17 kb bands was at P15.

(B) Sections from P5, P10, and P75 cerebellum were fixed and hybridized with α-^{32}P-labeled cDNA probes. A strong hybridizing signal was observed in the premigratory undifferentiated granule cells in the external granule cell layer (EGL) of P1 and P5 brains and later (P75) in the differentiating neurons in the inner granule cells layer.

(C) Adjacent sections from P5, P10, and P75 cerebellum were incubated with Piccolo rabbit antibody and processed for immunocytochemistry. The EGL is devoid of immunoreactivity. The molecular layer (ML) increases in thickness and intensity of immunoreactivity as the parallel fibers accumulate and mature and the dendritic trees of the Purkinje cells develop. At P10 and in the adult (P75), punctate staining around Purkinje cell bodies was observed (arrows). The glomeruli in the granule layer (GL) began to be apparent at P10 and was strongly immunoreactive in the adult cerebellum (arrowhead). PL, Purkinje cell layer.

(D and E) Sections from P5 cerebellum were double stained by Piccolo rabbit antibody (in green) and MAP2 monoclonal antibody (D, in red) or by synaptophysin monoclonal antibody (in green) and MAP2 rabbit polyclonal antibody (E) and were processed for immunofluorescent microscopy. Piccolo was found to exhibit a punctate pattern (D), similar to synaptophysin (E), on the surface of Purkinje cells dendrites labeled with MAP2. Scale bars in (C) (for [B] and [C]) and in (D) (for [D] and [E]) are 10 μm.

al., 1995), we next examined the temporal and spatial appearance of Piccolo in differentiating neurons by double-label immunofluorescent microscopy. Antibodies against MAP2 were used to mark dendrites at different stages of neuronal differentiation (Goslin and Banker, 1991). In stage 2 neurons, when minor processes first appear (2 days in vitro), Piccolo immunoreactivity was observed as fine puncta in the cell soma as well as in all fine processes, which were labeled by MAP2 antibodies (Figures 2A–2C). At 3 to 4 days in vitro, as the axonal outgrowth is initiated (stages 3–4), the distribution of Piccolo and MAP2 became polarized (Figures 2D–2F). Piccolo immunoreactivity was found primarily in the distal part of the axon and the growth cone as fine puncta (Figures 2D and 2F), while MAP2 immunoreactivity was detected in the dendrites and in the proximal axonal segments (Figures 2E and 2F). 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 (Figures 2A and 2D), which are then sorted and transported into axons. By 10 days in vitro, Piccolo immunoreactivity was observed as larger clusters along MAP2-containing dendritic profiles (Figures 2G–2I). Such clusters were also observed as early as 4 days in vitro (arrowheads in Figure 2F). The number of these immuno-positive clusters increased steadily during neuronal maturation (4–26 days in vitro). To confirm that these clusters along dendritic profiles are synapses, neurons were double stained with antibodies against Piccolo and other synaptic markers. Piccolo clusters along dendrites of stage 4 neurons were observed to colocalize with clusters of Bassoon (arrowheads in Figures 2J–2L) as well as with synaptophysin and AMPA receptor (data not shown), indicating that Piccolo is recruited to nascent synapses in these immature cultured neurons.

Accumulation Dynamics of Piccolo at Nascent Synapses

To estimate when Piccolo is recruited into individual nascent synapses, a retrospective immunohistochemical analysis was performed as described previously (Vardinon-Friedman et al., 2000). Activity-induced uptake of FM 4-64 was used to label SVs in presynaptic boutons of 11–14 days in vitro hippocampal neurons. The population of labeled vesicles was then followed by automated time-lapse confocal microscopy at 10-min intervals. After preparations were sampled at three to four time points, a second stimulus train was given in order to induce exocytosis of the labeled vesicles (destaining) to verify that the fluorescent puncta represented presynaptic boutons that displayed a capacity for activity-evoked exocytosis.

By repeating this procedure, we were able to detect the appearance of new presynaptic boutons that were not observed at prior time points. To determine whether such boutons contained clusters of Piccolo, the specimens were fixed at the end of each experiment and stained with Piccolo antibodies. We then determined, for each apparently new bouton recorded during the experiment, whether Piccolo was clustered at the same location. These data were then compared with the "age" of the bouton, defined as the time interval between the moment the bouton was first observed and the time at

