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# Electron microscopic morphometry of isolated rat brain porosome complex

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ABSTRACT

Porosomes are the universal secretory portals at the cell plasma membrane where secretory vesicles dock and transiently fuse via the kiss-and-run mechanism of cellular secretion, to release intravesicular cargo to the outside of the cell. During last two decades discovery of porosome and a great volume of work from different laboratories provide molecular insights on the structure, function, and composition of the porosome complex, especially the neuronal porosome. In rat neurons 12–17 nm cup-shaped lipoprotein porosomes present at presynaptic membrane. They possess a central plug and sometimes are with docked synaptic vesicles. Although earlier studies have greatly progressed our understanding of the morphology and the proteome and limited lipidome of the neuronal porosome complex, the current study was carried out to determine the morphology of the bare protein backbone of the neuronal porosome complex. Results from our study demonstrate that although the eight-fold symmetry of the immunoisolated porosome is maintained, and the central plug is preserved in the isolated structures, there is a loss in the average size of the porosome complex, possibly due to a loss of lipids from the complex.



## 1. Introduction

In all cells, cellular cargo destined for secretion are packaged and stored within membranous vesicles that transiently dock and establish continuity at the base of cup-shaped plasma membrane structures called 'porosomes' (Cho et al., 2002a,b,c, 2004; Cracium, 2004; Hamnel and Meilijson, 2012; Matsuno et al., 2008) and neurons are no exception (Cho et al., 2007, 2008, 2009, 2010, 2011; Drescher et al., 2011; Elshenawy, 2011; Jena et al., 2003; Jeremic et al., 2003; Lee et al., 2009; Okuneva et al., 2012; Shneider et al., 1997; Siksou et al., 2007; Wheatley, 2007; Zhao et al., 2010). The 'porosome' therefore has been called the universal secretory machinery at the plasma membrane in cells where secretory vesicles transiently dock and fuse to release intravesicular contents to the outside during secretion. In the past 18 years since the porosome discovery, solving the molecular mechanism of transient or kiss-and-run mechanism of cell secretion, over a thousand research papers have been published, confirming this mechanism, a new paradigm in our understanding of the secretory process (Allison and Doktyez, 2006; Anderson, 2006; Hamnel and Meilijson, 2012; Jeftinija, 2006; Jeremic, 2008; Leabu, 2006; Matsuno et al., 2008; Paknikar, 2007; Paknikar and Jeremic, 2007). It is reported that different cells have different speed of release, which is reflected on both the size of the secretory vesicle and the porosome. Small secretory vesicles fuse faster since they have higher curvature and the membrane is under high surface tension. Hence neurons have small 30–80 nm synaptic vesicles with 12–17 nm porosomes for fast fusion and release in contrast to the 1200 nm secretory vesicles and the 100–180 nm porosome in the slow-secreting exocrine pancreas (Jena et al., 2003; Jeremic et al., 2003; Shneider et al., 1997). In neurons and astrocytes, representing fast secretory cells, porosomes range in size from 10 to 17 nm. In an earlier study, using atomic force microscopy (AFM) and electron microscope (EM), it was demonstrated that in the neurons 40–50 nm synaptic vesicles are docked at roughly 10 nm in diameter neuronal porosomes (Cho et al., 2004). Recent EM 3D tomography in rat brain also reveals the presence of 12–17 nm permanent presynaptic densities to which

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35–50 nm synaptic vesicles are found docked (Siksou et al., 2007). Moreover, the inside-out ultrahigh-resolution AFM study of presynaptic membrane preparations of isolated synaptosomes clearly displays the presence of the inverted cup-shaped 10–17 nm neuronal porosomes. Results from more recent studies using AFM, EM, electron density and 3D contour mapping, provided additional nanoscale information on the structure and assembly of proteins within the neuronal porosome complex (Cho et al., 2008). Particularly, it has become clear that neuronal porosomes possesses a central plug that is absent in porosomes in other kinds of secretory cells. This central plug interacts with proteins at the periphery of the structure, conforming to its 8-fold symmetry; each one of them is connected with spoke-like elements to the central plug that is involved in the rapid opening and closing of the neuronal porosome to the outside (Cho et al., 2009). The neuronal central plug has been further examined at various conformational states, providing its gate-keeping role in neurotransmitter release during neurotransmission. Thus, the central plug at various conformational states: fully retracted, halfway retracted, and completely pushed into the porosome cup, have been elegantly demonstrated (Cho et al., 2010). Although it is easy to observe porosomes in intact synaptosomes and in inside-out synaptosome preparations using the AFM, in fixed cells it becomes difficult, since artifacts due to fixation, dehydration and tissue processing for EM, and compounded by the fact that the presynaptic membrane contain a high density of plasma membrane proteins resulting in heavy metal staining, rendering it difficult to observe porosomes from among the other structures. Nonetheless, porosomes are clearly identifiable in electron micrographs in numerous reported studies (Cho et al., 2004, 2007, 2008, 2009; Drescher et al., 2011; Lee et al., 2009; Okuneva et al., 2012; Siksou et al., 2007). To further understand the structure of the neuronal porosome complex, and the bare protein backbone of the complex for future single-particle cryo-EM studies, the current study was carried out on immunoisolated porosome complexes from high-detergent solubilized synaptosome membrane preparations.

