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Synaptic vesicle exocytosis in hippocampal synaptosomes correlates directly with total mitochondrial volume

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Abstract

Synaptic plasticity in many regions of the central nervous system leads to the continuous adjustment of synaptic strength, which is essential for learning and memory. In this study, we show by visualizing synaptic vesicle release in mouse hippocampal synaptosomes that presynaptic mitochondria and specifically, their capacities for ATP production are essential determinants of synaptic vesicle exocytosis and its magnitude. Total internal reflection microscopy of FM1-43 loaded hippocampal synaptosomes showed that inhibition of mitochondrial oxidative phosphorylation reduces evoked synaptic release. This reduction was accompanied by a substantial drop in synaptosomal ATP levels. However, cytosolic calcium influx was not affected. Structural characterization of stimulated hippocampal synaptosomes revealed that higher total presynaptic mitochondrial volumes were consistently associated with higher levels of exocytosis. Thus, synaptic vesicle release is linked to the presynaptic ability to regenerate ATP, which itself is a utility of mitochondrial density and activity.

Introduction

Diversity of the informational content processed by the brain, and its transformation into a compact language requires neuronal integration, ultimately fed by the synaptic diversity. These diverse synaptic structures arise in part from variations in the molecular makeup on both the presynaptic and postsynaptic sides of the synapse (Robbe *et al*, 2002; Franks and Isaacson, 2005; Enoki *et al*, 2009). Although most of the previous studies focused on the postsynaptic mechanisms, such as the effects of changes in the expression of AMPA and NMDA receptors on synaptic strength (Rao and Finkbeiner, 2007), the presynaptic mechanisms are equally important in neuronal integration.

Some of the presynaptic mechanisms affecting synaptic activity that have been sufficiently well characterized include: changes in the number of releasable synaptic vesicles (Xu-Friedman *et al*, 2001) and neurotransmitter amounts stored in them (Bekkers *et al*, 1990; Frerking *et al*, 1995), and variations in the density and type of calcium channels expressed (Ludwig *et al*, 1997; Stotz and Zamponi, 2001). Differences in the presynaptic mitochondrial density or activity may also have an impact on synaptic vesicle release via a number of different mechanisms. Mitochondrial ATP production powers synaptic vesicle release and recycling (Heidelberger *et al*, 1998, 2001, 2002; Sakaba and Neher, 2003). Mitochondria are also biosynthetic hubs for the production of most of the neurotransmitter

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molecules (Shupliakov *et al*, 1997). Additionally, they are capable of sequestering and buffering of cytosolic calcium, which has been shown to affect synaptic transmission at mammalian central synapses (Billups and Forsythe, 2002).

Here we report the role of mitochondria in synaptic vesicle exocytosis and endocytosis. Mitochondrial poisoning with respiratory chain inhibitors produced a substantial drop in cytosolic ATP, and was accompanied by a severe reduction in the amplitude of exocytosis. On the other hand, suppression of glycolysis had little effect on either ATP levels or exocytosis. However, none of the used inhibitors significantly affected the amplitude of calcium influx. 3D reconstruction of hippocampal synaptosomes with electron microscopy revealed that the number of endocytotic events (clathrin-coated vesicles) in stimulated synaptosomes correlates strongly with total mitochondrial volume.

Results

The roles of mitochondria and glycolysis in synaptic vesicle release were investigated using total internal reflection fluorescent microscopy (TIRFM) of mouse hippocampal synaptosomes. One of the main advantages of TIRFM is that it enables imaging of thin layers of the plasma membrane, which allows visualization of membrane-vesicle fusion events and in certain cases even single vesicles (Toomre and Manstein, 2001). Synaptosomes were loaded with a lipophilic membrane dye FM1-43, which in most protocols results in labeling of 1–2% of synaptic vesicles (Rouze and Schwartz, 1998). These vesicles are readily released upon a challenge with high potassium solution. A typical fluorescent response observed by TIRFM consists of two phases – the fast increase and slow decay (Figure 1A). Stimulation induced vesicle fusion results in a rapid accumulation of the dye in the plasma membrane with an average time course of ~ 450 ms, which is followed by its lateral diffusion in the membrane and slow dissociation into the solution (Figure 1A–1B). The fluorescence response is entirely abolished by the application of Cd^{2+} , a blocker of voltage-dependent calcium channels, indicating that the responses are calcium driven rather than osmotically-induced events (Figure 1C). Thus, the amplitude of the fluorescent response is proportional to the number of vesicle fusion events, and it can be used as a measure of exocytosis.