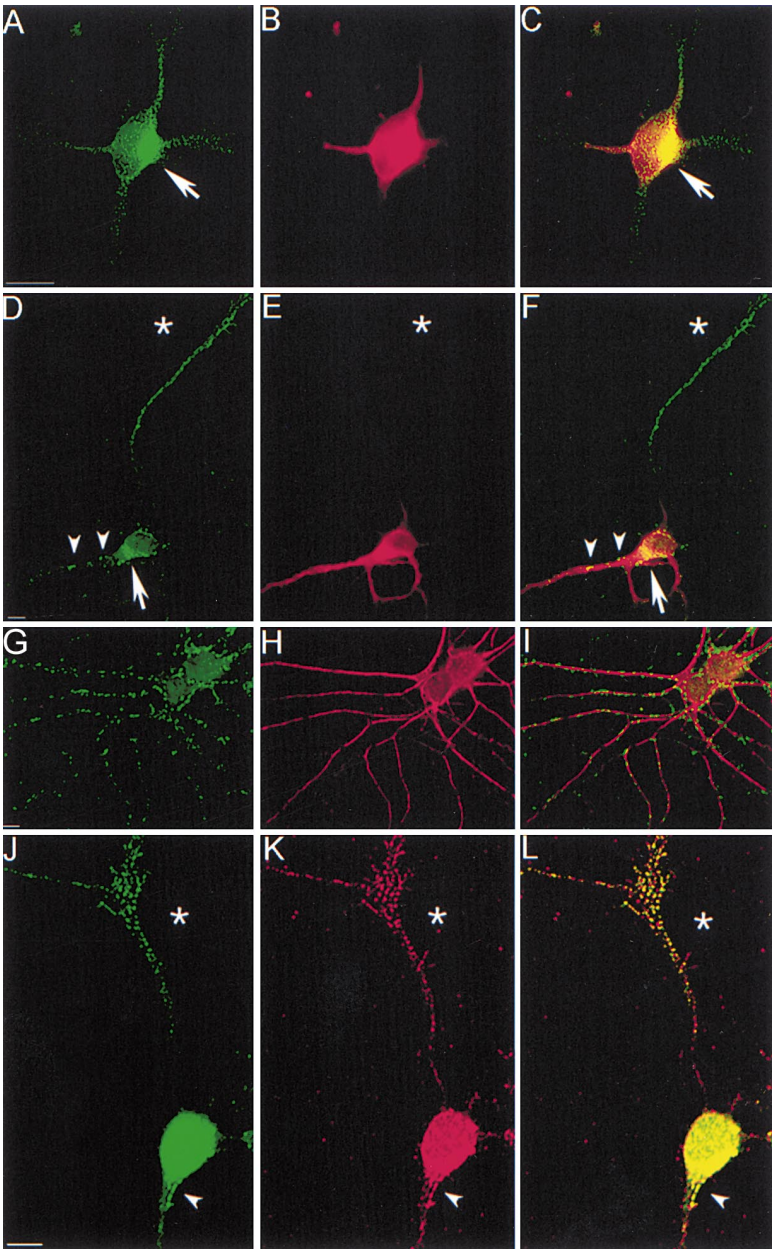

Figure 2. Spatial Distribution of Piccolo in Differentiating Hippocampal Neurons

(A–L) Piccolo is expressed in immature neurons and becomes synaptically localized at later stages. Cultured hippocampal neurons were fixed at 2 (A–C), 4 (D–F and J–L) and 10 (G–I) days in vitro and were immunostained for Piccolo (A, D, G, and J) and the dendritic marker MAP2 (B, E, and H) or Bassoon (K). In stage 2 cells (2 days in vitro), Piccolo is present in all processes as fine puncta (A); MAP2b is also present in all neurites but in a diffuse pattern (B). In stages 3 and 4, neurons as neurites begin to differentiate; MAP2b immunoreactivity becomes restricted to the somato-dendritic regions and proximal segment of the axon (E), and Piccolo puncta become concentrated in axons (asterisks in D–F). From stage 4, Piccolo immunoreactivity starts to be clustered along dendritic profiles and the cell soma (arrowheads in D and F), and the number of clusters greatly increased in 10-day in vitro neurons (G). Piccolo and Bassoon colocalized on the surface of dendrite (arrowheads in J–L), suggesting a synaptic localization. Interestingly, a perinuclear punctate staining of Piccolo is observed in stages 2 to 4 neurons (arrows in A and D). Scale bars, 10 μm in all panels.

which the preparation was fixed. Figure 3 shows an example of two new boutons that appeared ~56 min before fixing the preparation. Retrospective immunohistochemical analysis of the same region revealed the presence of discreet clusters of Piccolo at the same sites.

All boutons in which the appearance was recorded in these experiments were categorized according to their age at the moment of fixation (n = 23, 4 separate experiments, 44 fields of view; see Experimental Procedures). Eight out of nine boutons that appeared 1 hr or less before fixation were found to be associated with a discreet cluster of Piccolo. Likewise, 13 out of 14 boutons that appeared between 1 and 2 hr before fixation were also associated with Piccolo clusters. These results are similar to those found for Bassoon (Vardinon-Friedman et al., 2000), suggesting that Piccolo clusters at presynaptic sites before or concomitantly with the acquisition of a capacity for activity-induced recycling of SVs, and they support the possibility that the Piccolo may be involved in synapse assembly.

Piccolo Is Transported to Nerve Terminals in Association with an 80 nm Dense Core Granulated Vesicle

The punctate pattern exhibited by Piccolo in axonal growth cones could represent the association of Piccolo with vesicular membranes or a proteinecious particle. To understand the nature of the Piccolo puncta, we first examined the biochemical properties of Piccolo in embryonic day 18 (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 ro-

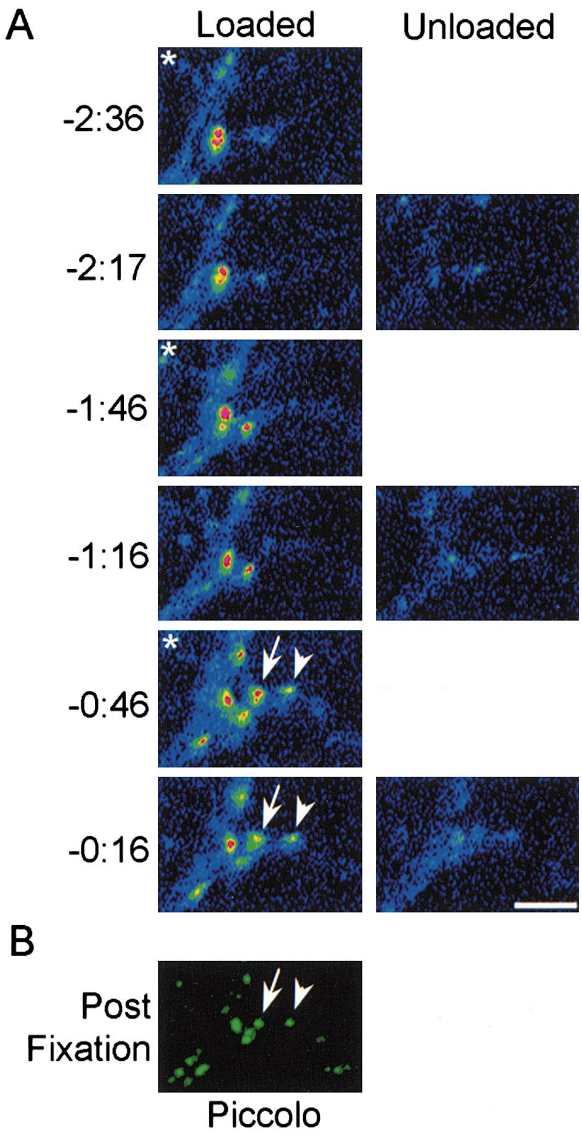

Figure 3. Clustering of the Piccolo at New Synaptic Sites

(A) Recurrent labeling with FM 4-64 and time-lapse imaging of labeled SVs were used to detect the appearance of apparently new presynaptic boutons (arrow and arrowhead, t = −0:46). Note that these new vesicle clusters were not observed prior to this time point; however, once they appeared, they remained at the same location until the end of the experiment. Images collected immediately after a FM 4-64 labeling episode are marked with asterisks. Time is given as hours and minutes relative to the moment of fixation and refers to images in the left panels. Images obtained after destaining stimulation trains (right panels) were collected ~10 min after those on their left.

