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Neuronal Porosome in the Rat and Cat Brain[1,2]

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Abstract—It is well established that during cell secretion, membrane-bound secretory vesicles dock and fuse at the base of supramolecular cup-shaped structures at the cell plasma membrane called "porosomes", to expel intra-vesicular contents to the outside. In neurons, it has been demonstrated that 12–17 nm cup-shaped lipoprotein structure possessing a central plug are present at the presynaptic membrane, where 50 nm in diameter synaptic vesicles transiently dock and fuse to release neurotransmitters. In the past decade, the neuronal porosome has been isolated and its major chemical composition determined. Additionally, the porosome has been both structurally and functionally reconstituted into artificial lipid membrane, establishing its role as the secretory portal in neurons. Studies utilizing atomic force and electron microscopy, combined with electron density and 3D contour mapping, provide at the nanoscale, the structure and assembly of proteins within the neuronal porosome. In the current study, ultrahigh resolution imaging of the presynaptic membrane of isolated brains from both rats and cats, demonstrate for the first time, the presence of neuronal porosomes in cat brain, and further confirms the presence of porosomes at the presynaptic membrane in rat brain synaptosomes. Results from the present study further confirm the cup-shaped morphology of porosomes in the rat brain, and demonstrates their similar shape and size in the cat nerve terminal. The study also demonstrates for the first time, the universal presence of similar porosomes in different species of mammals.

Keywords: neuronal porosome, electron microscopy, cat, rat.

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It is now well established that permanent supramolecular lipoprotein structures called "porosomes", are present at the cell plasma membrane in neurons, exocrine, endocrine, and neuroendocrine cells, where membrane-bound secretory vesicles transiently dock and fuse to expel their contents to the outside during cell secretion (Schneider et al., 1997; Jeremic et al., 2003; Siksou et al., 2007). Porosomes therefore have been demonstrated to be the universal secretory machinery in cells (Jena 2007; Paknikar, Jeremic, 2007; Allison, Doktyez, 2006; Anderson, 2006). The overall morphology, composition, and reconstitution of this universal secretory machinery in exocrine pancreas, neuroendocrine cells, and in neurons are well documented (Jena et al., 2003; Cho et al., 2007; Jeremic et al., 2003), and the 3D contour map of the assembly of proteins within the rat brain neuronal porosome complex, has also been previously determined (Cho et al., 2008). Porosomes are supramolecular lipoprotein structures, composed of cholesterol (Cho et al., 2007; Paknikar 2007) and several proteins (Cho et al., 2004; Jena et al., 2003; Jeremic et al., 2003), such as SNAP-23/25, syntaxin, synaptotagmin, the ATPase NSF, cytoskeletal proteins such as actin, α-fodrin, and vimentin, calcium channels β3 and α1c, and chloride ion channels CIC2, CIC3, and their isoforms. Cholesterol has been found to be an integral component of the porosome complex, required for retaining its biomolecular stability and integrity, and its intramolecular interactions among various constituent proteins (Cho et al., 2007; Wheatley 2007). Furthermore, studies demonstrate that t-SNAREs and calcium channels are present at the base of the porosome complex (Jena et al., 2003; Cho et al., 2005) and actin regulates the opening and closing of the structure during cell secretion (Schneider et al., 1997; Jena et al., 2003; Cho et al., 2010). Recent electron microscopy (EM) tomography of the presynaptic membrane also demonstrates the presence of 12–17 nm permanent presynaptic densities to which 45–50 nm synaptic vesicles are found docked (Siksou et al., 2007). However, since the resolution of the EM tomography in the experiment is <10 nm, it precludes a detailed analysis of the porosome structure in the study (Siksou et al., 2007). Nonetheless, this 3D EM tomograph of the presynaptic membrane clearly reveals the morphological outline of the porosome complex at the presynaptic membrane.

[1] The article was translated by the authors.

[2] **Abbreviations**: DiPR—Diameter of Porosome in Rat, DePR—Depth of Porosome in Rat, DiPC—Diameter of Porosome in Cat and DePC—Depth of Porosome in Cat.

