Published in final edited form as:

J Mol Struct. 2014 September 5; 1073: 187–195. doi:10.1016/j.molstruc.2014.04.055.

Neuronal *Porosome*-The Secretory Portal at the Nerve Terminal: It's Structure-Function, Composition, and Reconstitution

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Abstract

Cup-shaped secretory portals at the cell plasma membrane called *porosomes* mediate secretion from cells. Membrane bound secretory vesicles transiently dock and fuse at the cytosolic compartment of the porosome base to expel intravesicular contents to the outside during cell secretion. In the past decade, the structure, isolation, composition, and functional reconstitution of the neuronal porosome complex has been accomplished providing a molecular understanding of its structure-function. Neuronal porosomes are 15 nm cup-shaped lipoprotein structures composed of nearly 40 proteins. Being a membrane-associated supramolecular complex has precluded determination of the atomic structure of the porosome. However recent studies using small-angle X-ray solution scattering (SAXS), provide at sub-nanometer resolution, the native 3D structure of the neuronal porosome complex associated with docked synaptic vesicle at the nerve terminal. Additionally, results from the SAXS study and earlier studies using atomic force microscopy, provide the possible molecular mechanism involved in porosome-mediated neurotransmitter release at the nerve terminal.

Keywords

Neurotransmitter Release; Neuronal Porosome Complex; Atomic Force Microscopy; Electron Microscopy; X-ray solution scattering; Electrophysiology; Mass Spectrometry

INTRODUCTION

Secretion is a fundamental cellular process that occurs in every organism, from the yeast to humans. For example, secretion of neurotransmitters at the nerve terminal enable neurotransmission, allowing thought, movement, and coordination. Similarly after a meal, secretion of digestive enzymes from the exocrine pancreas help digest food. The consequent elevation of blood glucose following digestion, triggers secretion of insulin from β -cells of the endocrine pancreas. Similarly, exposure to certain types of pollen, or to a parasite, elicits

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an allergic inflammatory immune response, stimulating mast cells to secrete histamine and other compounds. Secretory products such as neurotransmitters, hormones, or digestive enzymes, are packaged and stored within membranous sacs or vesicles following synthesis, and are released from the cell during secretion.

Until recently, it was believed that during cell secretion, membrane-bound secretory vesicles completely collapse at the cell plasma membrane, resulting in the diffusion of intra-vesicular contents to the cell exterior and the compensatory retrieval of the excess membrane by endocytosis. Complete vesicle merger however fails to explain the generation of partially empty vesicles observed in electron micrographs in cells following secretion, suggesting the involvement of an additional mechanism that would enable the release of a portion of the vesicle content. The partial emptying of vesicles during secretion is possible if vesicles were to temporarily establish continuity with the cell plasma membrane, expell a portion of their contents, then detach, reseal, and withdraw into the cytosol (endocytose). Utilizing this mechanism, secretory vesicle could be reused for subsequent rounds of exo-endocytosis, until completely empty of contents. Synaptic vesicles have the advantage of rapidly refilling, utilizing neurotransmitter transporters present at the synaptic vesicle membrane. Hence, in 1973, 'transient' or 'kiss-and-run' mechanism of secretory vesicle fusion at the cell plasma membrane enabling fractional discharge of intravesicular contents was proposed (Ceccarelli, 1973). In 1990 it was hypothesized that the fusion pore, a continuity established between the vesicle membrane and the cell plasma membrane, results from a "preassembled ion channel-like structure that could open and close" (Almers and Tse, 1990). A 1992 review (Monck and Fernandez, 1992) opined that the principal difficulty in observing these structures and fusion pore formation at these structures, was the lack of adequate imaging tools to directly observe their presence and study their activity in live cells. Immediately thereafter, this goal was realized (Schneider et al, 1997) employing atomic force microscopy (AFM) (Alexander et al, 1989), and subsequently confirmed using electron microscopy (EM) (Jeremic et al, 2003; Cho et al, 2004) and small angle X-Ray solution scattering (SAXS) analysis (Kovari et al, 2014).

In the mid 1990's, employing the then newly developed technique of AFM, nanometer scale pore structures and their dynamics were discovered at the apical plasma membrane in live pancreatic acinar cells. Circular pit-like structures containing 100–180 nm cup-shaped depressions or pores were observed at the apical plasma membrane of pancreatic acinar cells where secretion is known to occur (Schneider et al, 1997). During secretion, the depression or pore opening grew larger, returning to its resting size following completion of cell secretion. Studies next established the observed depressions to be the secretory portals at the plasma membrane in cells (Cho et al, 2002a; Jena et al, 2003). Following stimulation of cell secretion, gold-conjugated amylase antibodies (amylase being one of the major intra-vesicular enzymes secreted by pancreatic acinar cells) accumulate at depressions. These results established depressions to be the long sought-after secretory portals in cells. The study further reported (Jena et al, 2003) the presence of t-SNAREs at the porosome base facing the cytosol, firmly establishing depression structures to be secretory portals where vesicles transiently dock and fuse for intra-vesicular content release during secretion (Jena et al, 2003). Subsequently depressions and their dynamics at the cell plasma membrane in chromaffin cells (Cho et al, 2002b), and in growth hormone (GH) secreting cells of the