(B) Retrospective immunohistochemistry of the same region. Apparently new boutons were associated with discreet cluster of Piccolo. Scale bar, 5 μm.

bust synaptogenesis (Altman and Das, 1965). E18 brain homogenate was hypotonically lysed and subjected to 100,000 g centrifugation to yield the supernatant (S100) and pellet (P100) fraction. Although Piccolo is present in S100, more than 90% of Piccolo was found in P100 (Figure 4A). A 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 SV protein, by Western blotting. As shown in Figure 4B, Piccolo in the P100 fraction was found in 0.3 and 0.8 M layers together with synaptophysin, indicating that Piccolo is associated with light membranes similar to SVs. 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 4B). As a control, when P100 fraction was treated with Triton X100 before the flotation assay, Piccolo and synaptophysin remained at the bottom of the gradient, indicating that the ability of both Piccolo and synaptophysin to float is dependent on membrane integrity. Interestingly, when adult rat brain (P30) was used in the experiment, Piccolo stayed at the bottom of the gradient, whereas synaptophysin was found in 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 (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.

To examine further whether Piccolo in immature neurons is associated with a vesicle, electron microscopy (EM) was used to study the ultrastructure of the putative Piccolo transport vesicle (PTV). Piccolo containing light membrane fractions immunoisolated from E18 with Piccolo rabbit polyclonal antibody–coated beads (Piccolo-rAb beads) was processed for EM. Samples processed with 3% paraformaldehyde and 1% glutaraldehyde (Fix 1) revealed spherical structures with electron-dense cores surrounded by clouds of less dense material (Figure 5A1). Using 4% glutaraldehyde 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; Figures 5A2 and 5A3). Vesicles of 50–60 nm with clear center were found on synaptophysin-mAb beads (n = 25; Figure 5C). No vesicular elements were attached to the control IgG beads (Figure 5D). As described later, this immunoisolation procedure is highly specific, leading to the selective isolation of Piccolo or synaptophysin, respectively, as assessed by Western blotting (Figure 7). These data support the hypothesis that Piccolo in immature axons is associated with a transport vesicle (referred to as the PTV).

To examine whether granulated vesicles of ~80nm are actually present in immature axons, 3 days in vitro hippocampal cultures were processed for EM. Interestingly, granulated vesicles with the similar size (80 nm) and morphology as the granulated vesicle isolated with our Piccolo antibodies were found in axons in the vicinity of nascent synapses (Figure 6A). These granulated vesicles are distinct from the clear-centered vesicles, presumably SVs, and clathrin-coated vesicles (Figure 6A). The association of Piccolo with these 80 nm granulated vesicles in vivo was confirmed by silver-enhanced immunogold EM with Piccolo antibodies (Figures 6D–6F). In this experiment, 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

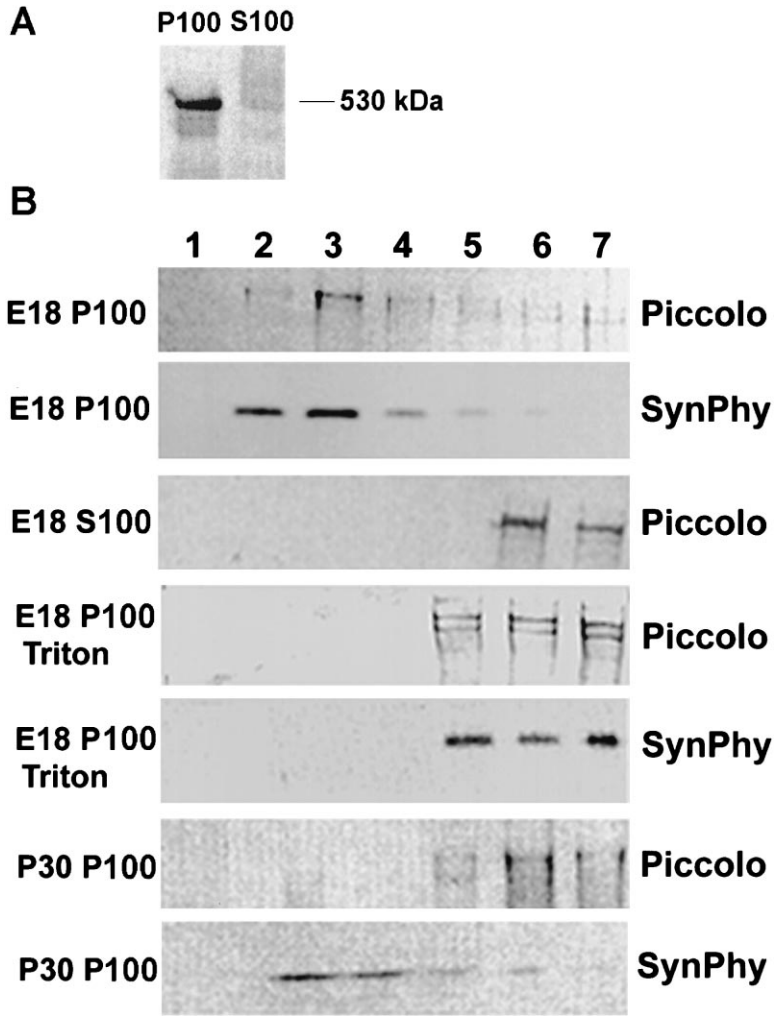

Figure 4. Piccolo Is Associated with Light Membrane in Embryonic Rat Brain

(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 to the bottom of sucrose gradient of 0.3, 0.8, 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 adjusted to 2 M sucrose.

vesicle). Of the 184 gold particles observed on these sections, 88.9% were found to be associated with dense core granulated vesicles; 2.6% were found to be labeled with other types of vesicular organelle, and 8.5% were found to be associated with nonvesicular material. In the control experiment, 78% of the gold particles were not found to be 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. 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 6B), as well as in a perinuclear region in association with Golgi stacks (Figure 6C), 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 2) indicate that shortly after synthesis, Piccolo becomes associated with Golgi-derived 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 diagramed in Figure 6H.