In recent studies (Cho et al., 2010), using atomic force microscopy, ultrahigh resolution imaging of the presynaptic membrane of isolated synaptosome preparations from rat brain has demonstrated the presence of neuronal porosomes in both their open, partially open, and close conformations. Results from this study further suggests that the central plug is retracted into the porosome cup when it is in its fully open conformation, and pushed outward to seal the porosome opening in its close status, supporting the hypothesis that the central plug operates as an exit door for the structure. Numerous articles and reviews have been written on the subject, and some are cited here for the benefit of the reader (Jena et al., 2009).

In further evaluation of the porosome structure at the presynaptic membrane in the rat brain, and to determine if similar structures are present in the cat, the current study was undertaken. Results from our study demonstrate for the first time, the presence of neuronal porosomes in cat brain, and further establishes their presence at the presynaptic membrane in the rat. Results from the current study further confirm the cup-shaped morphology of porosomes in the rat brain, and demonstrates their similar shape and size in the cat nerve terminal. The study also demonstrates for the first time, the universal presence of similar porosome structures in different species of mammals for neurotransmitter release.

MATERIALS AND METHODS

Isolation of Rat and Cat Brain Electron Microscopic Examination

Following pentobarbital injection (100 mg/kg), animals to have EM examination of their brains underwent transcardiac perfusion with heparinized 0.9% NaCl, followed by 500 mL of 4% paraphormaldehyde (Sigma Aldrich, USA) and 2.5% glutaraldehyde (Sigma Aldrich, USA) in 0.1 M phosphate buffer (PB), pH 7.4, at a perfusion pressure 120 mm Hg. The brains were removed from skull and placed in the same fixative overnight. The right hemispheric tissue blocks containing hippocampi, were cut into 400 micron-thick coronal slices. Slices were washed in cold 0.1 M PB and kept in 2.5% glutaraldehyde in 0.1 M PB until processing; When processing, the slices were washed in cold PB, post-fixed in 1% osmium tetroxide in cold PB for 2 h and again washed in 0.1 M PB. The hippocampus was identified with a microscope, cut out from the coronal slices, dehydrated in graded series of ethanol and acetone and embedded in araldite. Blocks were trimmed and 70–75-nm-thick sections were cut with an ultramicrotome (Reichert), picked up on 200-mesh copper grids, double-stained with uranyl-acetate and lead-citrate and examined with a JEM 100°C (JEOL, Japan) and Tesla (Czechoslovakia) transmission electron microscopes. For each case 115 sections were observed.

Morphometric Analyses

A morpometric analysis of the porosomes at the presynaptic membrane of synaptic endings was performed to identify any differences in size with regard to diameter and depth of the cup-shaped porosomal structures in the two mammalian species—rat and cat. The following abbreviations are used in reporting our study: A total of 184 synaptic terminals were studied in different areas of the rat ($n = 91$) and cat ($n = 93$) brain; 64 neuronal porosomes were clearly identified and carefully measured using the ImageJ software (version 1.41).

Statistical Analyses

One-way ANOVA was performed on the obtained data, to determine whether parameters of neuronal porosomes—diameter of opening and depth—differ from each other in the brain of different species of animals—rat and cat. The statistical significance of differences between the two groups of measurements was calculated by two sample t-test with a p-value threshold of ≤ 0.05.

RESULTS

In view of the 10–18 nm size of neuronal porosome structure, it has been difficult to observe these structures in electron micrographs. The difficulty in imaging neuronal porosomes is further compounded by the presence of high concentration of proteins at the presynaptic membrane. Therefore our strategy was to quickly and optimally fix the brain tissue in preparation for high resolution electron microscopy. As can be observed in Fig. 1, the cup-sgaped neuronal porosome is clearly visible, and in several micrographs seen with docked synaptic vesicle. The one-way ANOVA revealed that there was species differences in the morphometric parameters of neuronal porosomes: ($F(3, 131) = 22.85$, $p < 0.001$). According to our data the range of DiPR are from 9 nm up to 19 nm and DiPC—from 10 nm up to 18 nm; the dimensions of DePR—from 7 nm up to 21 nm, and of DePC—from 5 nm to 18. The mean value of DiPR (15.36 ± 0.49) nm exceed DiPC (14.06 ± 0.40) by 8.5% ($t = 2.08$, $p < 0.05$). The mean value of DePR goes above DePR by 26% ($t = 4.11$, $p < 0.001$) (Fig. 2).