pituitary gland (Cho et al, 2002c), was reported. In 2003, following immunoisolation of the depression structures from acinar cells of the exocrine pancreas, their composition was determined, and they were both structurally and functionally reconstituted into artificial lipid membranes (Jeremic et al, 2003). Morphological details of depressions associated with docked secretory vesicles were revealed using high-resolution EM (Jeremic et al, 2003). In the past decade, employing a combination of approaches such as AFM, biochemistry, electrophysiology, conventional EM, mass spectrometry, and small angle X-Ray solution scattering (SAXS) analysis, this specialized portal has been found to be present in all secretory cells examined, including neurons (Cho et al, 2004; Jena 2012b; Kovari et al, 2014; Lee et al, 2012). Consequently, these structures were named 'porosomes' (Jena 2007,2009,2012a,2012b) or secretory portals in cells. Our studies and studies from other laboratories (Craciun and Barbu-Toduran, 2013; Elshennay, 2011; Hammel and Meilijson, 2012; Japaridze et al, 2012; Matsuno et al, 2008; Okuneva et al, 2012; Paredes-Santos et al, 2012; Savigny et al, 2007; Siksou et al, 2007) established porosomes to be secretory portals that perform the specialized task of fractional discharge of intravesicular contents from cells during cell secretion. The significance of the porosome discovery is reflected by several publications involving its structure (Craciun and Barbu-Toduran, 2013; Elshennay, 2011; Hammel and Meilijson, 2012; Japaridze et al, 2012; Matsuno et al, 2008; Okuneva et al, 2012; Paredes-Santos et al, 2012; Savrgny et al, 2007; Siksou et al, 2007), and the associated transient fusion mechanism (Aravanis et al, 2003; Taraska et al, 2003; Thorn et al, 2004) accompanied by fractional discharge of intravesicular contents from cells. Studies in endocrine cells report secretory granules to be recaptured largely intact following stimulated exocytosis (Taraska et al, 2003); in neurons, single synaptic vesicles fuse transiently and successively without loss of vesicle identity (Aravanis et al, 2003); and in the exocrine pancreas, secretion is characterized by long fusion pore openings and preservation of secretory vesicle lipid identity (Thorn et al, 2004). The past two decades have witnessed great progress in our understanding of Ca^{+2} and SNARE-mediated membrane fusion (Trimble et al, 1988; Oyler et al, 1989; Bennett et al, 1992; Weber et al, 1998; Cho et al, 2002d, 2005a; Jeremic et al, 2004a,b, 2006; Cook et al, 2008; Shin et al, 2010a; Issa et al, 2010) and on secretory vesicle volume regulation required for regulated fractional expulsion of intravesicular contents from cells (Jena et al, 1997; Cho et al, 2002e; Kelly et al, 2004; Jeremic et al, 2005; Shin et al, 2010b; Lee et al, 2010; Chen et al, 2011). These findings have greatly contributed to the progress in our understanding of porosome-mediated secretion, resulting in a paradigm shift in our understanding of the secretory process in cells. In the current review, the structure, composition, and reconstitution of the neuronal porosome complex, and the molecular mechanism of its involvement in neurotransmitter release is discussed.

Structure-Function of the Neuronal Porosome Complex

Examination of the presynaptic membrane at the nerve terminal using high resolution AFM (Cho et al, 2004), EM (Cho et al, 2004), and SAXS (Kovari et al, 2014), demonstrate the presence of approximately 15 nm cup-shaped porosomes, each possessing a central plug [Figure 1,2]. The lip of the porosome opening to the outside is lined by eight equally spaced protein densities [Figure 1D,E]. The eight protein densities are observed both in the native

