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SERIAL ULTRATHIN SECTIONING AND 3D RECONSTRUCTIONS OF SYNAPSES DURING LONG-TERM POTENTIATION IN THE RAT DENTATE GYRUS IN VIVO

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Inroduction

Learning and memory are believed to involve in synapse changes. The cellular mechanisms for storing memory in the brain are not known. Long-term potentiation (LTP) (Bliss and Lomo, 1973; Bliss and Collinridge, 1993) represents unique physiological model for the search of structural correlates of synaptic transmission. However, the structural basis of LTP expression still represents the subject of considerable debates (Muller et al., 2000; Sorra and Harris, 1998; Stewart et al., 2000; Toni et al. 1999, 2001). There is evidence for LTP remodeling occurring both at the pre- and posynaptic synaptic levels (Bliss and Collinridge, 1993), although last findings strongly argue for an important role played by postsynaptic receptors (Malenka and Nicoll, 1999). The most prominent role in postsynaptic mechanism of LTP plays the remodeling of dendritic spines and dendritic filopodia (Engert and Bonhoeffer, 1999; Fiala et al., 1998; Harris, 1999; Kirov and Harris, 1999; Segal, 2001; Segal and Andersen, 2001; Sorra and Harris, 1998; Toni et al. 1999, 2001;Yuste and Bonhoeffer, 2001).

The most part of the structural studies has been done in the dendate gyrus of the hippocampal formation in vivo and involved repeated stimulation of the perforant path input. In this region the changes in synapse number and structure of dendritic spines have been reported to occur as early as 2-30 min after induction of LTP (Desmond and Levy, 1986a,b, 1988, 1990; Trommald and Hulleberg, 1997) and to have lasted for hours to days (Geinisman et al., 1991, 1994; Stewart et al., 2000).

Remodeling of synapses in vivo showed experimental proofs regarding such natural biological models: in the course estrus of the rat (Wooley et al., 1990; Yankova et al., 2001), hibernation of ground squirrel (Popov and Bocharova, 1992; Popov et al., 1992), induced epilepsy after recurrent seizures in rat infancy (Jiang et al., 1998), in the course rat learning (Geinisman et al., 2001) and postnatal development of rat brain (Fiala et al., 1998). Recent image studies suggest that dendritic spines emerge or dissaaapear depending on the age and activity of the neuron. Immature dendrites have more protrusions when the neurons have less synaptic activation, or when there is more local activation (Harris, 1999). In vitro synaptic activation of hippocampal neurons can induce outgrowth of dendritic filopodia and spines(Engert and Bonhoeffer, 1999; Maletevic-Savatic et al., 1999; Segal, 2001; Yuste and Bonhoeffer, 2001).Conversely mature dendrites in vitro become increasingly spiny with reduction in synaptic activity (Kirov and Harris, 1999). These data show high lability of synaptic structure. However, real remodeling of both presynaptic and postsynaptic structure requires more intensive 3D reconstructions serial thin sections to understand synapse organization. So far, such studies are very rare.

Postsynaptic density (PSD) is single marker for identification of synapses at ultrastructural level.

Knowledges about the density of synapses and fine details (volume, area, shape) of both PSDs and dendritic spines are very important in the search of morphological correlates of synaptic transmission. In last years there is a big progress for the study dendritic spines and presynaptic boutons using high resolution fluorescent microscopy (Engert and Bonhoefer, 1999; Segal, 2001). Nevertheless, there are objective both limitations and difficulties for confocal microscopy usage: (i) it is possible to use only cultured neurons/organotypic cultures or surviving brain slices which must be placed in artificial medium; (ii) in contrast to electron microscopy (EM) there is limitation for resolution that does not allow to estimate correct curvature of surface of small both spines and boutons; (iii) impossible to distinguish neighboring spines because of their overlapping; (iiii) the absence of control for penetration fluorescent dye and its interactions with cytosol ingredients that depends from functional state of nervous tissue. Usefulness of the confocal microscopy for synaptic details and for a study of the presynaptic elements was described previously (Harris, 1994; Moser et al., 1997). Single-section analysis are no recognized to be accurate because individual synapses might be identified incorrectly or missed and variability in synapse density, shape and size, including synapse orientation substantially influences the probability of viewing them on a random single sections (Braendgaard and Gundersen, 1986; Coggeshall and Lecan, 1996; Fiala and Harris, 2001; Harris, 1994) Advances in computer technology, particularly in personal computer hardware and software allow to use serial electron microscopic image registration for next stereological analysis and 3D reconstruction of any synaptic organelles (for details see: Fiala and Harris, 2001). Fiala and Harris (2001) has developed new relatively simple software (IGL Trace) for unbiased reconstruction of synaptic structures usinf serial sections (for details see: "http://synapses.bu.edu/index.asp").