The Piccolo Transport Vesicle Is a Precursor of Active Zones

The presence of a presynaptic cytomatrix protein, Piccolo, on the outside of a granulated vesicle suggests that this vesicle may be a precursor to the active zone. If so, one would predict that other components of active zones would be present. To test this hypothesis, immunoisolated PTV preparations were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and evaluated for the presence of SV, active zone, or perisynaptic proteins by Western blotting. As shown in Figure 7, when Piccolo antibody–coated beads were used, not only did we affinity purify Piccolo but also the CAZ protein Bassoon, the synaptic plasma membrane SNARE proteins syntaxin and SNAP25, and the synaptic junctional cell adhesion molecule N-cadherin. The SV proteins VAMP 2/synaptobrevin, synaptophysin, and synaptotagmin were not found, and neither was the perisynaptic GABA transporter GAT1. In reciprocal experiments with synaptophysin antibody–coated beads, the CAZ proteins Piccolo and Bassoon were not isolated, and neither was GAT1. None of these proteins were isolated with beads coated with an irrelevant control IgG.

Double-label immunofluorescent microscopy on the axons and growth cones of 3 days in vitro neurons was

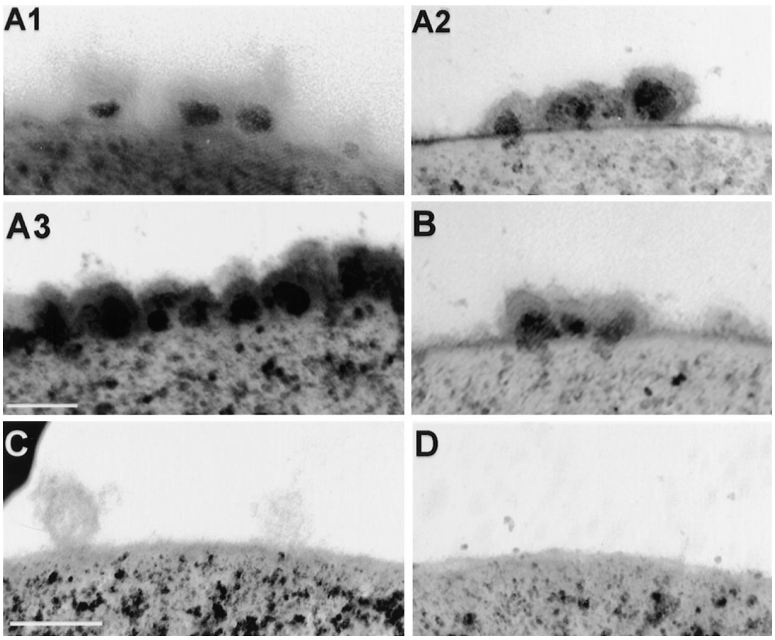

Figure 5. Piccolo Transport Vesicle Is a Dense Core Granulated Vesicle with ~80 nm Diameter

(A–D) The light membrane fractions collected from the flotation assay (Figure 4B, 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 Experimental Procedures), and in (A2–D), the beads were fixed by Fix 2 to enhance the membrane. Scale bars in (A3) (for [A1], [A2], [A3], and [B]) and in (C) (for [C] and [D]) are 100 nm.

used to confirm these results. Cultures were double stained for Piccolo and Bassoon, synaptotagmin, VAMP 2/synaptobrevin II, syntaxin, SNAP25, or N-cadherin. As shown in Figure 8, Piccolo and Bassoon puncta revealed a high degree of colocalization (>95%; Figures 8A–8C). In contrast, Piccolo puncta did not colocalize with either synaptotagmin (Figures 8D–8F) or VAMP 2/synaptobrevin II (Figures 8G–8I). A moderate level of colocalization was observed with N-cadherin (50%; Figure 8J–8L). Although some colocalization was observed between Piccolo puncta and syntaxin 1A or SNAP25 (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.

The presence of a second CAZ protein, Bassoon, on the PTV was confirmed both by immunoisolation (Figures 5B and 7) and immuno-EM (Figure 6G). In immunoisolation experiments, beads coated with Bassoon antibodies purified the same collection of proteins as beads coated with Piccolo antibodies (Figure 7). Furthermore, EM analysis of magnetic bead coated with our Bassoon antibodies revealed the presence of 80 nm dense core vesicles (Figure 5B) similar to those observed when the beads were coated with Piccolo antibodies (Figure 5A). The association of Bassoon with 80 nm dense core granulated vesicles was confirmed by immunogold EM analysis of cultured hippocampal neurons (Figure 6G). Here, 72 dense core vesicles were found to be decorated with gold particles (1.33 gold particles per dense core vesicle). Of the 112 gold particles detected, 91.5% were found to be associated with dense core granulated vesicles; 2.1% were found to be labeled with other types of vesicular organelle, and 6.4% were found to be associated with nonvesicular material. Taken together, these results strongly suggest that the CAZ proteins Piccolo and Bassoon, as well as other active zone proteins, are associated with dense core granulated vesicles in vivo and that these vesicles are precursors of the active zone.

The association of active zone proteins with dense core granulated vesicles is unexpected and suggests a novel potential role for classic peptide containing dense core vesicles during synaptogenesis. To begin to address this important issue, we examined whether the classic dense core vesicle protein chromogranin B is present in the PTV. Chromogranin B immunoreactivity was selectively found on Piccolo and Bassoon antibody–coated beads but not on synaptophysin or IgG-coated beads (Figure 7). Furthermore, chromogranin B and Piccolo immunoreactive puncta were found to colocalize in immature axonal growth cones (Figures 8M–8O). Together, these results demonstrate that numerous components required for the assembly of active zones are transported in association with 80 nm granulated vesicles, which are likely to serve as a precursor for the presynaptic active zone.