neuronal porosome complex [Figure D top left] as well as in isolated porosomes reconstituted in lipid bilayers prepared using brain phosphatidylethanolamine (PE), phosphatidylcholine (PC), dioleoylphosphatidylcholine (DOPC), and dioleoylphosphatidylserine (DOPS) [Figure D top right]. Similar to AFM micrographs, approximately 8 interconnected protein densities are observed at the lip of the porosome complex in EM micrographs of purified neuronal porosome preparations [Figure 1E]. Electron density and contour maps and resultant 3D topology profiles of the porosome complex, provide further details of the arrangement of proteins, and their connection to the central plug region of the complex via distinct spoke-like elements [Figure 1E lower left]. The 3D topography of the porosome complex [Figure 1E lower right] obtained from electron density maps, further demonstrate the porosome to exhibit a circular profile with a central plug connected via spokes. AFM micrographs of inside-out presynaptic membrane, demonstrate inverted cup-shaped porosomes, some with synaptic vesicles docked to their base [Figure 1F,G]. AFM, EM, and photon correlation spectroscopy [Figure 1H,I] demonstrate porosomes to range in size from 12–17 nm. Interestingly, high-resolution AFM micrographs of the neuronal porosome complex present at the presynaptic membrane demonstrates the central plug at various conformation states: pulled out, intermediate, and fully withdrawn into the porosome complex [Figure 3], suggesting its capability for vertical motion, and its possible involvement in the rapid opening and closing of the porosome complex [Figure 4]. The detailed structure of the porosome complex in association with synaptic vesicle obtained from SAXS studies (Kovari et al, 2014), provides further structural information to speculate on the possible involvement of the central plug in neurotransmitter release at the nerve terminal. First, it appears from the SAXS structure that the SV needs to dock extremely tightly with the cup-shaped porosome complex, that would result in the SV forming a cap-like or mushroom-head (Figure 3,5). Second, following stimulation of neurotransmission, the arm of the central plug that appears to be connected to the lip of the porosome opening as a cantilever (Figure 4C2, 4C3), could be pushed inward, resulting in a small bump that would be close in size to the diameter of the foot of the central plug which from the SAXS image (Figure 4,5) appears to be approximately 3–4 nm. Consequently, the membrane at the porosome base constituting this 3–4 nm in diameter bump must be under enormous tension and simultaneously have established tight apposition and contact with the outer leaflet of the SV membrane via SNARE proteins. The establishment of t-/v-SNARE complex in a ring or rosette pattern (Cho et al., 2002b; Cho et al., 2005a; Jeremic et al., 2006; Cho et al., 2009; Cho et al., 2011) at this contact site in the presence of Ca^{2+} ions (Jeremic et al., 2004a; Mohrmann et al., 2010; Jeremic et al., 2004b; Cho et al., 2005b; Potoff et al., 2008) would result in the fusion of the opposing lipid membranes. The resultant fusion pore would establish continuity between the SV lumen and the porosome for the release of neurotransmitters at the nerve terminal. Subsequently, the movement of the porosome plug toward the porosome opening to the outside would release tension off the membrane at the porosome base, resulting in a rapid closure or resealing of the fusion pore that had been transiently established [Figure 4]. Since actin, myosin, and several structural proteins constitute the neuronal porosome proteome (Lee et al., 2012), the motile central plug element is likely composed of these motor proteins requiring ATP for motion.

Composition of the neuronal porosome complex

Determination of the composition of the neuronal porosome proteome using immunoisolation and gel filtration chromatography, followed by tandem mass spectrometry and immuno analysis, demonstrate nearly 40 proteins to constitute the complex. Furthermore, interaction between proteins within the porosome complex and their resulting arrangements, have been predicted using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database search (Szklarczyk et al, 2011). The association and dissociation of proteins at the porosome following stimulation of cell secretion demonstrates both its stable presence and reflects on the dynamic nature of the organelle.

Immunisolated porosomes from solubilized rat brain synaptosome resolved using 12.5% SDS-PAGE, followed by electrotransfer of the resolved proteins onto nitrocellulose membrane and immunoblot analysis using various antibodies, have demonstrated the presence of 9 proteins, namely SNAP-25, the P/Q-type calcium channel, actin, syntaxin-1, synaptotagmin-1, vimentin, the *N*-ethylmaleimide-sensitive factor (NSF) (Jena et al, 2003; Jeremic et al, 2003), the chloride channel CLC-3, and the alpha subunit of the heterotrimeric GTP-binding $G_{\alpha o}$ (Cho et al, 2004). Some of the identified proteins have been previously implicated (Jena et al, 2003; Jeremic et al, 2003; Faigle et al, 2000; Nakano et al, 2001; Ohyama et al, 2001; Prekereis and Terrian, 1997). Immunisolated porosomes from solubilized rat brain synaptosome resolved using 12.5% SDS-PAGE, followed by fluorescent SYPRO Ruby staining demonstrate 15 bands ranging in molecular weights from >205 kDa to >6.5 kDa, yielded an additional 9 proteins to be associated with the neuronal porosome complex using mass spectrometry [Table 1] (Lee et al, 2012).

Tubulin, myosin 7b, spectrin beta chain, creatine kinase, dystrophin, langerin, GTPase activating protein (GAP), intersectin1, and myosin heavy chain 1, were identified [Table 1]. Since mass spectrometry demonstrated the association of intersectin1 with the porosome complex, and since intersectin1 is known to interact with dynamin (Evergren et al, 2007), the association of dynamin with the porosome was hypothesized and has been confirmed [Figure 5] (Lee et al, 2012). Immunoblot analysis of the SNAP-25 immunoisolate from solubilized rat brain homogenate, demonstrate the presence of dynamin and its increased association with the porosome complex following stimulation of neurotransmitter release [Figures 5, 6] (Lee et al, 2012). An interesting aspect of the neuronal porosome complex that emerges from these studies is the dynamic nature of its composition during the neurotransmitter release process. Immunoblot analysis of the SNAP-25 immunoisolate from solubilized rat brain homogenate obtained from control and KCl-stimulated brain slices demonstrate the presence of dynamin and its increased association with the neuronal porosome complex following stimulation of neurotransmitter release [Figure 5] (Lee et al, 2012). In contrast, a dissociation of $G_{\alpha o}$ immunoreactivity is demonstrated following neuronal stimulation in the study, since the same blot used for determining the presence of dynamin was used to probe for $G_{\alpha o}$ immunoreactivity [Figure 5] (Lee et al, 2012).