The phase of LTP which requires enhanced protein synthesis (both in vivo and in vitro) is believed to begin 2-6 h after induction of potentiation (Frey and Morris, 1997). This argues that more prominent structural changes, if any, are likely to occur after this period. Previously (Sorra and Harris, 1998) the stereological analysis including 3D reconstructions of various synapses showed that LTP did not cause an overall formation of new synapses at 2 hr post-tetanus in hippocampal area CA1 in vitro and this study supported the hypothesis that LTP could involve a redistribution of synaptic weights among existing synapses. Controversial data were observed by Toni et al.(2001) CA1 synapses using organotypic slice cultures. These authors showed the increase in the proportion of synapses with perforated PSDs and formation multiple spine boutons. However, the absence of quantitative stereological analysis of distributions and density of different categories synapses similar to Sorra and Harris (1998) does not allow to estimate the changes in number of synapses (density) after LTP induction to compare with confocal micrsopy data (Engert and Bonhoeffer, 1999; Korkotian and Segal, 2001).

To understand better the functional implication of the different types of synapses observed after LTP induction we used both stereological analysis and 3D reconstructions of serial 70nm-thick sections according to methodology of Harris (1994). Hippocampus from left brain hemisphere was subjected to tetanic electrical stimulation while the hippocampal formation in right brain hemisphere was used as "control". Besides, we used electrical stimulation without LTP as additional "control".

MATERIALS AND METHODS

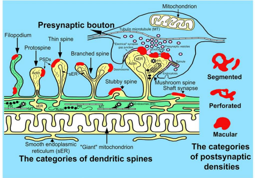
Electrophysiological induction of long term potentiation in vivo. Each rat was anesthetized with urethane (1.5 mg/kg, i.p.). All experimentsal procedures were carried out under procedures designed to minimize animal suffering. The animals was placed in a stereotaxic holder, in a recording chamber and small holes were drilled in the skull to allow the insertion of electrodes into brain. Recording electrodes (insulated nichrome wire, diameter 125 µm), were lowred into the dentate gyrus (2.5 mm lateral and 4 mm posterior to bregma). Bipolar stimulating electrodes (twisted nichrome wire), were advanced into the angular bundle to stimulate axons of the perforant path (4.0 mm lateral and 8.0 mm posterior bregma). The depth of the lectrodes was adjusted to maximize the slope of the population excitatory postsynaptic potential. After satisfactory potentials had been obtained, electrodes were cemented in place with acrylic cement, and anchored to jewellers screws in the skull. Constant intensity test stimuli were delivered at 30-s intervals throughout the experiment on the tetanized side. Tetanic stimulation of perforant path consisted of three trains (200 Hz for 200 ms), with an intertrain interval of 30 s. Responses were digitised at 10 kHz and stored to disk for off-line analysis. Stimulus timing, data collection and analysis were under computer control (CED, Cambridge), and test responses were monitored at intervals throughout. Responses were sampled for 30 min before and 1 hr after the last train.