Mitochondrial function and glycolysis were pharmacologically manipulated to assess their relative contributions to synaptic vesicle exocytosis. Mitochondrial depolarization with 10 μM FCCP (Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone), a proton ionophore that uncouples the respiratory chain from the oxidative phosphorylation, reduced the amplitude of 30 mM KCl evoked synaptic vesicle release as measured by FM1-43 fluorescence to $40 \pm 17\%$ of KCl control ($n = 28$, synaptosomes) in hippocampal synaptosomes (Figure 1C–1D). Similarly, pretreatment of synaptosomes with 10 μM rotenone, an inhibitor of the mitochondrial complex I, produced a dramatic reduction in the response, $31 \pm 6\%$ of control ($n = 28$, synaptosomes). On the other hand, a blockade of glycolysis with 10 mM D-deoxyglucose (DG) produced no significant changes in exocytosis, with only a slight insignificant increase in the amplitude to $112 \pm 19\%$ of control ($n = 28$, synaptosomes), see Figure 1C–1D. These results show that changes in the rate of mitochondrial oxidative phosphorylation produce a profound and significant effect on the amplitude of exocytosis ($P < 0.05$, one-way ANOVA with Bonferroni correction). We also explored the effects of mitochondrial calcium sequestration on synaptic vesicle release, wherein mitochondrial calcium uptake followed by its subsequent efflux into the cytosol is capable of facilitating vesicle fusion. Incubation of synaptosomes with 100 μM ruthenium red (RRed), which blocks mitochondrial calcium uniporter and prevents mitochondrial calcium uptake, had little effect on the release, $85 \pm 22\%$ of control ($n = 28$, synaptosomes) (Figure 1D).

Changes in presynaptic calcium influx or cytosolic ATP level, which would affect either synaptic vesicle fusion or docking of new vesicles, might be responsible for the observed reduction in synaptic release with mitochondrial inhibitors. Stimulation of calcium green-1 (CG-1) loaded synaptosomes pretreated with 10 μ M FCCP resulted in calcium influx, which was similar in amplitude and decay kinetics to the control synaptosomes (Figure 2A). Calcium influx in DG treated synaptosomes had a slightly higher amplitude and significantly prolonged decay kinetics than in control (Figure 2A). These findings demonstrate that neither mitochondrial nor glycolytic inhibition significantly alters presynaptic calcium influx and thus (one-way ANOVA, $F=0.6$, $F_{crit}=5.1$, $P=0.6$, $\alpha = 0.05$), it does not directly interfere with triggering of synaptic vesicle fusion. We also assessed changes in the ATP content in synaptosomes by the luciferin-luciferase assay. Synaptosomes were pretreated with drugs, briefly stimulated with 30 mM KCl, and then lysed and assayed for ATP. Both FCCP and rotenone pretreated synaptosomes had their ATP levels significantly reduced ($P<0.05$, one-way ANOVA with Bonferroni correction) when compared to the control synaptosomes, $33 \pm 14\%$ and $41 \pm 15\%$, respectively, while DG and RRed pretreated synaptosomes showed only modest declines in ATP to $82 \pm 12\%$ of control for DG and $80 \pm 9\%$ of control for RRed, $n=5$ for all assays, (Figure 2B). This indicates that synaptic release requires mitochondrial ATP even if calcium influx remains unperturbed.

We also examined the effects of mitochondrial ATP production on endocytosis. We used serial electron microscopy (EM) to reconstruct a number of synaptosomes incubated in either normal 4 mM K^+ or 15 mM K^+ saline. 3D reconstruction allowed us to measure such presynaptic characteristics as synaptosomal volume, mitochondrial volume, the total number of vesicles, and the number of clathrin-coated vesicles (CCVs) (Figure 3A).

EM examination of hippocampal synaptosomes revealed that $\sim 46\%$ contained a single or multiple mitochondria. In our 3D reconstructions, only synaptosomes with mitochondria were used. To differentiate between induced and spontaneous synaptic activity, some of the reconstructed synaptosomes were incubated in 15 mM K^+ saline for ~ 1 minute before fixation. 15 mM K^+ saline, in general, is sufficient to induce activity (TIRFM data, not shown). However, our attempts to use a stronger stimulus such as 30 mM K^+ saline led to the formation of endosome-like structures, hampering our ability to get accurate estimates of synaptic vesicle numbers.