Thus, a comparison of the parameters of a bowl-shaped structure porosomalnoy presynaptic membrane (JP and GP) in the cat and rat found, in the first place, their interspecies differences, and, secondly, their variability within a species.

DISCUSSION

Porosomes have been identified as the universal secretory machinery in cells and its presence has been well documented in various secretory cells, such as acinar cell of the exocrine pancreas, endocrine cell,

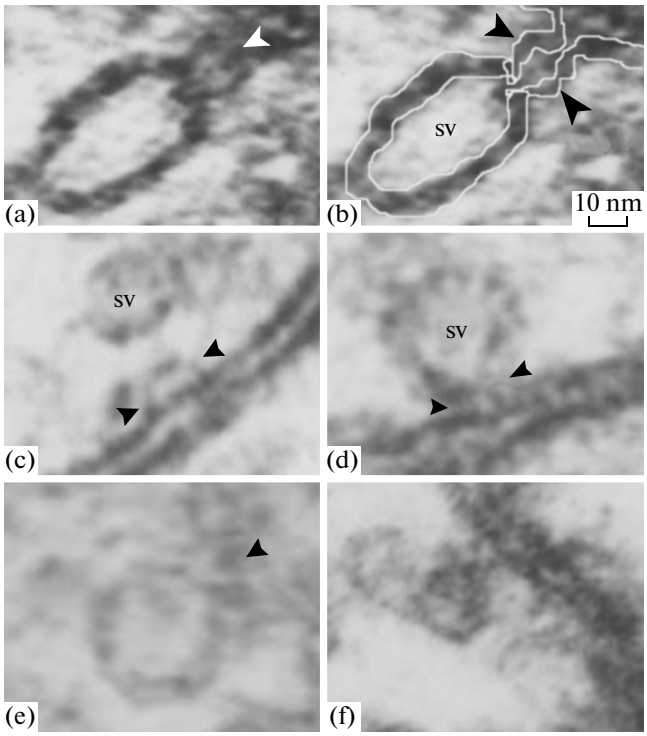

Fig. 1. Electron micrographs of neuronal porosome complex in rat brain. Note the 10–17 nm cup-shaped porosome at the pre synaptic membrane, where 50 nm in diameter synaptic vesicles are found docked. (a)—the central plug at the porosome opening (white arrowhead); (b)—the porosome structure (black arrowhead) and synaptic vesicle (SV) are allocated by a white contour; (c)—the porosome structures (black arrowheads) are also observed at the presynaptic membrane, away from synaptic vesicles (SV), demonstrating that these are permanent structures at the presynaptic membrane, and do not form as a result of synaptic vesicle docking; (d)—a synaptic vesicles docked to a cup-shaped porosome; (e)—central plug of porosome (black arrowhead); (f)—a small approximately 25–30 nm clear vesicle docked to the porosome complex at the presynaptic membrane. The small size of the synaptic vesicle suggests that the vesicle has discharged its contents.

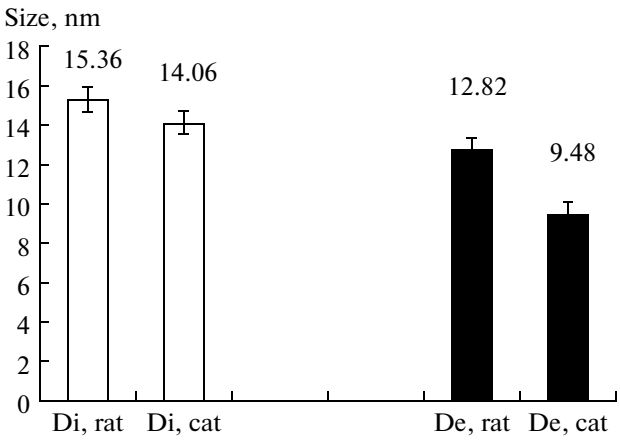

Fig. 2. The diameter (Di), and depth (De) of neuronal porosomes in rat and cat. Differences of values for rat and for cat are significant at $p \le 0.05$ (Di) or at $p \le 0.001$.

astrocytes, and or neuron (Jena et al., 2009). Also it is become clear that despite many structural and compositional similarities of porosomes in different secretory cells, their size could differ in these cells. Among other reasons, this fact could be related with different size of secretory vesicles in fast and slow secretory cells, as well as different porosomes for different cargo containing vesicles in the same cell.

