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Characterization and plasticity of the double synapse spines in the barrel cortex of the mouse

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Abstract. The somatosensory barrel cortex of rodents and its afferent pathway from the facial vibrissae is a very useful model for studying neuronal plasticity. Dendritic spines are the most labile elements of synaptic circuitry and the most likely substrate of experience-dependent alterations in neuronal circuits in cerebral cortex. We characterized morphologically and numerically a specific population of spines, i.e. double synapse spines, which have two different inputs – one excitatory and the other inhibitory, in the B2 barrel of mouse somatosensory cortex. We also described changes in morphology of double synapse spines induced by classical conditioning in which stimulation of vibrissae was paired with a tail shock. The analysis was carried out by means of serial EM micrograph reconstruction. We showed that double spines account for about 10% of all analyzed spines. The morphology of a typical double synapse spine is similar to the morphology of single synapse spine and both consist of two parts – a large head and a narrow, long neck. Excitatory synapses are preferentially located on the head of double synapse spines and inhibitory synapses are usually located on the neck of these spines. The length of the double synapse spine neck decreases and the cross-section area of the spine neck increases significantly as a result of sensory conditioning.

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Key words: barrel cortex, dendritic spine, electron microscopy, morphological plasticity, sensory conditioning

INTRODUCTION

The barrel cortex of rodents, which receives specific thalamocortical input associated with large whiskers, possesses several important advantages for studying plastic changes of cerebral cortex. Each whisker is represented anatomically and functionally by a single barrel (cytoarchitectonic specialization) in layer IV of the primary somatosensory cortex (Woolsey and Van der Loos 1970).

Neuroplasticity connected with morphological development of the barrels is especially pronounced in neonatal animals. However, the barrel cortex of adult rodents displays considerable plasticity in response to sensory manipulations such as vibrissae deprivation, pairing inputs from two whiskers, long-lasting sensory stimulation and sensory conditioning. Previous studies reported changes in response properties of cortical neurones and an alteration of cortical body maps visualized with 2-deoxyglucose (Diamond et al. 1993, Kossut 1992, Siucinska and Kossut 1996, Welker et al. 1992).

More than 90% of excitatory synapses in the rodent cerebral cortex occur on dendritic spines, which are small protrusions characterized by high motility. Inhibitory synapses are usually located on dendritic shafts, but a sparse population of this type of synapses was found on dendritic spines (Harris and Kater 1994).

Dendritic spine typically consists of a spherical head and a cylindrical, narrow neck that is directly connected with dendritic shaft (Harris and Kater 1994). Spines without any synapse do not exist. Spines have a smaller volume of endoplasmatic reticulum than dendrites, and its cytoplasm does not contain the mitochondria or microtubules that are usually present in dendritic shafts (Spacek and Harris 1997). A lot of spines have a specific structure, a specialization of the smooth endoplasmic reticulum called the spine apparatus – that can be easily identified using electron microscopy (Spacek 1985). A variety of spine shapes have been described as thin, stubby, mushroom spines or branched spines (Jones and Powell 1969).

The enormous and experience-dependent motility of spines at least in young rodents and their large number suggest that they could play a role in synaptic transmission and especially in plasticity. It has been shown that the number of spines increases after induction of long-term potentiation (LTP) in area CA1 of hippocampus (Engert and Bonhoeffer 1999) and after long-term sensory stimulation in the cerebral cortex (Jones et al. 1996, Knott. et al. 2002).

Double synapse spines, which are characterized by the presence of two synapses – one excitatory and the other one inhibitory – were first described by Jones and Powell (1969). Double synapse spines form a very small population of spines but appear to be easily affected by sensory experience (Knott et al. 2002).

The aim of this study was to characterize the double synapse spines in the barrels of mouse somatosensory cortex and changes in their morphology induced by sensory conditioning involving unilateral activation of selected vibrissae (CS) paired with a mild tail shock (UCS). This procedure was used in the previous study and resulted in an enlargement of the cortical representation of the row of vibrissae involved in the training, visualised with 2-deoxyglucose autoradiography (Siucinska and Kossut 1996).

METHODS

Animals and treatment

The animals used were ten adult Swiss-Webster mice (older than 8 weeks), of both sexes, which were housed under standard conditions in a day/night cycle LD 12:12 with free access to water and food. Before the onset of the conditioning procedure the animals were habituated in the home-made restraining apparatus (10 min a day for 3 weeks). The animals were then divided into two groups: control – 6 naive mice; and experimental one – 4 mice that were conditioned according to Pavlovian paradigm. All experimental protocols were approved by the Ethics Commission of the Government of Poland and were in accordance with The European Communities Directive (86/609/EEC).