Out of the total 17 reconstructed hippocampal synaptosomes, 10 were incubated in 15 mM K^+ saline and the rest was prepared in normal saline containing 4 mM K^+ . In reconstructed synaptosomes, mitochondrial volume ranged from 7% to 35% of the total synaptosomal volume, with the average value of 19%. On the other hand, the volume occupied by synaptic vesicles was in the range of 8 – 41%. Surprisingly, mitochondrial volume and the total number of synaptic vesicles were anti-correlated ($R^2 = 0.5924$, $P=0.0003$, $\alpha = 0.05$), e.g. larger presynaptic total mitochondrial volumes were typically associated with smaller populations of synaptic vesicles (Figure 3B). This could be the result of permanent fusion of some of synaptic vesicles to provide extra space for mitochondria.

We next quantified the rate of endocytosis by counting the number of CCVs in stimulated and non-stimulated synaptosomes. In general, the rate of vesicle membrane retrieval is much faster ~ 1 s/vesicle (Sankaranarayanan and Ryan, 2000) than the rate of subsequent clathrin uncoating with an estimated half-life of a CCV of 10 s at 37°C (Edeling *et al*, 2006). This time is expected to be longer in our experiments carried out chiefly at RT. Thus, the number of CCVs reflects the average number of endocytotic events. Synaptosomes incubated in 4 mM K^+ solution had quite similar numbers of CCVs with an average of ~ 4 vesicles per synaptosomes (Figure 3C). The linear regression analysis between the values of number of

CCVs and mitochondrial volume in synaptosomes resulted in $R^2 = 0.0058$ ($P=0.9$, $\alpha =0.05$), implying that two variables behave independently. On the other hand, synaptosomes stimulated with 15 mM K^+ solution showed substantially higher activity with an average number of CCVs per synaptosomes of ~ 22 . We found that that presynaptic mitochondrial volume and the number of CCVs in those synaptosomes were positively correlated with each other with $R^2 = 0.7941$ ($P=0.002$, $\alpha =0.05$) (Figure 3C). Since the total mitochondrial volume is likely related to the presynaptic ability to regenerate ATP, the positive relationship between mitochondria and the number of clathrin coated vesicles in stimulated synaptosomes suggests that the magnitude of synaptic vesicle release is in part determined by the average presynaptic ATP level maintained by mitochondria.

Discussion

Neuronal ability to communicate with each other by means of chemical neurotransmission is the basic coinage for brain's function. The synapse is a major part of that mechanism, and is the point where the actual communication between cells takes place. One of the amazing properties of a synapse is its ability to transmit a variety of messages by adjusting its strength both presynaptically by changing the amount of neurotransmitter released for a given signal (O'Connor *et al*, 2005), and postsynaptically, by altering its signal amplification properties.

Changes in synaptic transmission are often the result of structural changes in the presynaptic and postsynaptic parts of the synapse. While many CNS synapses display differences in the expression of voltage gated calcium channels (Ludwig *et al*, 1997; Stotz and Zamponi, 2001) and postsynaptic receptors (Rao and Finkbeiner, 2007), they also show a considerable variation in their presynaptic mitochondrial contents (Shepherd and Harris, 1998). However, at present, it remains unknown whether differences in postsynaptic mitochondrial contents have any impact on synaptic release.

An axon terminal is an isolated neuronal compartment with its own unique calcium and ATP dynamics, which is mostly concerned with an accurate translation of action potentials into chemical signals. Mitochondria constitute part of the presynaptic structure, yet not all synapses have mitochondria (Shepherd and Harris, 1998; Waters and Smith, 2003). It is quite possible that those mitochondria-free presynaptic terminals are inactive or temporarily without mitochondria, since mitochondria are known to relocate into and out of the synapse depending on its activity (Hollenbeck, 1996; Rintoul *et al*, 2003; Kang JS *et al*, 2008). Experiments in cultured hippocampal neurons have shown that presynaptic terminals without mitochondria can be readily stained and destained with synaptic FM dyes for a prolonged time (Waters and Smith, 2003). This suggests that mitochondria-free terminals are likely capable of synaptic release. However, this still does not eliminate the possibility of mitochondrial translocation into and out of the terminal, which may happen too fast to be detected by regular time-lapse microscopy.