When rat brain porosomes purified using gel filtration chromatography [Figure 7] were analyzed by LC-MS/MS on both LTQ and QSTAR XL, several new proteins constituting the neuronal porosome complex were identified, and the ones that were detected in both

experiments are listed in Table 2 (Lee et al, 2012). Interestingly, plasma membrane calcium-transporting ATPase 1 and 2 are both found to be present in the neuronal porosome complex, suggesting that they may be involved in ATP-mediated expulsion of the extracellular calcium that had entered the cell during stimulation of cell secretion. Similarly, the presence of sodium/potassium-transporting ATPase subunit alpha-3 with the porosome suggests the role of porosome in ATP coupled exchange of sodium and potassium across the plasma membrane (in opposite directions), creating the required electrochemical gradient required for maintaining the membrane resting potential as well as for the regulation of cell volume during secretion. Additionally, 2',3'-cyclic-nucleotide 3'-phosphodiesterase which has been reported to associate with microtubules, and have microtubule-associated protein-like activity, are present in the neuronal porosome. At the porosome, the 2',3'-cyclic-nucleotide 3'-phosphodiesterase can also link tubulin to the cell membrane and participate in regulating the distribution of microtubules in the cytoplasm (Bifulco et al, 2002). Hence, the presence of 2',3'-cyclic-nucleotide 3'-phosphodiesterase at the porosome would therefore be critical to the structural integrity of the porosome complex. Dihydropyrimidinase-related protein present in the porosome proteome is known to play a role in cytoskeletal remodeling and cell polarity (Goshima et al, 1995). Exactly, how this protein would be involved in neurotransmitter release at the porosome, remains to be established. Similarly, NSF and dynamin at the porosome complex would be required for t-/v-SNARE complex disassembly by NSF, and fission of the established continuity between the lipid vesicle membrane and the porosome base by dynamin, following the transient fusion of synaptic vesicles at the porosome [Figure 6].

Since SNAREs are present at the porosome base (Jeremic et al, 2003; Cho et al, 2009), and calcium channels have been demonstrated to physically interact with SNAREs at the porosome (Cho et al, 2005b), suggests the presence of both proteins at the porosome base. Similarly, NSF and dynamin would also be present at the porosome base, since they would both be required for the dissociation of the SV from the porosome base following secretion [Figure 7]. Similarly, as previously discussed, the centrally located plug in the neuronal porosome complex regulating the opening and closing of the organelle (Cho et al, 2010) may involve cytoskeletal as well as motor proteins.

To further understand the molecular architecture and interaction of proteins within the neuronal porosome complex, known interactions between proteins identified in the porosome were examined using the STRING 9.0 database search [Figure 8] (Szklarczyk et al, 2011). STRING 9.0 is a database of known and predicted protein interactions. The interactions include direct (physical) and indirect (functional) associations; derived from four sources: namely, genomic context, high-throughput experiments, conserved co-expression, and from previous knowledge. STRING quantitatively integrates interaction data from these sources for a large number of organisms, and transfers information between these organisms where applicable. The database currently covers 5,214,234 proteins from 1133 organisms. Using the STRING 9.0 database search (Szklarczyk et al, 2011), the STRING maps generated, clearly identifies two clusters of protein-protein interactions in the porosome proteome [Figure 8] (Lee et al, 2012). The protein-protein interaction cluster to the left, represent primarily cytoskeletal and signaling proteins, where as the cluster to the right in figure 8 is representative of proteins that are primarily involved in membrane fusion.

Not surprisingly, this second cluster include both SNAREs, their associated regulatory proteins, as well as calcium channels, suggesting their location to be at the base of the porosome cup, facing the cytosol [Figure 9] (Lee et al, 2012). Heterotrimeric GTP-binding protein and the GTP-binding membrane fission protein dynamin (Dnm2), are present in the left cluster. The presence of dynamin in the left cluster is natural, since it is a microtubule-associated protein. The involvement of Dnm2 in fission of the neck of fused vesicles at the porosome base however, would require their presence at the porosome base. The functional interaction values in the STRING diagram predicted in Figure 8, represents >99% confidence, however future electron crystallography and molecular simulations studies will further provide the precise molecular arrangement of proteins within the neuronal porosome complex.

Besides proteins, the composition of lipids constituting the neuronal porosome complex is beginning to be explored. Earlier studies demonstrate the critical role of cholesterol on the integrity and function of the neuronal porosome complex, and its influence on the assembly of neuronal t-/v-SNARE complex has been determined (Cho et al, 2007). Results from the study (Cho et al, 2007) demonstrate a significant inhibition in interactions between porosome-associated t-SNAREs and calcium channels following depletion of membrane cholesterol. Since calcium is critical to SNARE-induced membrane fusion (Cho et al, 2002d; Jeremic et al, 2004a,b), the loss of interaction between SNAP-25, Syntaxin-1, and calcium channel observed in the absence of cholesterol, would seriously compromise or even abrogate neurotransmission at the nerve terminal.