Behavior training (CS + UCS)

The training consisted of three daily sessions lasting 10 minutes each. During each session, row B of vibrissae on the left side of the snout was stimulated by a stroke of paintbrush (conditioned stimulus, CS). Strokes were repeated 3 times during 9 seconds and during the last 0.5 s a mild tail shock (unconditioned stimulus, USC; 0.5 mA, 0.5 s) was applied. The pair of stimuli (CS + UCS) were repeated four times a minute (Siucinska and Kossut 1996).

Transmission electron microscopy study

Twenty-four hours after completion of the training, the mice were anesthetized with Vembutal (25–30 mg/kg body weight) and perfused through the heart with 20 ml of rinse buffer (0.2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) followed by 200 ml of fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The brains were removed immediately after perfusions and left in the same fixative for 24 h at 4°C.

The next day, after washing in 0.1 M phosphate buffer pH 7.4, 60 μm tangential sections were cut from the barrel cortex affected by the training. Sections were examined under a binocular and the ones containing layer IV and the barrel field cortex were collected for processing. These sections were washed in 0.1 M cacodylate buffer (pH 7.4), postfixed in 1% osmium tetraxide in 0.1 M cacodylate buffer (pH 7.4) twice (the first time with 1.5% potassium ferrocyanide), washed in 70% alcohol with 1% uranyl acetate, and after dehydration in the series of ethanol concentrations, embedded in Epon resin (Polysciences) between silicon-coated glass slides.

After identification of the barrel cortex the region of the B2 barrel hollow was prepared for ultrathin sectioning. The series of 30 to 50 silver sections (65–70 nm thick) were cut. Sections were collected on formvar-covered single-slot copper grids and stained with lead citrate.

Using transmission electron microscopy (Joel 100SX) a picture (magnification 10 K) from the center (hollow) of the B2 barrel of each serial section was taken, preferring area without cell's body, myelinated axons and large dendrites. Finally, the negatives were scanned into the computer at 600 dpi resolution using Snap Scan Agfa 1236.

Identification of synapses and the double synapse spines

Synapses were defined as regions between two separate membranes, containing clearly visible vesicles at the presynaptic side and an electron dense zone at the postsynaptic side, i.e. the postsynaptic density (PSD). A distinction between inhibitory (symmetric) and excitatory (asymmetric) synapses was made on the basis of the thickness of the PSD and shape and size of the presynaptic vesicles. Axonal ending with round and large vesicles was defined as the excitatory axon. Excitatory synapses also had thick PSDs compared with thin PSDs in inhibitory synapses. The vesicles of the inhibitory synapses were more flattened and smaller than in the excitatory synapses.

Dendritic spines (seen in the 6–12 adjacent micrographs) were identified by their small size relative to the larger dendrites and by the presence of the characteristic spine apparatus.

Quantitative analysis of double synapse spines

The series micrographs were initially aligned in Adobe Photoshop CS, in which stacks of serial pictures were also prepared (one stack from one series of sections).

Spine density was calculated according to stereological formula. The stacks consisted of 30 to 50 serial electron micrographs taken at the initial magnification of 10000× and aligned in Adobe Photoshop CS, were analyzed with image J software at the final magnification of 30000×. The area for counting spines was then determined. In these samples, all spines were counted and expressed as number per 1 μm. The spine density per volume was obtained from formula: $N_V = \Sigma Q^- / a \times h$, where ΣQ^- is the number of spines found in the entire volume (excluding spines present at the last pictures from stacks), a is the sampled area, and h is the mean thickness of the ultrathin section using for building stacks (Weibel 1979).

Sixteen double synapse spines of each group of mice selected for measurements had two synapses – one excitatory and another one inhibitory – and they had a transversal cross-section. Dendritic spines which were cut obliquely or longitudinally were not taken into consideration in this study. Three area measurements from every micrograph of chosen spines were made using image J software (National Institutes of Health, Bethesda, MD), and then the micrographs in which they were seen were counted. The location of excitatory and inhibitory synapses on spines was estimated.

Statistical analysis

The differences in length of the double synapse spines and in the cross-sectional area of the double synapse spine's neck between experimental and control mice, were evaluated using ANOVA, followed by Tukey's *post-hoc* test.

RESULTS

Number of double synapse spines

All double synapse spines in the hollow of the B2 barrel had one excitatory and one inhibitory synapse (Figs 1A–D, 2A–D). No double synapse spines with two synapses of the same type (two excitatory or two inhibitory synapses) were found. No spine was seen to have more than two synapses.