Mitochondria are capable of directly affecting synaptic vesicle release. Firstly, mitochondria are essential for ATP production, and thus, for a proper functioning of such ATP – dependent synaptic processes as exocytosis, endocytosis, and neurotransmitter reuptake. Synaptic ATP concentration is especially critical for several important steps in neurotransmission: NSF mediated synaptic vesicle priming (Dunant and Israël, 2000), the initial step of vesicle retrieval (endocytosis) (Heidelberger, 2001), synaptic vesicle clathrin uncoating by Hsp70 (Morgan *et al*, 2001), and vesicle transportation by the actin-myosin based molecular motors back to active zone (Soldati and Schliwa, 2006). Therefore, a change in synaptic ATP concentration profile either through inhibition of mitochondria or their departure would have a dramatic effect on synaptic vesicle turnover and synaptic

vesicle release. Mitochondria offer additional dimension for synaptic release regulation via cytosolic calcium uptake. High mitochondrial membrane potential favors mitochondrial calcium accumulation (Rizzuto *et al*, 2004). Its subsequent release elevates the resting synaptic calcium concentration, which in turn increases the probability of synaptic release (Billups and Forsythe 2002; David and Barrett, 2003; Kuromi. *et al*, 2004). Therefore, given the isolated nature of the synapse, a change in mitochondrial population size due to their relocation is expected to have significant effects on synaptic ATP and calcium concentration profiles, and thus, on synaptic vesicle release.

In this study we show that changes in the presynaptic terminal ability to regenerate ATP achieved either by having more or fewer mitochondria or by varying the rate of mitochondrial oxidative phosphorylation, produce a considerable effect on synaptic vesicle exocytosis. Inhibition of the mitochondrial respiratory chain results in a dramatic reduction in evoked synaptic vesicle fusion events (Figure 1C–1D). This reduction in release is likely the consequence of the reduced presynaptic ATP level, since the amplitude of calcium influx remains unchanged (Figure 2). A slight increase in calcium influx amplitude observed with DG is likely due to the loss of plasma membrane calcium pump modulation by glycolysis rather than a change in mitochondrial ATP (Dhar-Chowdhury *et al*, 2007; Ivannikov *et al*, 2010). Although our EM measurements find that only ~ 46% of hippocampal synaptosomes have mitochondria, which is consistent with earlier studies (Shepherd and Harris, 1998), TIRFM did not show a significant variability in responses from groups of imaged synaptosomes. This might be due to the fact that synaptosomes without mitochondria have a much weaker release, and thus, they do not contribute significantly to the signal. 3D reconstruction of stimulated synaptosomes show that synaptosomes with more mitochondria release more synaptic vesicles, as measured by the number of CCVs (Figure 3C). Synaptic vesicle docking at the release sites and uncoating of CCVs by Hsp70 are the only ATP sensitive steps in endocytosis and exocytosis (Zinsmaier and Bronk, 2001). Thus, higher numbers of CCVs observed consistently with higher mitochondrial volumes suggest vesicle docking rather than clathrin uncoating as a step most affected by changing ATP levels. On the other hand, numbers of CCVs in unstimulated synaptosomes show no correlation with mitochondrial volume, which is in agreement with the fact that spontaneous vesicle release is a stochastic event presumably operating well below the energetic limits of the terminal (Attwell and Laughlin, 2001). While in this study we used total mitochondrial volume as a measure of the presynaptic terminal ability to regenerate ATP, terminals with similar total mitochondrial volumes may have different capacities to produce ATP. Heterogeneity of mitochondrial morphology and enzyme expression profiles between parts of the CNS is a well-established fact. However, mitochondria within the same cellular structures such as dendrites or axons are typically less variable (Perkins and Ellisman, 2007).

Our results suggest that presynaptic mitochondria may indeed affect the magnitude of synaptic vesicle exocytosis at synapses, primarily via ATP synthesis. Nevertheless, many questions still remain. One of them is the question about the pattern of synaptic release at mitochondria-free synapses, which account for over half of all synapses in the CNS. It would be interesting to learn if mitochondrial absence makes those synapses silent, and subsequent mitochondrial arrival at the presynaptic terminal commences synaptic communication.