Thus, because of different fusing of vesicles of different size (smaller vesicles fuse more efficiently than larger ones) (Jena, 2007, 2008, 2009), it was supposed that curvature of both, secretory vesicles and porosome base, would dictate the efficacy of vesicle fusion at the cell plasma membrane (Jena, 2007, 2008, 2009). In agreement with earlier studies (Jena, 2007, 2008, 2009), different porosome sizes are observed in the current study. In an earlier collaborative study, using AFM and EM, we demonstrated in the rat brain 40–50 nm synaptic vesicles transiently dock with 8–10 in diamter neuronal porosomes (Cho et al., 2004). In contrast, in a slow secretory cells, such as in acinar cells of the exocrine pancreas, secretory granules measuring approximately 1000 nm in diameter, expel their content following transient fusion at the porosome base measuring 100–180 nm (Schneider et al., 1997; Cho et al., 2002). Based on these findings, we suggest that depending on the size of secretory vesicles, the size of the porosome-cup is expressed. In nature, different speed of release, as well as different volume of content release dictates different size of porosomes. In the proposed research we extend our observation of natural design of neuronal porosome, by demonstrating heterogeneity in size not only within a species, but also between species. Using high-resolution EM micrographs we were able to study the size of neuronal porosome in the hippocampus of both the rat and cat, and demonstrate heterogeniety. To obtain a more complete evaluation of neuronal porosome, we measured not only porosome diameter as in our early studies (Cho et al., 2004), but also measured the porosome depth.

First at all, the results point out for the first time the existence of porosome in the cat brain. Then, they point out that diameter and depth of porosome significantly differ in rat and cat. Especially big difference was observed in the case of porosome depth measurement. At this step it is quite difficult to explain such difference. However, we suggest that one factor that may contribute to this distinction could be possible difference in the biomolecular composition of the neuronal porosome. This possibility could be present even in the porosome structure in all species, since we observe porosome heterogeneity in the same species. Additional factor that could be unidentified even on high-resolution EM micrographs and possibly can

provide difference and heterogeneity of porosomal dimensions is highly dynamic structure of neural porosomes, that is obvious in AFM, electron density and 3D contour mapping evidences (Cho et al., 2010) and displays difference in the conformational state (open, partially open or closed) of central plug and a whole porosome too.

Future studies could provide the explanation regarding this depth heterogeniety. However, besides new direct documentation of synaptic vesicles docked at the porosome base in both the rat and cat synaptic terminal, our data clearly indicates that fine tuning of porosome size to corresponding secretory processes take place not only in various secretory cells but also in the same region at the nerve terminal in different species. According to our measurements, the depth of porosomes oscillates from 5 nm up to 21 nm in both types of experimental animals.

The evidences of last years that demonstrate the structure and assembly of proteins within the neuronal porosome complex obtained by different methodological approaches (atomic force microscopy, electron microscopy, electron density and 3D contour mapping) (Cho et al., 2008, 2010) provide new insight into porosome functioning. Atomic force microscopy ultra high-resolution imaging of the presynaptic membrane of isolated synaptosomes preparations demonstrate the presence of the neuroporosome plug at various conformations (open, partially open and closed) and allows authors to suggest the involvement of the central plug in the rapid opening and closing of the structure to the outside during neurotransmission via its vertical movement (Cho et al., 2010). The different positions of the plug specify neuroporosome as a highly dynamic structure. The wide range of porosomal depth on EM micrographs alongside with methodical features could be connected with this dynamism. To reveal the appropriate depth of porosome to its different conformational state (open, partially open and closed), also to separate methodological features significant amount of porosomes should be measured in future studies. However, these studies will be very difficult using EM studies (which are 2D), compared to the 3D imaging using the AFM. Comparisons of the parameters of cup—shaped porosomal structure at the presynaptic membrane between rat and cat revealed significant species-dependent difference and size heterogeneity of diameter and the depth of porosomes within the animals of different species.

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