The total analyzed volume of the sample in which spines density was estimated was 642.06 ± 13.43 μm^3. The density of single synapse spines in the B2 barrel of control mice was close to the total spine density in the research region (single spines density, 0.935 ± 0.20 in μm^3; total spine density, 1.045 ± 0.196 in μm^3) and single spines accounted for 89.18% of all spines. Double synapse spines were a sparse population of spines (double spines density, 0.111 ± 0.033 in μm^3) and they accounted for only 10.82% of all spines in this region.

Size and shape of double synapse spines

Double synapse spines had a relatively large head compared to their narrow, long neck but generally they were not larger than single spines. Their spine apparatus was clearly seen, and formed typically two or three (rarely more) electronic dense, linear structures, found in the spine head or in the border region between the head and neck of the spine. No other organelles were observed in these spines.

The morphological analysis revealed a significant decrease in the length of double synapse spines in the conditioned group of mice (0.520 ± 0.155 μm) when compared with control, not conditioned, animals (0.763 ± 0.351μm; Fig. 3). The cross-sectional area of the spine's neck was significantly larger, however, in

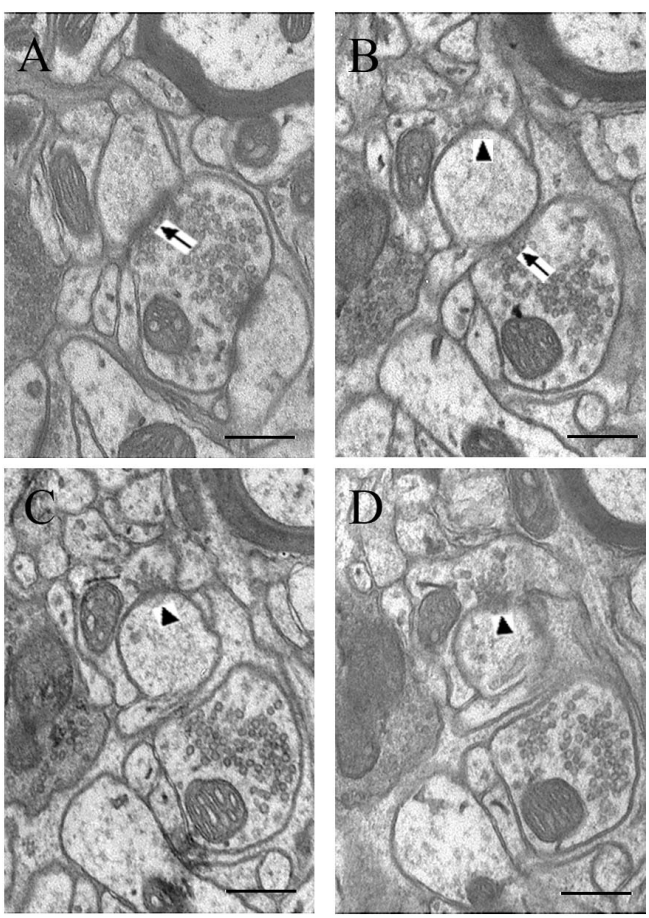

Fig. 1. An example of double synapse dendritic spine (with two different synapses) in the barrel cortex of mice. Series of four (distance 65 nm) electron micrographs shows two synapses, one inhibitory synapse (arrowhead) and the another excitatory synapse (arrow). Scale bars are 0.5 μm.

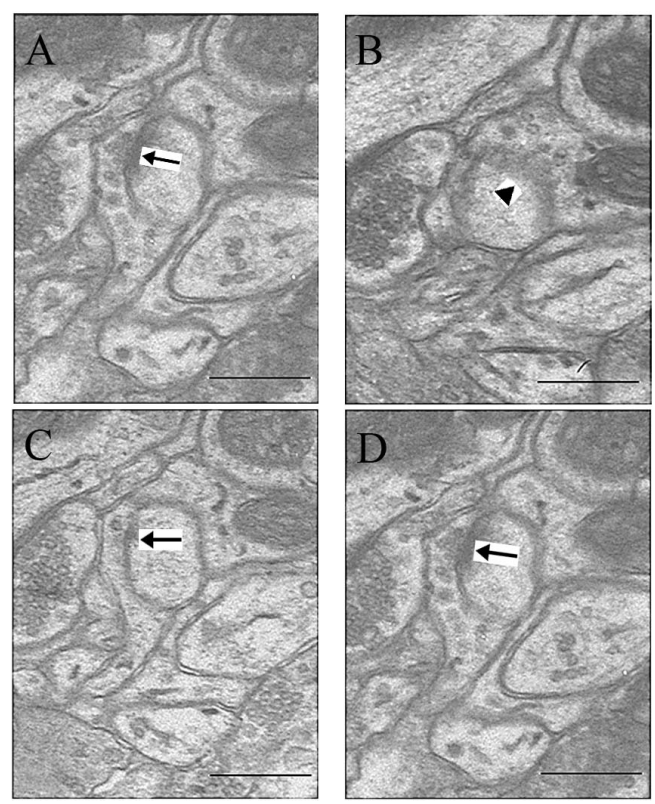

Fig. 2. An example of double synapse dendritic spine (with two different synapses) in the barrel cortex of mice. Series of four (distance 65 nm) electron micrographs shows two synapses, one inhibitory synapse (arrowhead) and the one another excitatory synapses (arrow). Scale bars are 0.5 μm.