Experimental Procedures

Preparation of crude fraction of synaptosomes

Mice used for all experiments were purchased from TACONIC, and were housed and cared for in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Postnatal *C57BL/6* mice two to four weeks old were anesthetized and decapitated. After removal of the skull, the brain was isolated and immediately placed in ice-cold Hank's balanced salt solution (Sigma-Aldrich). All of the following procedures for isolation of synaptosomes with some modifications were previously described and published by Breukel *et al*, 1997. Briefly, the hippocampus was excised and transferred into glass homogenizer filled with 3 ml of ice cold homogenization buffer (320 mM sucrose, 4 mM HEPES, pH 7.3), and 3 μ l of 200 mM PMSF solution to inhibit protease activity. The tissue was homogenized by ~ 5–10 strokes of the glass plunger until the uniform consistency. The homogenate was then centrifuged in the *Eppendorf 5415C* centrifuge for 15 min at 2,000 rpm. The resulting pellet was resuspended in 3 ml of ice cold homogenization buffer, and centrifuged for another 15 min at 11,000 rpm. The final pellet was re-suspended in 0.5 ml of ice cold artificial cerebrospinal fluid -ACSF (in mM, NaCl 124, KCl 4, NaH₂PO₄ 3, CaCl₂ 3, MgSO₄ 2, NaHCO₃ 23, D-glucose 10, pH 7.4) solution, and either plated on glass poly-D-lysine covered coverslips for TIRF imaging, or fixed for electron microscopy.

ATP measurements in synaptosomes

Synaptosomal ATP was determined with the luciferin/luciferase reporting system. The luciferase solution was prepared in a stock solution from one vial of the luciferin-luciferase assay kit (Sigma- Aldrich Inc.) diluted in 450 μ l of the isotonic solution and 50 μ l distilled water. This stock solution was stored at –20°C.

Synaptosomes from the hippocampus were prepared as described above. 100 μ l aliquots of synaptosomal suspension were treated with various mitochondrial inhibitors for 5 min at room temperature, and then briefly stimulated with 30 mM K-ACSF. Controls were treated with a blank solution. After the stimulus synaptosomal samples were quickly transferred to ice, and lysed for 10 min by adding 100 μ l of a solution containing mM: 25 Tris-phosphate pH 7.8, 2 DTT, 2 1,2-diaminocyclohexane- N,N',N'-tetraacetic acid, 10% glycerol 1% Triton® X-100. The lysed samples were either used for ATP measurements immediately, or frozen for later study.

To determine the ATP content of the lysates, a luciferase working solution was prepared by diluting 40 μ l of the stock solution described above in 1 ml isotonic buffer. 100 μ l of each lysate sample was placed in a single well of a 96-well plate, which was in turn put into the microplate luminometer (TR717, Applied Biosystems, USA), and 50 μ l of the luciferase working solution was injected in each well. Luminescence measurements were taken at room temperature every 30 sec for 10 minutes with an integration time of 100 ms per measurement.

Electron Microscopy of Synaptosomes

The synaptosomes prepared as described above were centrifuged again, and resuspended in elevated 15 mM potassium ACSF (in mM, NaCl 110, KCl 15, NaH₂PO₄ 3, CaCl₂ 3, MgSO₄ 2, NaHCO₃ 23, D-glucose 10, pH 7.4). For 3D reconstruction synaptosomes were fixed immediately after the re-suspension in either normal saline or 15 mM potassium ACSF at RT. Synaptosomes were fixed by adding an equal volume of cold EM fixative (4% (w/v) paraformaldehyde, 5% (v/v) glutaraldehyde, 0.1M sodium cacodylate, 3.4 mM CaCl₂, pH 7.2), and incubated on ice for 2 hours. Synaptosomes were washed twice with water and incubated in osmium tetroxide fixative (2% (w/v) OsO₄ in 0.1 M cacodylate buffer) for 1 hour on ice. The pellets were washed four times with distilled water, and incubated in 2% uranyl acetate solution at 4°C overnight. Dehydration and embedding procedures were performed according to a detailed protocol described in Breukel *et al*, 1997.

Blocks of the tissue embedded into *Epon* epoxy resin were trimmed into the trapezoid shape with a razor blade and cut with an *LKB* ultramicrotome into a ribbon of ~ 80 nm thin sections (yellow to silver reflection). The floating sections were then carefully placed on copper grids covered with formal film (50 mesh), and dried in air. The grids were further counterstained in lead citrate, and observed under a transmission electron microscope (*Philips CM12*) at 120kV acceleration voltage.

For 3D reconstruction, the images of synaptosomes of similar magnification in the adjacent sections were taken with a CCD camera. The reconstruction of 3D structures from the acquired images and volume rendering were performed by using a public domain program – *The Reconstruct* (<http://synapses.clm.utexas.edu/tools/index.stm>). Total of 17 synaptosomes were reconstructed (all of which had mitochondria, the ones without mitochondria were not used in the analysis), with the number of sequential sections per single reconstruction ranging from 5 (with the total thickness of ~ 500 nm) to 12 (with the total thickness of ~ 1.2 μ m).