Discussion

In this study, we have investigated the potential involvement of the CAZ molecule Piccolo in the assembly of CNS synapses and examined how this molecule is recruited into nascent synapses. Our primary objective was to gain insights into the mechanisms underlying presynaptic active zone assembly. Our data show that Piccolo is expressed both in situ and in cultured neurons at early stages of neuronal differentiation. Moreover, once synthesized, 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 SV recycling. Our initial characterization of the PTV revealed that it contains other constituents of active zones as well as chromogranin B but not SV proteins or the perisynaptic protein GAT1. These data suggest that active zone proteins are transported to nascent synapses on precursor vesicles and support the hypothesis that the

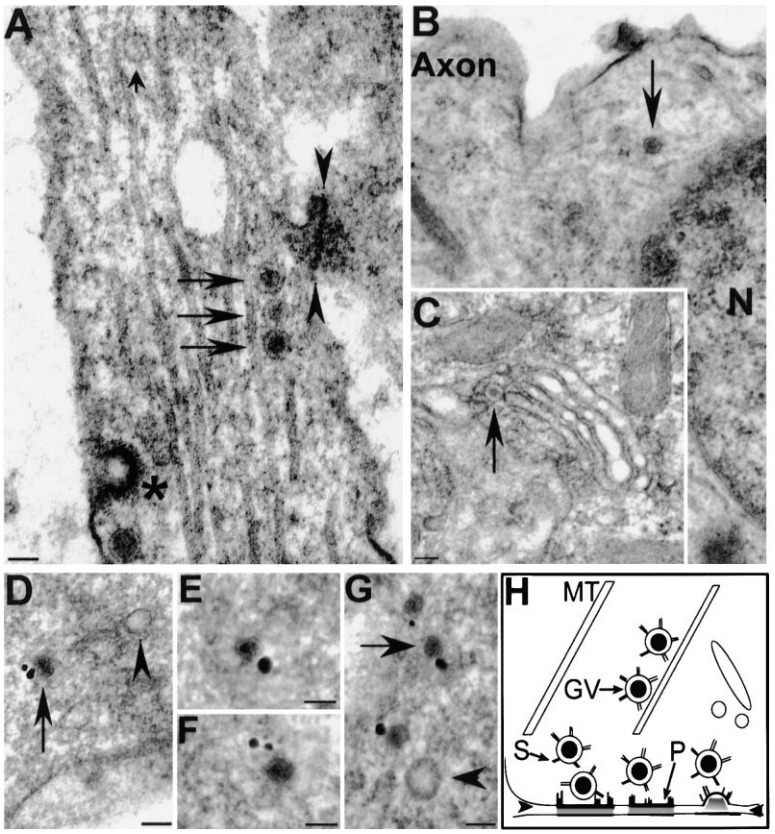

Figure 6. Dense Core Granulated Vesicles Are Present in Axons and Cell Soma of 3 Day In Vitro Hippocampal Neurons

(A–C) EM micrographs of hippocampal neurons cultured for 3 days in vitro. Eighty nm dense core granulated vesicles are observed along microtubules (MT) in axons ([A]; 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 representing a nascent synapse (arrowheads) can be observed between the axon and an adjacent cell soma. Dense core granulated vesicles were also observed in cell soma close to the initial segment of axon (arrow in [B]) and in close association with Golgi stacks (arrow in [C]).

(D–G) Immunogold EM micrographs of 3 day in vitro hippocampal neurons. The presence of Piccolo (D–F) and Bassoon (G) on these granulated vesicles was visualized by immunogold labeling followed by silver enhancing. Gold particles labeled dense core vesicles (arrows in [D and G]), but not other type of vesicles (arrowheads in [D and G]).

(H) Schematic diagram of a nascent synapse based on EM micrographs of E15 spinal cord (Vaughn, 1989). GV, granulate vesicle; S, spicule; P, patches of newly forming active zone; MT, microtubule. Scale bars, 100 nm in (A–H).

active zone is assembled wholly or in part by the fusion of this precursor vesicle with the presynaptic plasma membrane.

Piccolo Is a Marker of Nascent Active Zone Assembly

Recent advances in the molecular analysis of CNS synapses have lead to the identification and characterization of numerous constituents of synaptic junctions (Craven and Bredt, 1998; Garner et al., 2000a, 2000b). To date, four presynaptic proteins have been identified that are potentially involved in the assembly of active zones (Garner et al., 2000a). These include the structurally related CAZ proteins Piccolo and Bassoon (tom Dieck et al., 1998; Wang et al., 1999; Fenster et al., 2000), the rab3 effector protein RIM (Wang et al., 1997), and phorbol ester binding protein Munc13-1 (Betz et al., 1998). All four appear to be structural components of active zones and are thought to be involved in defining the site of SV docking, fusion, and recycling (see Garner et al., 2000a). In this study, we have investigated the utility of Piccolo as a marker for the formation of active zones. Our developmental studies both in situ and in culture show that Piccolo appears in neurons at early stages of differentiation and thus is available to participate in active synaptogenesis. For example, in developing neurons, Piccolo is found to cluster along dendritic profiles as early as 4 days in vitro together with other presynaptic and postsynaptic components consistent with a role for Piccolo in the assembly of CNS synapses. This conclusion is supported by our retrospective studies showing that the appearance of Piccolo (discussed in this article) as well as Bassoon (Vardinon-Friedman et al., 2000) at nascent synapses is concurrent with the acquisition of activity-induced recycling of SVs. These data and the presence of Piccolo and Bassoon on the same transport vesicle demonstrate a fundamental role for these CAZ proteins in the establishment of functional presynaptic active zones and their utility as excellent markers for nascent active zones.