Fixation by perfusion. Four male Sprague-Dawley rats (300-400 g, 2-3 months old) (three animals for the LTP and 1 animal for electrical stimulation withoutLTP) were anesthetized with an intraperitoneal injection of chloral hydrate (3.5% in saline, 1 ml/100g). The thorax was opened, the left cardiac ventricle cannulated and then right cardiac auricle opened. One hundred milliliter of phoshate buffered physiological saline was perfused through the animal. Subsequently, about 100 ml of 3% paraformaldehyde and 0.5% glutaraldehyde 0.1M Na-cacodilate buffer (pH 7.2-7.4) was perfused transcardially at room temperature. Hippocampi were dissected. Using razor blade about 500mm-thick slices transverse to the long axis of dorsal hippocampal portion were cut. The slices were fixed by immersion in cacodylate buffer (pH 7.2-7.4) containing 2.5% glutaraldehyde for 1-2 h at room temperature, followed by three washes in cacodylate buffer without aldehydes. Post-fixation was done with 1% osmium tetroxide and 0.01% potassium dichromate in cacodylate buffer for 1-1.5 h at room temperature. Processing for microscopy. For dehydration, aqueous solutions of ethanol at 40,50, 60, 70, 80 and 96% (each for 10 min) and 100% acetone (three changes, each for 10 min) were used. Specimens were infiltrated with a mixture of 50% epoxy resin, 50% pure acetone for 30 min at room temperature. Specimens were embedded in pure Epon 812/AralditeM epoxy resin for 1 h at 60oC and polymerized for night at 80oC. Best cutting was done in epoxy resin mixture: 22.5 ml of Epon 812, 22.5 ml of Araldite M (CY 212, 506), 60 ml of DDSA and 0.5 ml DMP-30.Given mixture was mixed for day and preserved for 3 days at room temperature. Each slice was placed on Teflon support and was covered capsule with epoxy resin. Embedded slices on surface of block were trimmed by glass knife along all surface of hippocampal slice and 1-2µm-thick sections were prepared. The sections stained with toluidine were examined in light microscope. Using left side of glass knife as cutter, trapezoid area was prepared as rectangle: one side was 20-25 µm in length and another one included CA1 area, fimbria, dentate gyrus and CA3/CA4 area. Using Diamond knife the serial sections were done on surface water/ethanol solution (5% ethanol in water) in knife bath. Series of serial sections in such solution were very stable in contrast to pure water in bath. Serial sections were catched on Pioloform-coated slot grids and counterstained with saturated ethanolic uranyl acetate, followed by Reynolds lead citrate, each 15-20 min. Grids were placed in rotating grid holder to obtain uniform orientation of sections on adjacent grids. Sections were photographed at 6,000x magnification with JEOL 1010 electron microscope. A grating replica (d=0.463 µm, Electron Microscopy Sciences Inc., Fort Washington, PA, USA) was used for calibration of electron microscope magnifications. Usually cross-sectioned myelinated axon or dendrite spanning all sections furnished a fiduciary reference for maintaining preliminary alignment of serial sections. Section thickness was determined using approach of Shepherd and Harris (1998). During cutting, the ribbons consisting 60-70nm-thick sections had grey-white color. 90-130 serial sections per series were used.

Stereology of synapses. In present investigation we have selected next target areas with synapses: for medial projection of perforant path in dendate gyrus target area was in disttance of 50-80 □m from granular cell bodies; and lateral projection of perforant path in dentate gyrus was localized in distance of 130-150 µm from granular cell bodies border. Stereological analysis was done according to Harris (1994).

Digital reconstructive analysis. Digitally scanned EM negatives with resolution 900 dpi were aligned as JPEG images using IGL Trace software developed by Dr. John Fiala (Boston University, MA, USA: "http://synapses.bu.edu/index.asp"). Alignments were done for full-field images. Contours of individual dendrites, axons, dendritic spines, PSDs, and mitochondria were traced digitally. Volumes, areas, and total numbers of structure were computed. Contours were displayed three-dimensionally using software recommended by John Fiala ("http://synapses.bu.edu/index.asp").

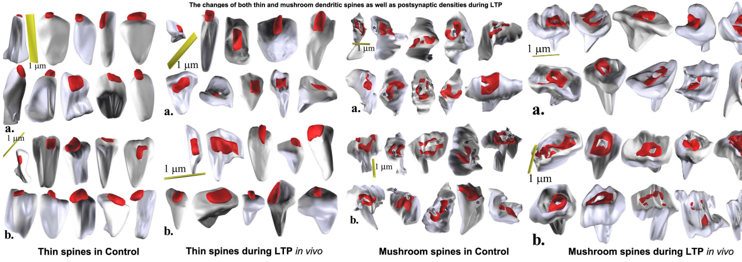
Statistical analysis. Micrococal Origin (Micrococal) software was used to graph, to obtain means and SDs, and to perform the tests of significance described in Results. Stastistical analysis were performed using the Student's *t* test. Data are presented as a mean +/- SD. N shows the number of analyzed structures/series of serial sections. The significance criterion was set at p< 0.05. One-Way ANOVA was used for detemination of significance criterion.