Reconstitution of the neuronal porosome complex

To assess the functionality of the isolated porosome preparations, an electrophysiological bilayer setup (EPC9) has been utilized [Figure 10], where membrane capacitance and conductance is continually monitored. Reconstitution of the bilayer membrane with isolated neuronal porosomes, followed by exposure to isolated SV by adding SV to the cis compartment of the bilayer chamber, results in transient SV docking and fusion at the bilayer membrane. A large number of SVs are found to fuse at the bilayer, which is demonstrated by the large stepwise increases in membrane capacitance [Figure 10d]. As expected, addition of 50 μ M ATP enables t-/v-SNARE disassembly and the release of docked SVs, resulting in the return of the bilayers membrane capacitance to resting levels. Addition of recombinant NSF has no further effect on membrane capacitance, since NSF is present in the isolated neuronal porosome complex. Thus, the associated NSF at the t-/v-SNARE complex is adequate for complete disassembly of the SNARE complex for release of SVs following transient fusion and the completion of a round of neurotransmitter release. To assess biochemically the release of docked SVs following exposure to ATP, synaptosomal membrane preparations are exposed to 50 μ M ATP and the supernatant fraction assessed for the presence of SVs by monitoring levels of the synaptic vesicle proteins SV2 and VAMP-2 [Figure 10e]. Results from these experiments demonstrate that both SV2 and VAMP-2 proteins are enriched in supernatant fractions following exposure of isolated synaptosomal membrane to ATP, further confirming the results presented in Figure 10d (Cho et al, 2004). Collectively, these functional reconstitutions, and the structural

reconstitution studies presented in Figure 1, demonstrate the complete and intact isolation of the neuronal porosome complex.

Summary

Results from studies in the past decade demonstrate that neuronal porosomes are 15 nm cup-shaped lipoprotein structures composed of nearly 40 proteins. Being a membrane-associated supramolecular complex has precluded determination of its atomic structure. Recent studies however, using small-angle X-ray solution scattering (SAXS) provide at nanometer resolution, the native 3D structure of the neuronal porosome complex with docked synaptic vesicle. Furthermore, results from AFM and SAXS studies provide the possible molecular mechanism involved in porosome-mediated neurotransmitter release at the nerve terminal. The neuronal porosome, although an order of magnitude smaller than those in the exocrine pancreas and in neuroendocrine cells, possess many similarities both in structure and composition. Though nature has designed the porosome as a universal secretory portal, it has fine-tuned the complex in each cell type to suite its various secretory needs, and hence porosome size may reflect such fine-tuning. For example, it is well known that smaller vesicles fuse more efficiently and effectively than larger ones (Ohki, 1984; Wilschut et al, 1981), hence the curvature of both the secretory vesicle and the porosome base would dictate the efficacy and potency of vesicle fusion at the cell plasma membrane. Neurons being fast secretory cells therefore possess small 30–50 nm in diameter secretory vesicles or synaptic vesicles and a porosome base measuring approximately 2–4 nm in diameter, for rapid and efficient fusion. In contrast a slow secretory cell like the exocrine pancreas, possess larger secretory vesicles measuring >1000 nm in diameter, that transiently fuse at porosomes that have bases measuring nearly 20 nm in diameter. Therefore within the small volume that the fusion reaction is known to take place, it is suggested (Hammel and Meilijson, 2012, 2013) that classical biophysics may play a minor role and that the approach of statistical mechanics should be considered.

To better understand the function of the porosome at the molecular level requires an understanding of the distribution of the identified proteins within the complex. Unlike individual proteins or lipids, determination of the atomic structure of dynamic macromolecular lipoprotein complexes such as the neuronal porosome, poses a difficult challenge, requiring the use of several experimental and computational approaches to maximize resolution and accuracy. Therefore, EM, SAXS, and AFM analyses will be complemented by techniques from structural mass spectrometry and proteomics to obtain molecular details of porosome structure including subunit stoichiometry, interacting subunits, and site of contact between subunits. Changes to porosome subunit composition and subunit interactions during the secretion process, also need to be addressed to gain an understanding of porosome structure-function. Therefore experiments will involve biochemical and molecular approaches including CXL-MS and multiple quantitative mass spectrometry techniques. Determination of the protein-protein interactions within the native porosome is central to building a structural model. Since isolated porosomes are functional, demonstrate good stability and tight association of the core constituent proteins within the complex, isolated porosomes will be utilized to determine the interaction and distribution of proteins within the structure, thereby limiting the number of false positives. New cross-

linkers combined with tandem mass spectrometry will provide identities of interacting subunits and provide the identities of specific residues cross-linked both between and within subunits. The latter will provide information on interaction domains and distance constraints on protein structures. Similarly quantitative mass spectrometry or iTRAQ, will provide information on changes in porosome subunits as a function of secretion status and absolute quantification of proteins or AQUA (Clifford-Nunn et al, 2012) will be used for absolute quantification of key subunits in order to determine the number of each subunit present in the complex. Quantitative CXL-MS analysis using heavy isotope cross-linkers will identify changes in molecular interactions in resting and stimulated porosomes and iTRAQ analysis will be used to identify changes in subunit composition. Immuno-AFM, immuno-EM, single particle cryo-EM tomography (Zang and Ren 2012), and SAXS studies on isolated neuronal porosomes will help determine the distribution of some of the major proteins within the complex. Computational approaches employing coarse-grain molecular docking studies (Gray et al, 2003a; Smith and Sternberg, 2002; Janin et al, 2003; Schneidman-Duhovny et al, 2003; Katchalski-Katzir et al, 1992; Gabb et al, 1997; Moont and Sternberg 2002; Jackson et al, 1998; Vakser 1995; Mandell et al, 2001; Chen and Weng 2003; Ritchie and Kemp 2000; Fernandez-Recio et al, 2002; Grey et al, 2003b; Gabdouline and Wade 2001; Fitzjohn and Bates 2003), homology modeled interactions (Aloy et al, 2004; Pieper et al, 2004; Marti-Renom et al, 2000), and fitting of known atomic structures of protein-protein interactions and complexes (Volkmann et al, 2000; Roseman 2000; Wriggers and Birmanns 2001; Ceulemans and Russell 2004; Volkmann and Hanein 1999; Rossmann et al, 2001; Chiu et al, 2002; Chacon and Wriggers 2002), will further understanding of the molecular structure of the neuronal porosome complex. Such ultrastructural and mass spectrometry methods will provide complementary information and the high degree of cross-validation required to build an accurate structural model of this complex organelle, -the neuronal porosome complex.