Active Zone Proteins Are Transported to Nascent Synapse in Association with Golgi-Derived Granulated Vesicles

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, but the relationship between these vesicles, their cargoes, and the assembly of presynaptic subdomains such as the active zone is unclear. In this study, we have followed the changing spatial distribution of Piccolo during neuronal differentiation and have found that it is transported to nerve terminals together with other components of the mature active zone in

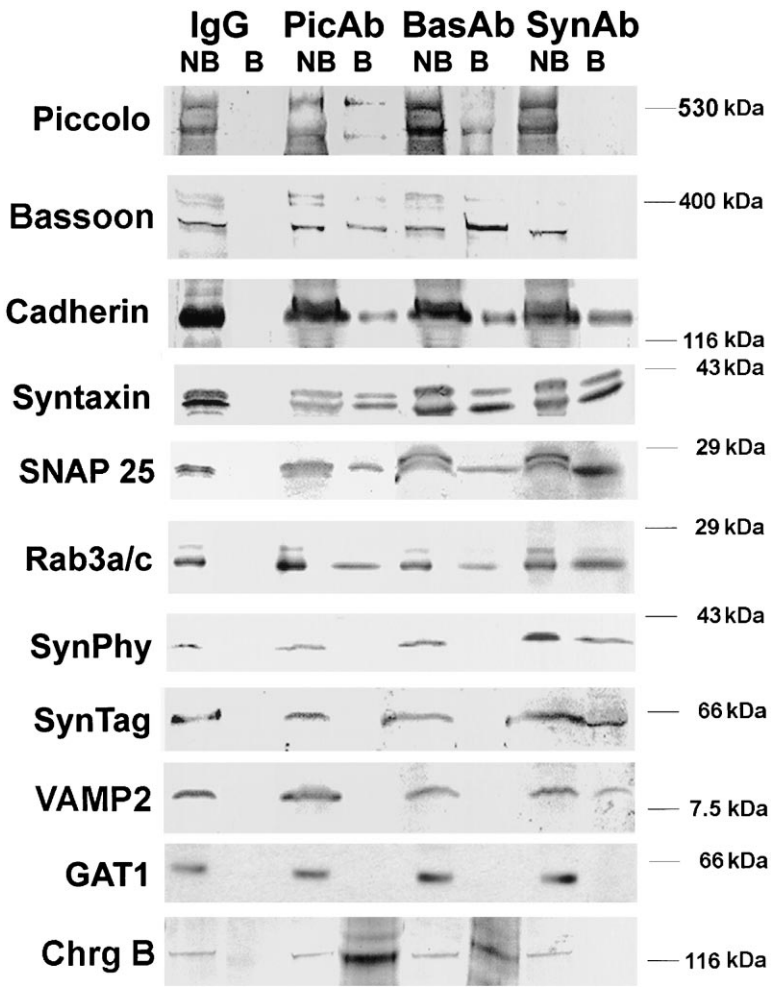

Figure 7. Piccolo Transport Vesicle Contains Components of Presynaptic Active Zone but Not Those of SVs or Perisynaptic Plasma Membrane

(A and B) 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to Western blotting to detect the presence of Piccolo, Bassoon, N-Cadherin, syntaxin, SNAP 25, Rab3a/c, synaptophysin (SynPhy), synaptotagmin (SynTag), VAMP2/synaptobrevin2 (VAMP2), GABA transport 1 (GAT1), and chromogranin B (Chrg B).

association with 80 nm vesicles that are morphologically distinct from SVs. This conclusion is supported by biochemical, immunofluorescent, and EM studies. The proteins found thus far to be associated with the PTV include the synaptic cell–cell adhesion molecule N-cadherin, the synaptic t-SNARE proteins syntaxin, and SNAP-25, as well as the presynaptic CAZ protein Bassoon. Components of the perisynaptic plasma membrane and SVs were not present on the PTV. Taken together, these data indicate that at least some components of active zones are transported together on a common vesicle. Furthermore, these data suggest that cytoskeletal components of the active zone are not necessarily recruited to nascent synapses from cytosolic pools but from preformed vesicular protein complexes. Assessing whether other components of active zones are also on the PTV will be necessary to test this hypothesis further.

Surprisingly, EM analysis of the immunoisolated PTV from immature rat brain revealed that in contrast to the 50 nm clear SVs, the 80 nm PTV had an electron-dense core reminiscent of classic neuropeptide containing large (120 nm) dense core vesicles (LDCVs) (Huttner et al., 1995). Several lines of evidence support the conclusion that Piccolo is associated with a dense core vesicle. First, it was possible to use antibodies against Piccolo or Bassoon to selectively and reproducibly immunoisolate 80 nm granulated vesicles. Second, in the axons of cultured hippocampal neurons, granulated vesicle could be specifically immunogold labeled with antibodies against Piccolo or Bassoon. Third, chromogranin B, a marker for classic dense core vesicles, was immunoisolated with Piccolo and Bassoon antibodies and colocalized with Piccolo by double-label immunofluorescent microscopy. These data clearly indicate that PTV is a dense core vesicle. Traditionally, dense core vesicles are thought to be electron dense because the lumen of these vesicles contains proteins. Other than chromogranin B, the identities of the proteins that make up the core of the PTV are currently unknown. Several interesting possibilities include neuropeptides, components of extracellular matrix (ECM), and/or trophic factors. Based on studies of agrin, neuregulin, and laminin-β1 at the neuron muscular junction (NMJ) (Nastuk and Fallon, 1993; Sanes and Scheller, 1997), such proteins deposited into the cleft of newly forming CNS synapses may play fundamental roles in the differentiation of the postsynaptic and perhaps also the presynaptic sides of synaptic junctions. With regard to agrin, neuregulin, and laminin-β1, which are all found in the CNS (Sanes and Scheller, 1997; Gautam et al., 1999), it is unclear whether any of them play a direct role in the differentiation of