Synaptic vesicle count and mitochondrial volumes measurements were performed in *ImageJ*. The correlation and regression analyses were carried out in *Microsoft Excel*. Statistical analysis was performed on all data sets in Microsoft Excel. Distribution of data points was checked for normality by applying 3σ rule. Standard deviations and confidence intervals were then calculated with the standard *t*-test with $p < 0.05$. Significance of differences between the groups of data in question was determined by ANOVA single factor analysis followed by Bonferroni's post hoc tests (<http://www.graphpad.com/quickcalcs/posttest1.cfm>).

Total Internal Reflection Fluorescence Microscopy and Data Analysis

Synaptosomes were plated on coverslips and stained with FM 1-43 dye or Calcium Green-1AM. The TIRF setup, staining procedures and data analysis were described in full detail in our previous publication (Serulle *et al*, 2007), and were followed exactly.

In brief, a typical TIRF experiment consisted of a brief high potassium stimulation (30 mM KCl in ACSF) followed by washout with normal ACSF, and incubation with drug solution at RT (or normal ACSF in case of control). After 10 min incubation, the synaptosomes were challenged with high potassium solution again. The amplitude of FM1-43 fluorescence peak was identical for up to 3 consecutive challenges with elevated KCl ACSF. The images were acquired every 25 ms for 10 s, and analyzed in ImageJ and BrainVision software packages as described in Serulle *et al*, 2007.

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Figure 1. Imaging of synaptic vesicle release with total internal reflection microscopy

(A) Diagram of synaptic vesicle and associated FM1-43 fluorescence changes.

Synaptosomes with FM1-43 loaded vesicles (1) are stimulated with high potassium solution. Vesicle fusion (2) results in a rapid and localized accumulation of the dye in the plasma membrane followed by its lateral diffusion and slow dissociation into the surrounding solution (3).

(B) Selected time-lapse color coded frames of evoked FM1-43 release from hippocampal synaptosomes stimulated with 30 mM K⁺ solution at 0 ms.

(C) Representative fluorescent changes $\Delta F/F_0$ of FM1-43 in control hippocampal synaptosomes and synaptosomes pretreated for 5 min with 100 μM Cd²⁺, 10 μM FCCP, and 10 mM deoxyglucose (DG) stimulated with 30 mM K⁺ solution at 0 ms. Each trace is an average response from n=7 synaptosomes.

(D) Quantification of FM1-43 responses. Amplitudes of FM1-43 responses from drug treated synaptosomes are shown as % of the responses in control 30 mM K⁺ solution treated synaptosomes. Each experiment included n=28 synaptosomes. Error bars are t-test confidence intervals, p<0.05.

Figure 2. Synaptosomal calcium and ATP level changes in response to metabolic inhibitors

(A) Fluorescent changes $F-F_o/F_o$ ($\Delta F/F_o$) of calcium green-1 (CG-1) in control synaptosomes and synaptosomes incubated with 10 μ M FCCP and 10 mM DG upon stimulation with 30 mM K^+ solution at 0 s. Each trace represents an average response from 3 synaptosomal preparations.

(B) Quantification of ATP levels in control and drug treated synaptosomes. ATP levels measured with the luciferin/luciferase assay are shown as % of the ATP level in control 30 mM K^+ solution treated synaptosomes. Error bars are t-test confidence intervals, $p < 0.05$, $n = 5$ assays for all groups.

Figure 3. 3D-reconstruction of hippocampal synaptosomes

(A) Shown are 6 sequential sections, each ~ 80 nm thick, from a hippocampal synaptosome incubated in 15 mM K⁺ saline. The boxed is the enlarged view showing clearly distinguishable clathrin-coated synaptic vesicles. To the right are volume rendered 3D reconstructions of the above shown synaptosome. Colors: dark/light blue – mitochondrion, yellow – mitochondrial cristae, red spheres – synaptic vesicles, red spheres with yellow circles – clathrin-coated vesicles, green – post-synaptic densities, grey – the plasma membrane.

(B) Chart depicting relationship between the values of mitochondrial and synaptic vesicle pool volumes in reconstructed hippocampal synaptosomes (n=17) and shown as % of the total synaptosomal volume. The straight line is the linear fit approximation shown together with its R² value.

(C) Chart depicting relationship between mitochondrial volume and the number of clathrin-coated vesicles in synaptosomes prepared in either 4 mM K⁺ (n=7) or 15 mM K⁺ saline (n=10). The straight lines are linear fits with their respective R² values.