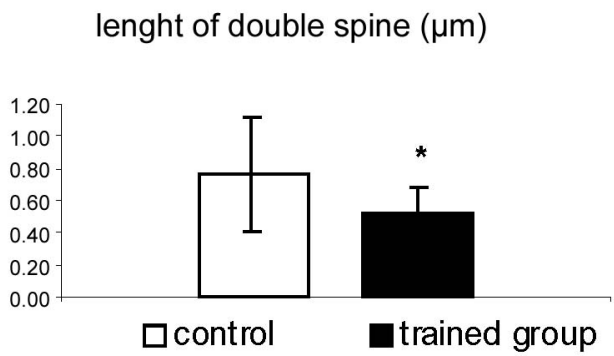

Fig. 3. Histogram showing the mean of the length of double synapse spine and standard deviation in the B2 barrel of mice. The significant increase (asterisk) by 52.5% was observed in the length of double spine in the trained group (black bars) vs. control (white bars), $P<0.05$.

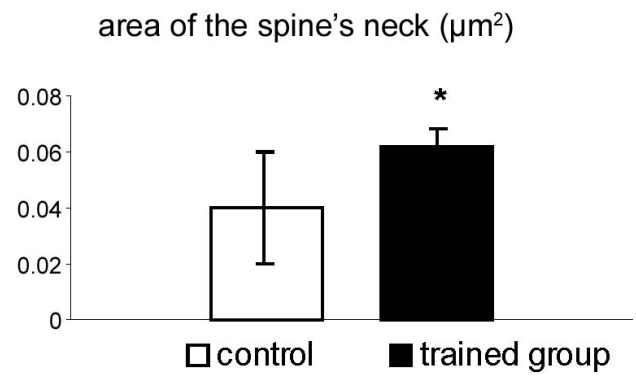

Fig. 4. Histogram showing the mean of the area of the spine's neck and standard deviation in the B2 barrel of mice. The significant decrease (asterisk) by 31.85% was observed in the area of the spine's neck in the trained group (black bars) vs. control (white bars), $P<0.05$.

the experimental (0.061 ± 0.007 μm²) than in the control group (0.040 ± 0.020 μm²; Fig. 4).

Location of synapses on spines

Synapses were found on the top and the sides of the spine heads, on the neck of the spine and on the border region between neck and head of spine. Most excitatory synapses were located at the spine head – especially on the top of the head – and there were a few synapses placed at the border region between head and neck of spine. Inhibitory synapses were generally located on the neck of the spine or in the neck/head border region. There was a small population of inhibitory synapses located on the side of head and a few inhibitory synapses placed on top of the spine head. Location of the synapses on the double synapse spines in the B2 barrel was found unchanged after conditioning.

CONCLUSIONS

In our study we characterized double synapse spines in the hollow of the barrels. Double synapse spines form a small population of spines having a large head and narrow neck. In general, their morphology is similar to the morphology of single synapse spines. Excitatory synapses are typically located on the spine head, as opposed to inhibitory synapses, which are usually found on the spine neck or on the border region between neck and head spine. The downstream location of inhibitory synapses on a double spine seems to confirm that inhibitory synapses can modify excitatory input of the other synapse and in this way be associated with special intracortical inhibitory mechanism (Dehay et al. 1991). Moreover, the location of synapses on the double spines is not modified by sensory training.

In the ultrastructural study by Yuste and Bonhoeffer (2001), it has been shown that changes in neuronal activity induce pronounced morphological alternations of dendritic spines. In the present study we found that the short–term conditioning can also modulate morphology of the double spines. As a result of sensory training their length was decreased while the area of spine's neck was increased, suggesting a constant volume of the double spine regardless of the training.

Our results confirm and expand on the data of Knott and coauthors (2002) which showed that the double synapse spines in the sensory cortex are easily affected by sensory experience. In our previous study, we observed that the changes induced by sensory conditioning were especially associated with inhibitory transmission (Gierdalski et al. 2001, Siucinska 2003, Siucinska et al. 1999), and future studies will focus on the analysis of inhibitory and excitatory synapses involved in this process.

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