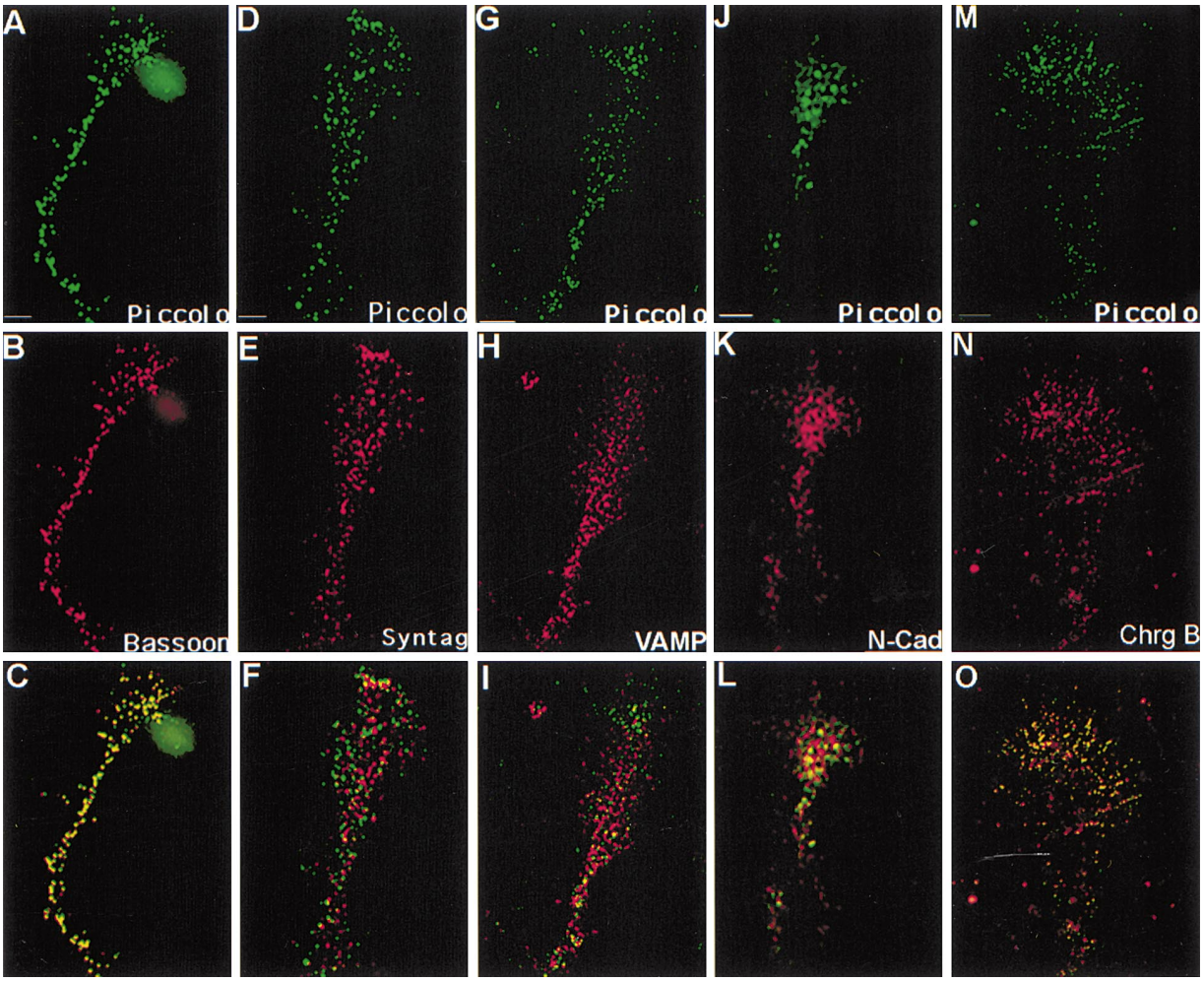

Figure 8. In Axonal Growth Cones, Piccolo Colocalizes with the Components of Active Zone but Not SVs

(A–O) Neurons cultured for 4 days in vitro were double labeled for Piccolo (A, D, G, J, and M) in green and Bassoon (B), synaptotagmin (E), VAMP2/synaptobrevin2 (H), N-cadherin (K), or chromogranin B (N) 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), but almost no colocalization can be observed between Piccolo and synaptotagmin (F) or between Piccolo and VAMP2 (I). Piccolo partially colocalized with N-cadherin (L) and with chromogranin B (O). Scale bars, 10 μm in all panels.

CNS glutamatergic or GABAergic synapses. It is also not clear in motor neurons, where they are potent inducers of postsynaptic differentiation of the NMJ, how they are transported to nascent synapses. Nonetheless, our ability to biochemically purify the PTV and analyze its contents should begin to provide clues to the identities and function of these important inducers of CNS synapse differentiation.

An interesting issue raised by our studies concerns the biogenesis of the PTV. Recent studies by Ahmari et al have observed the presence of vesicular tubules as well as dense core granulated vesicles at nascent synapses (Ahmari et al., 2000). 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). Currently, it seems unlikely that the PTV buds from these vesicular tubules, but rather derives from Golgi. This conclusion is supported both by the punctate perinuclear appearance of Piccolo and Bassoon in immature neurons as well as 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 SVs as well as 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 SV proteins VAMP2/synaptobrevin II, synapsin, 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 GFP-VAMP 2/synaptobrevin II to tag the cluster of vesicles associated with nascent synapses as observed by Ahmari et al, it should be noted that we did not find VAMP2/synaptobrevin II 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.

The Role of the PTV in the Assembly of Presynaptic Active Zones

Other than timing (Vardinon-Friedman et al., 2000), our understanding of the cellular mechanisms that orchestrate the assembly of synaptic junctions is very crude at best. Our unexpected finding that components of the presynaptic active zone are transported to nerve terminals in association with 80 nm granulated vesicles provides exciting new clues to the cellular mechanisms that are likely to underlie the synapse assembly. In particular, our studies suggest that 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. For example, the presence of cell adhesion molecules such as N-cadherin 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). Moreover, the insertion of CAZ proteins such as Piccolo and Bassoon as well as components of the SV exocytotic machinery such as syntaxin and SNAP-25 at the nascent active zone site would facilitate the rapid acquisition of activity-induced SV recycling as well as confine their fusion to this site. Finally, 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. Assuming this core is composed of neuropeptides, neurotrophic factors, and ECM proteins, we would predict that the fusion of the PTV at nascent synapses would play a direct role in the differentiation of the PSD, such as promoting the synapse specific recruitment of subclasses of neurotransmitter receptors and their associated PSD proteins.

A compelling argument that PTV-like granulated vesicles play a direct role in the formation of active zone comes from studies on the developing mouse spinal cord. Here, 80 nm granulated vesicles were observed in direct contact with the presynaptic plasma membrane of nascent synapses (Vaughn, 1989). Intriguingly, the cytoplasmic surface of these spinal cord granulated vesicles were coated with tuffs of electron-dense material, referred to as spicules (Vaughn, 1989). These spicules resemble the cytoskeletal matrix present at the active zones of both nascent and mature synapses (Landis et al., 1988; Hirokawa et al., 1989; Vaughn, 1989). Taken together, these data strongly support our conclusion that these dense core granulated vesicles associated with the formation of active zones carry structural components of active zones.