## 2. Material and methods

### 2.1. Synaptosome preparation

Synaptosomes were prepared from rat brains according to earlier published methods (Cho et al., 2004). Sprague-Dawley rats weighing 120–150 g were euthanized using preapproved animal use protocol. The entire rat brain is isolated and placed in ice-cold buffered sucrose solution containing 5 mM Hepes, pH 7.4, 0.32 M sucrose, supplemented with protease inhibitor cocktail obtained from Sigma–Aldrich, St. Louis, MO. The brain tissue is then sliced and homogenized using a Teflon-glass homogenizer. The homogenate obtained is subjected to centrifugation for 3 min at 2500 × *g*, and the resulting supernatant fraction is again centrifuged for 15 min at 14,500 × *g* to obtain a pellet. The pellet is then re-suspended in buffered sucrose solution, and layered onto a 3–10–23% Percoll gradient, centrifuged at 28,000 × *g* for 6 min, and the enriched synaptosome fraction at the 10–23% Percoll gradient interface is collected for the study.

### 2.2. Immunoisolation of the neuronal porosome complex

The neuronal porosome complex was immunoisolated using SNAP-25-specific antibody conjugated to protein A-sepharose. Synaptosomes isolated from rat brain tissue were used for immunoisolation of the neuronal porosome complexes. For each immunoisolation of the neuronal porosome complex, 5 mg of a 2% Triton-Lubrol-solubilized synaptosome preparation was used. The Triton-Lubrol solubilization buffer contained 1% Triton and 1% Lubrol; 1 mM benzamidine; 5 mM Mg-ATP; and 5 mM EDTA in PBS at pH 7.5. Ten micrograms of SNAP-25 antibody conjugated to the protein A-sepharose was incubated with approximately 5 mg of the solubilized synaptosomes for 1 h at room temperature followed by five washes of 10 vol/wash, using the wash buffer (500 mM NaCl, 10 mM Tris, 2 mM EDTA, pH 7.5). The immunoisolated sample attached to the immunosepharose beads was eluted using a pH 3.0 buffer to obtain the neuronal porosome complex for EM analysis.

### 2.3. Transmission electron microscopy

To perform transmission electron microscopy, the isolated neuronal porosomes were fixed in 0.1% paraformaldehyde and 0.1% glutaraldehyde in Hepes-buffered saline (pH 7.2) and stored at 4 °C. Formvar and carbon coated copper grids used were first treated with 1% alcian blue for 10 min at room temperature, followed by four brief rinses in distilled water and air drying. Approximately 5 l of sample solution were then deposited onto the grid surface, followed by two rinses with 0.1 M cacodylate buffer and two rinses

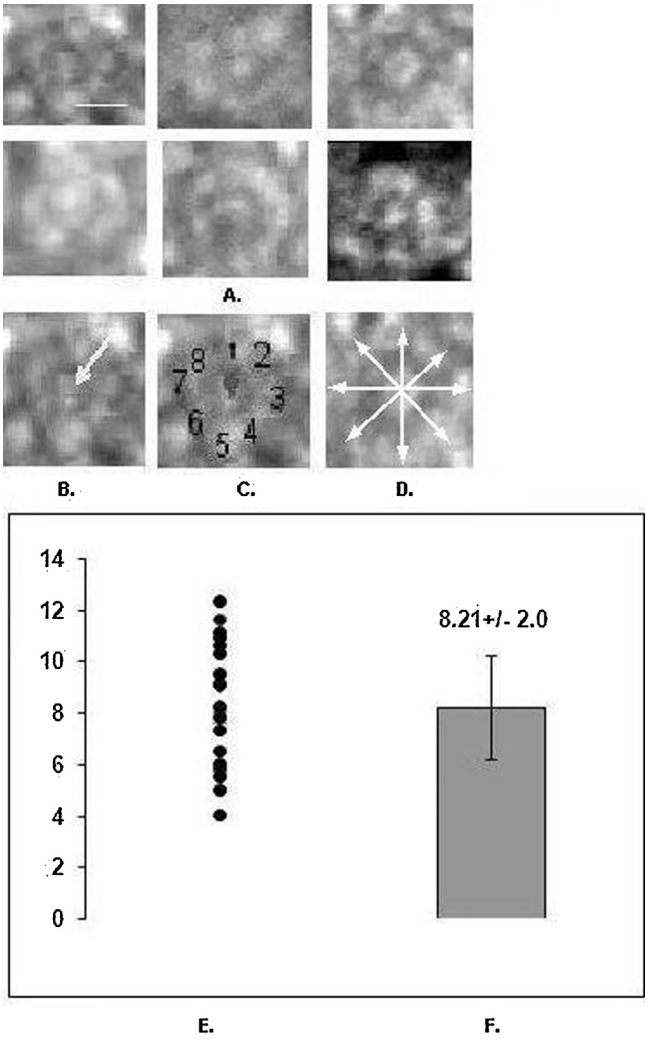


**Fig. 1.** Electron micrographs of negatively stained immunoisolated neuronal porosome complexes from rat brain tissue. (A–I) Transmission electron micrographs of immunoisolated neuronal porosome complexes. (J–L) The presence of eight-fold symmetry and a central plug is demonstrated in the immunoisolated neuronal porosome complex (white arrow, bar = 5 nm). (M) Size distribution of isolated porosome demonstrating the presence of 5–12.3 nm porosomes. (N) The mean size of neuronal porosomes measure 8.41 nm and a value of 0.32 as standard error of the mean value ($n = 32$).

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