Acknowledgments

The work described in this manuscript was supported by grants from the NIH R01 DK56212 and NS39918 to BPJ.

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Highlights

- Porosome, the 15 nm cup-shaped secretory portal at the nerve terminal
- Porosome is involved in the release of neurotransmitters from within synaptic vesicles
- Porosome has been isolated and its proteome determined
- Porosome structure has been determined using EM, AFM, and SAXS
- Porosomes have been both structurally and functionally reconstituted in lipid membranes

Figure 1.

Structure and organization of the neuronal porosome complex at the nerve terminal (Jena 2011). (A) Low resolution AFM amplitude image Bar=1 μ m (A) and high-resolution AFM amplitude image Bar=100 nm (B) of isolated rat brain synaptosomes in buffered solution. (C) Electron micrograph of a synaptosome (Cho et al, 2004), Bar=100 nm. (D) Structure and arrangement of the neuronal porosome complex facing the outside (Fig. D top left), and the arrangement of the reconstituted complex in PC:PS membrane (Fig. D top right). Lower panels are two transmission electron micrographs demonstrating synaptic vesicles (SV) docked at the base of cup-shaped porosome, having a central plug (red arrowhead) (Cho et al, 2008). (E) EM, electron density, and 3D contour mapping (Fig. E), provides at the nanoscale, the structure and assembly of proteins within the complex (Cho et al, 2008). (F) AFM micrograph of inside-out membrane preparations of isolated synaptosome. Note the porosomes (red arrowheads) to which synaptic vesicles are found docked (blue arrow head) (Cho et al, 2004). (G) High-resolution AFM micrograph of a synaptic vesicle docked to a porosome at the cytoplasmic compartment of the presynaptic membrane (Cho et al, 2004). (H) AFM measurements (n=15) of porosomes (P, 13.05 $\pm$ 0.91) and synaptic vesicles (SV, 40.15 $\pm$ 3.14) at the cytoplasmic compartment of the presynaptic membrane (Cho et al, 2007). (I) Photon correlation spectroscopy (PCS) on immunisolated neuronal porosome complex demonstrate their size to range from 12 to 16 nm (Cho et al, 2007). (J) Schematic

illustration of a neuronal porosome at the presynaptic membrane, showing the eight peripheral ridges connected to the central plug (Cho et al, 2007). ©Bhanu Jena.

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Figure 2.

Docked synaptic vesicles at neuronal porosome complex in the presynaptic membrane of the nerve terminal, observed using atomic force microscopy (AFM), electron microscopy (EM), and small angle X-ray solution scattering (SAXS). (a) AFM micrograph obtained in fluid of a synaptic vesicle (SV) docked at the cup-shaped porosome complex (P) at the cytosolic compartment of the presynaptic membrane. Note the 35 nm SV docked to a 15 nm porosome complex. (b) An EM micrograph of a 35 nm SV docked to a 15 nm P at the presynaptic membrane (Cho et al, 2004). Note the central plug of the porosome complex in the electron micrograph. (c) The averaged SAXS 3-D structure of synaptic vesicle (purple) docked at the cup-shaped neuronal porosome complex (pink) at the presynaptic membrane in isolated synaptosomes, is presented (Kovari et al, 2014). Note that the AFM, EM, and SAXS images, all demonstrate similarity in the docking and interaction of synaptic vesicles with the neuronal porosome complex at the presynaptic membrane. ©Bhanu Jena.

Figure 3.

AFM micrographs of the presynaptic membrane of isolated synaptosome preparations demonstrating the presence of the neuronal porosome plug at various conformations. (A) Low resolution AFM height image Bar=1 μ m (B) and high resolution AFM height image Box=100 nm² of isolated rat brain synaptosomes in buffered solution. (C) The area in (B) has been further imaged at higher resolution showing the central plug when fully pushed outward (Fig. C red rings; D, G), suggesting close conformation, when half-way retracted (Fig. C green ring; E, H), suggesting the semi-open conformation, and completely retracted into the porosome cup (Fig. C yellow ring; F, I), suggesting fully open status (Cho et al, 2010). ©Bhanu Jena.

Figure 4.