PTVs May Represent Functional Units of Active Zones

Assuming that each PTV has all or most of the components needed to establish a SV release site, in the correct stoichiometry, it is interesting to speculate how many SV fusion sites that a single PTV may contain. Quantitative analysis of hippocampal excitatory synapses has revealed that the area of a single SV docking site at active zones is 3844 nm^2 (62 $\times$ 62 nm) in brain and 5929 nm^2 (77 $\times$ 77 nm) in hippocampal culture (Schikorski and Stevens, 1997). The surface area of an 80 nm dense core granulated vesicle is approximately 20,160 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). With 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 SV 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.

In conclusion, our studies 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 SVs acquire the capacity to recycle at new axodendritic sites within less than one hour of axodendritic contact (Ahmari et al., 2000, Vardinon-Friedman et al., 2000).

Experimental Procedures

Materials

The rabbit polyclonal and mouse monoclonal Bassoon antibodies against a 75 kDa GST-Bassoon fusion protein from clone sap7f were raised and purified as described previously (tom Dieck et al., 1998). The other antibodies used were as follows: rabbit anti-MAP2b antibody (Kindler et al., 1990), mouse monoclonal anti-synaptotagmin I antibody (Cl 41.1; gift from R. Jahn, Max Planck Institute, Goettingen, Germany), mouse monoclonal anti-synaptophysin antibody and mouse monoclonal anti-SNAP25 antibody (Roche Diagnostics GmbH, Mannheim, Germany), rabbit polyclonal anti-syntaxin antibody (gift from M. Quick, University of Alabama), and mouse monoclonal anti-Rab3a antibody (Transduction Laboratories, Lexington, KY).

Hippocampal Cultures and Immunofluorescent Microscopy

Primary cultures of hippocampal neurons were prepared from Sprague-Dawley rat embryos at E18 as described previously (Zhai et al., 2000). Immunofluorescent staining was performed as described previously (Zhai et al., 2000). 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.

Flotation and Immunoisolation

For flotation assay, E18 brains were dissected 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 nine volumes of H_2O. The crude membrane was then centrifuged at 100,000 g for 1 hr. The pellet is P100, and the supernatant is S100. P100 or S100 fraction is then adjusted to 2 M sucrose and loaded as a layer of a discontinuous sucrose gradient underneath layers of 1.2, 0.8, and 0.3 M sucrose. The sucrose gradient is centrifuged at 350,000 g for 3 hr. Fractions are taken from the top of the gradient to the bottom. The immunoisolations were done as described by Henley and McNiven (1989). Briefly, tosylated superparamagnetic beads (Dynabeads M-500 Subcellular; Dynal Inc., NY) 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 phosphate-

buffered saline (PBS)/0.1% bovine serum albumin (BSA) and were 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% fetal bovine serum). 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.

Electron Microscopy

Beads bound fractions were fixed either 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 days in vitro were fixed by 0.1% glutaraldehyde, 4% paraformaldehyde, and 1% tannic acid in PBS and processed for EM. Immuno-EM on cultured neurons was done as described by Grosse et al. (2000). Briefly, after fixation, neurons were rinsed with 10% methanol, 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 were 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, PA). After washing, neurons were postfixed with 2% glutaraldehyde 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 the 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 categorized based on their location. If the gold particle is within 20 nm of an organelle, the gold particle is 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.

Functional Labeling of Presynaptic Boutons and Retrospective Immunohistochemistry

Functional labeling of presynaptic boutons, multisite, multisection time-lapse fluorescent/differential interference contrast confocal microscopy, retrospective immunohistochemistry, and data analysis were performed as described previously (Vardinon-Friedman et al., 2000). Briefly, hippocampal cell cultures, prepared from 1–3 day old Sprague-Dawley rats, were placed in a heated perfusion chamber and maintained in enriched minimum essential media. Functional presynaptic boutons in the field were visualized by selectively loading them with FM 4-64 (N-(3-triethylammoniumpropyl)- 4-(p-dibutylaminostyryl)pyridinium, dibromide (15 μM; Molecular Probes, Eugene, OR) using field stimulation (30 s at 10 Hz) followed by 30 additional seconds in the dye, a 1 min wash with ADVASEP 7 (β-Cyclodextrin Sulfbutyl Ether, 1 mM; Cydex, KS; Kay et al., 1999), and a 9 min wash with dye-free-enriched MEM. Dye unloading was performed by stimulating the neurons for 120 s at 10 Hz. At the end of the experiments, the neurons were fixed with cold (−20°C) methanol for 10 min and were processed as described previously. The neurons were labeled with the rabbit polyclonal anti-Piccolo antibody described previously in this article followed by secondary labeling with Alexa 488 goat anti-rabbit (Molecular Probes).

All data analysis was performed as previously described. Briefly, maximal intensity projection images and digital movies were prepared from all image stacks, and these were used to detect new FM 4-64-labeled puncta. Such puncta were then examined carefully in the original image sets, and any bouton for which punctate staining, however faint, was observed in the first image of the time-lapse series was rejected. A newly appearing bouton was scored as such only if it complied with three additional conditions: (1) It had to be at least 2 μm away from a preexisting bouton. (2) It had to persist until the end of the time-lapse session, and (3) it had to release the dye in response to a train of action potentials. Images of the fixed and immunolabeled tissue were then aligned and examined as previously described to determine whether a discreet cluster of piccolo was observed at the same sites. Because of the strict limitations placed on scoring new boutons, we were left with rather small numbers of new boutons per site. It was thus meaningless to calculate the mean and standard deviation for each site or experiment, and all results for all experiments were aggregated.

Acknowledgments

We are grateful to Edward Phillips for his invaluable technical assistance and to Dr. Stefan Kindler for his support and many stimulating discussions. This work was supported by grants from the National Institutes of Health (RO1 NS39471, PO1 AG06569) to C. C. G., the Deutsche Forschungsgemeinschaft (SFB 426/A1) and the Fonds der Chemischen Industrie to E. D. G., and the Israel Science Foundation (139/98) to N. E. Z.

Received May 30, 2000; revised December 1, 2000.

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