The categories of dendritic spines

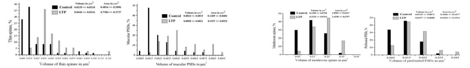
The categories of postsynaptic densities

The changes of both thin and mushroom dendritic spines as well as postsynaptic densities during LTP

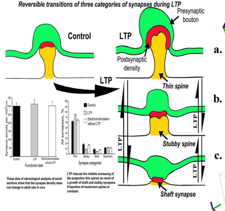


Thin spines in Control | Thin spines during LTP *in vivo* | Mushroom spines in Control | Mushroom spines during LTP *in vivo*

a, proximal; and b, distal portions of rat dentate gyrus dendrites; red color shows PSDs



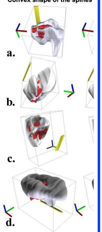
Reversible transitions of three categories of synapses during LTP

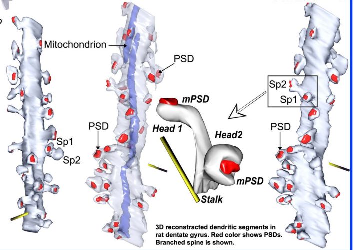


These data of stereological analysis of serial sections show that the synapse density does not change in adult rats in vivo

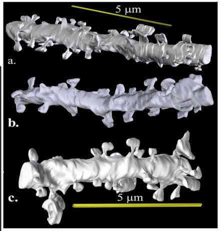
LTP induces the reliable increasing of the proportion thin spines as result of a growth of shaft and stubby synapses. Proportion of mushroom spines is constant.

Convex shape of the spines



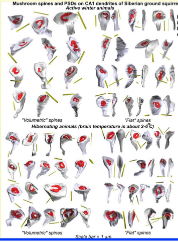


3D reconstracted dendritic segments in rat dentate gyrus. Red color shows PSDs. Branched spine is shown.

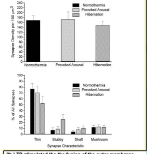


3D reconstructed dendritic segments during LTP

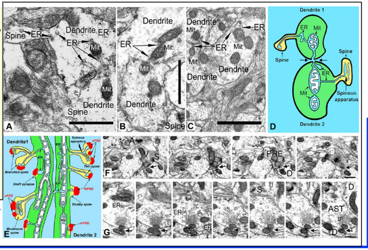
Mushroom spines and PSDs on CA1 dendrites of Siberian ground squirrel



Reversible retraction of dendritic thin spines were revealed in CA1 hippocampal area of ground squirrel during hibernation Besides, the retraction does not change the synapse density. Both 'volumetric' and 'flat' spines were revealed.



2h LTP stimulated the the fusion of the outer membrane of mitochondrial envelope with the cell (dendrite) membrane thorough smooth endoplasmic reticulum(sER) cisterns. Sites of membrane fusion were located directly in apposition to similar fusion sites in: adjacent dendrites; dendrites and presynaptic boutons; and dendrites and glial cell processes (For details see Poster 37.03 - Popov and Stewart)



RESULTS

Each synapse was identified according to the presence of PSD. There are two main categories of PSDs: macular and perforated. Segmented PSDs are a subset of perforated PSDs (Sorra and Harris, 1998). At present investigation the PSDs located on dendrites originating from nonspiny interneurons (Harris, 1994) were discarded. Only axo-spinous and shaft synapses were analyzed. Four categories of synapses were subjectively classified: 'mushroom', 'thin', 'stubby', and 'shaft'. Three categories of synapses were classified according to shape of dendritic spines. According to terminology of Peters and Kaiserman-Abramof (1970) a spine was classified as mushroom if its head was much wider than the neck; thin, if its length was greater than its neck and head; stubby, if the neck diameter was similar to its length. As rule, volume of thin spine was in about 10 times less than volume of the mushroom spine. Branched dendritic spines were classified as thin or mushroom spines according to their volumes.

We show synapse density for control (303 +/-20 PSDs per 100 □m³; N=12), LTP (315 +/- 26 PSDs per 100 µm³; N=12), and electrical stimulation without LTP (303 +/- 28 PSDs per 100 µm³; N=12). Student's t test revealed no differences between the LTP and both nonpotentiated states. Besides, we did not reveal reliable differences for synapse densities into different areas of dentate gyrus for all functional states of brain tissue (3 animals for both control and LTP, and 1 animal for electrical stimulation without LTP).

Distribution of four categories of synapses in the LTP and both control states.