Hypothetical model of the mechanism of involvement of the porosome central plug in neurotransmitter release at the nerve terminal. Top left shows the averaged SAXS 3-D structure of synaptic vesicle (purple) docked at the cup-shaped neuronal porosome complex (pink) at the presynaptic membrane. To the top right is a schematic drawing of the SAXS structure showing a synaptic vesicle-porosome complex. At the lower presynaptic membrane of the schematic synaptosome outline, drawing depicts of the possible mechanism of porosome (P) involvement in synaptic vesicle (SV) docking at the presynaptic membrane (PSM) and neurotransmitter release [1–5]. Note the arm (A) of the central plug (CP) that enables the vertical movement of the plug [1]. As the foot of the CP is pushed inwards following stimulation of neurotransmission, it results in a small bump (b) that would be close in size to the diameter of the foot of the CP, which is approximately 3–4 nm in diameter. Consequently, the membrane at the porosome base constituting this 3–4 nm in diameter bump is under enormous tension and simultaneously establishes tight apposition and contact with the outer leaflet of the SV membrane via SNARE proteins, resulting in the establishment of t-/v-SNARE complex in a ring or rosette pattern [3], resulting in fusion pore (FP) formation for neurotransmitter release. Subsequently, the lifting of the porosome plug back to its original resting position results in release of the tension off the membrane at the porosome base, and the rapid FP closure that had been transiently established [4]. The spent SV then undocks from the porosome, refilled with neurotransmitters via the neurotransmitter transporters (NTT) present at the SV membrane [5], and be ready for the next round of docking, fusion, and transmitter release. ©Bhanu Jena.

Figure 5.

Immunoblot analysis of total rat brain homogenate, and SNAP-25 immunoprecipitated porosomes from control (C) and KCl-stimulated (S) rat brain slices. Approximately equal amounts of NSF, syntaxin-1, dynamin and G α_o -immunoreactivity is seen in both control and stimulated brain tissue. However, the association and dissociation of dynamin and G α_o respectively from the neuronal porosome (5, 10, and 20 μ l of SNAP-25 immunoprecipitates) following stimulation, reflects the dynamic nature of the porosome proteome. Note NSF, syntaxin, dynamin, and G α_o , are greatly enriched in the neuronal porosome complex (Lee et al, 2012). ©Bhanu Jena.

Figure 6.

Schematic drawing depicting the presence and increased association of dynamin with the porosome complex following stimulation of neurotransmitter release. Following stimulation of secretion, synaptic vesicles would dock at the porosome base, develop intravesicular pressure via active transport of water through water channels or aquaporins (AQP) at the vesicle membrane, transiently fuse at the porosome base via SNAREs and calcium, and expel neurotransmitters. After secretion, NSF an ATPase, and dynamin a GTPase, would work synchronously to disassembly t-v-SNARE complexes and fission the neck of fused vesicles at the porosome base respectively. By this mechanism, partially empty vesicles could go through multiple rounds of docking-fusion-expulsion-dissociation. Unlike protein and peptide containing vesicles, synaptic vesicles have neurotransmitter transporters at the vesicle membrane to rapidly refill vesicles (Lee et al, 2012). ©Bhanu Jena.

Figure 7.

The elution profile of the neuronal t-SNARE proteins syntaxin-1 (33 kDa) and SNAP-25 (25 kDa) in solubilized synaptosomal membrane preparation resolved on a G-200 sizing column. Note the co-localization of the 44 kDa GTP-binding protein $G_{\alpha o}$ with t-SNAREs in the fractions. Synaptosomal membrane was solubilized in Triton/Lubrol (1% w/v) PBS, and loaded onto G-200 Sephadex gel filtration column. Fractions 20 to 50 were collected and assayed for t-SNAREs following SDS-PAGE and immunoblot analysis. Note the elution of syntaxin-1, SNAP-25, and $G_{\alpha o}$ in fractions 30 through 36. As previously demonstrated (Cho *et al.*, 2007), the neuronal porosome appears to be a >650 kDa complex eluted in fractions 30–32 (Lee *et al.*, 2012). ©Bhanu Jena.

Figure 8.

Schematic drawing depicting the evidence view of predicted interactions between identified proteins within the neuronal porosome proteome and other regulatory proteins. These interactions are generated from inputs of the identified proteins in the neuronal porosome, using STRING 9.0 (Szklarczyk et al, 2011). STRING 9.0 is a database of known and predicted protein interactions. The interactions include direct (physical) and indirect (functional) associations derived from genomic, high-throughput, conserved co-expression, and earlier knowledge. Note the two clusters of protein-protein interactions identified in the porosome complex. The one cluster to the left, and most likely present at the apical end of the porosome cup are cytoskeletal structure and signalling proteins. The cluster to the right represents proteins that are primarily involved in membrane fusion including SNARE proteins and calcium channels, and therefore their location would be at the basal part of the porosome cup facing the cytosol. Interestingly, heterotrimeric GTP-binding protein and the GTP-binding membrane fission protein dynamin (Dnm2), are present in the left cluster. The presence of dynamin in the left cluster is of little surprise since they are microtubule-associated proteins, and intersectin1 is also known to interact with dynamin. However their involvement in fission of the neck of fused vesicles at the porosome base would require their

presence at the base of porosomes. The confidence of the predicted functional interactions shown are >99% (Lee et al, 2012). ©Bhanu Jena.

Figure 9.

Schematic drawing of the neuronal porosome architecture, associated with a docked synaptic vesicle, and in the process of neurotransmitter release. The possible arrangement of some of the key proteins shown as 3D schematic Richardson diagrams within the cup-shaped neuronal porosome complex, is obtained from experimental results and from the evidence view of predicted interactions using the STRING 9.0 database search. Note most of the structural and signaling proteins are present at the apical domain of the porosome cup in contrast to the membrane fusion proteins and calcium channel found at the base of the organelle (Lee et al, 2012). ©Bhanu Jena.

Figure 10.

Functional reconstitution of the immunisolated neuronal porosome complex. (a) Schematic representation of a porosome at the presynaptic membrane, with a docked synaptic vesicle at its base. (b) Schematic drawing of an EPC9 electrophysiological bilayers apparatus, to continually monitor changes in the capacitance of porosome-reconstituted membrane, when synaptic vesicles are introduced into the cis bilayers compartment, followed by ATP and purified recombinant NSF protein. (c) Schematic representation of synaptic vesicle (SV) docking at the base of a neuronal porosome complex reconstituted into the lipid bilayer, fusing to release its contents, and finally disengaging in the presence of ATP. (d) Capacitance measurements of porosome-reconstituted bilayers demonstrate that exposure to SVs results in a dramatic increase in membrane capacitance, which drops to baseline following exposure to 50 μ M ATP. Recombinant NSF has no further effect ($n=6$). (e) Exposure of isolated synaptosomal membrane preparations to 50 μ M ATP results in the release of SVs from the membrane into the incubation medium, as demonstrated by immunoblot analysis of the incubating medium using the SV-specific protein antibodies, SV2 and VAMP-2 (Cho et al, 2004). ©Bhanu Jena.

Table 1

MALDI-TOF/TOF results on specific bands in SDS-PAGE-resolved SNAP-25 immunoisolated neuronal porosome complex, obtained using 1% Triton-Lubrol-solubilized rat brain synaptosome preparation (Lee et al, 2012).

Gel Band	Identified Proteins	Accession No.	Confidence Index % (C.I.)
3	Tubulin beta chain	3745822	C.I. % = 99.83
4	Similar to KIAA1512 myosin 7b Similar to Spectrin beta chain (Brain 4)	34859107 34856723	C.I. % = 99.39 C.I. % = 98.6
5	Creatine kinase Dystrophin	31542401 18150266	C.I. % = 99.73 C.I. % = 70.76
6	Langerin GTPase activating protein (GAP) Intersectin 1 isoform (ITSN-1)	17426713 3004867 47717123	C.I. % = 60.55 C.I. % = 42.98 C.I. % = 52
8	Myosin heavy polypeptide 1	7669506	C.I. % = 21.29

Table 2

Proteins identified in purified neuronal porosomes in multiple experiments

Gene symbol	MW	Protein Name	Found in earlier studies
ACTB	42 kDa	Actin, cytoplasmic 1	x, *
AT1A3	112 kDa	Sodium/potassium-transporting ATPase subunit alpha-3	
AT2B1	139 kDa	Plasma membrane calcium-transporting ATPase 1	
AT2B2	137 kDa	Plasma membrane calcium-transporting ATPase 2	
BASP1	22 kDa	Brain acid soluble protein 1	
CAP1	52 kDa	Adenylyl cyclase-associated protein 1	
CN37	47 kDa	2',3'-cyclic-nucleotide 3'-phosphodiesterase	
DPYL2	62 kDa	Dihydropyrimidinase-related protein 2	
DPYL3	62 kDa	Dihydropyrimidinase-related protein 3	
DPYL5	62 kDa	Dihydropyrimidinase-related protein 5	
GLNA	42 kDa	Glutamine synthetase	
GNAO	40 kDa	Guanine nucleotide-binding protein G(o) subunit alpha	x, *
NCAM1	95 kDa	Neural cell adhesion molecule 1	
NSF	83 kDa	Vesicle-fusing ATPase	*
RAB3A	25 kDa	Ras-related protein Rab-3A	
RTN3	102 kDa	Reticulon-3	
RTN4	126 kDa	Reticulon-4	
SNP25	25 kDa	Synaptosomal-associated protein 25	x, *
STX1A	33 kDa	Syntaxin-1A	*
STX1B	33 kDa	Syntaxin-1B	*
STXB1	68 kDa	Syntaxin-binding protein 1	
SYN2	63 kDa	Synapsin-2	
SYPH	33 kDa	Synaptophysin	
SYT1	47 kDa	Synaptotagmin-1	*
TBA1A	50 kDa	Tubulin alpha-1A chain	x
VAMP1	13 kDa	Vesicle-associated membrane protein 1	
VAMP2	13 kDa	Vesicle-associated membrane protein 2	
VATB2	57 kDa	V-type proton ATPase subunit B, brain isoform	

Purified rat brain porosomes from two separate experiments (Fig. 7, fractions 30–32) were analyzed by LC-MS/MS on both LTQ and QSTAR XL. Only proteins identified in both samples are reported here all of which had protein confidence 95% with at least two unique peptides each having 95% confidence or above. Proteins also found in earlier immunoisolation studies are marked in a separate column, × indicating proteins identified using MALDI-TOF/TOF;

* indicating proteins identified using immunoblot analysis (Cho et al, 2004; Lee et al, 2012).