We show distribution of fourth categories synapses for LTP and both control states. Reliable differences were revealed between portion of thin, stubby, and shaft synapses LTP (N=12) vs both control (N=12) and electrical stimulation without LTP (N=4; one hippocampus). No reliable differences for mushroom synapses for all states were revealed. We did not revealed reliable differences for four categories of synapses between control and electrical stimulation without LTP.

Quantitative analysis of 3D reconstructions

There are some examples 3D reconstructions of dendritic segments from both control. Similar to single-sections the visual analysis of 3D reconstructed dendritic segments did not allow to see some changes in dendrite morphology.

In this connection we have prepared 3D reconstructions of two categories of synapses which contained expressed spines with PSDs: thin and mushroom. For analysis, about 100 spines per each state were reconstructed.

Thin dendritic spines

To show fine structure dendritic spines we randomly selected 40 thin spines (20 spines with PSDs for both control and LTP). Our Figures show 3D reconstructions of the spines for control and LTP, respectively. Panels "a" on both Figures show the thin spines in distal portion of dentate gyrus while panels "b" show the spines in proximal area of granular layer. Comparative visual analysis of 3D images show that shape of the spine is more concave/or flat for LTP in contrast to control. Quantitative measurements support the visual analysis. Our Figures show that induction of the LTP induces the increasing of volume and area thin spines. One-Way ANOVA reveals reliable differences between control and LTP for both volume ($p=4.6 \times 10^{-4}$) and area ($p=2.5 \times 10^{-4}$).

We did not observe perforated/segmented PSDs for thin spines. Visual analysis of PSDs in both control and LTP did not reveal differences in macular PSDs (Fig.5 and Fig.6, respectively). Fig.8 shows quantitative measurements of volume and area of macular PSDs. The increasing in these parameters during induction LTP in contrast to control is very reliable for both volume ($p=3.8 \times 10^{-9}$) and area ($p=5.1 \times 10^{-10}$).

Mushroom spines

Figures show randomly selected subsets of mushroom spines for control and LTP, respectively. Panels 'a' show the spines located in distal portions of granule cell dendrites while Panels "b" represent the spines in proximal portions. Even visual analysis shows that LTP induces the changes in shape of the spine. In LTP the spines have concave shape in contrast to convex shape in control. Our Figure shows more prominent changes in shape of mushroom dendritic spines in LTP

We did not observe reliable differences in spinule distribution between control and LTP. For both control and LTP spinules could be localized in various regions of mushroom spine head. The presence of spinules does not correlate with functional state of brain tissue.

Graphs show that induction of the LTP induces the increasing of volume and area thin spines. Statistical analysis shows very reliable differences between control and LTP for both parameters: volume ($p=6.3 \times 10^{-9}$) and area ($p= 4.1 \times 10^{-9}$). LTP induces reliable differences in the increasing of PSDs in volume ($p=1.4 \times 10^{-4}$) and area ($p= 9.3 \times 10^{-4}$).

There is no exact classification of thin and mushroom spines because the part of the thin spines is on border of the mushroom spines and vice versa. Overlapping is 19.7% between thin and mushroom spines in control. However this overlapping is only 5.6% between thin spines in the control and mushroom spines in the LTP. For CA1 hippocampal region the overlapping is 8% (Sorra and Harris, 1998). Thus, it is possible to suggest that our criterion of classification of thin and mushroom spines during stereological analysis of fourth categories of synapses is convenient and relatively sufficient for such study (See: Scheme).

SUMMARY

- Dendritic spines are the most prominent postsynaptic structure with a marked postsynaptic density (PSD) consisting of filamentous material that anchors receptors for synaptic transmission; here the PSD was taken as a marker of synapse.
- Serial electron microscopy (EM) was used as unbiased approach for estimation of structural changes in the synapses of dentate gyrus after induction of LTP. Unbiased sampling by means of a volume dissector was used to distinguish various categories of axo-dendritic and axo-spinous synapses.
- Volume and surface area of PSDs were measured as an indicator of overall synapse size because it is well correlated with the dimensions of other components of the synapse.
- The data obtained suggest that as with the *in vitro* study of Sorra and Harris (1998) the total number of synapses does not change *in vivo* following LTP.
- However, LTP induces sharp transformations in the proportions of both thin and mushroom dendritic spines at 6h post-tetanic stimulation of perforant path *in vivo*.
- There are also increases in both volume and area of macular (unperforated) PSDs on thin spines following LTP, and in volume of thin spines; similarly there are increases in both volume and area of mushroom spines and of the segmented PSDs on these spines following LTP,