Dendritic Spine Density and LTP Induction in Cultured Hippocampal Slices

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Collin, Carlos, Katsuyuki Miyaguchi, and Menahem Segal. Dendritic spine density and LTP induction in cultured hippocampal slices. J. Neurophysiol. 77: 1614-1623, 1997. Transverse hippocampal slices were cut from 8- to 9-day-old rats and maintained in an interface chamber for periods of 1-4 wk, in tissue culture conditions. Neurons in the slice preserved their spatial organization and connectivity. Dendritic spine density in CA1 neurons was very low at 1 wk in culture, and long, filopod-like structures were abundant. Spine density increased in these neurons nearly threefold during the course of 3 wk in vitro, to approach values of those of the normal, in vivo hippocampus. The magnitude of long-term potentiation (LTP) of reactivity of CA1 to stimulation of CA3 inputs. Also, the density of spines in dissociated cultures is lower at 1 wk in culture, and long, filopod-like structures were abundant. Spine density increased in these neurons nearly threefold during the course of 3 wk in vitro, to approach values of those of the normal, in vivo hippocampus. The magnitude of long-term potentiation (LTP) of reactivity of CA1 to stimulation of CA3 neurons also increased during weeks in culture in parallel with the change in spine density. Chronic exposure of slices to drugs that interact with synaptic activity caused changes in their dendritic spine density. Blockade of the N-methyl-D-aspartate (NMDA) receptors with the receptor antagonist 2-aminophosphonvalerate (d-APV) or blockade of action potential discharges with tetrodotoxin (TTX) prevented dendritic spine development in immature cultures. Enhancing synaptic activity by blockade of GABAergic inhibition with picrotoxin did not affect spine density to a significant degree. d-APV-treated slices expressed larger LTP than controls. TTX-treated slices expressed smaller LTP than controls. Picrotoxin treated slices did not express LTP. It is proposed that LTP and dendritic spine density are correlated strongly during development, whereas they are not correlated in the more mature slice/culture of the hippocampus where spine density can be modulated by chronic exposure to blockers of synaptic activity, which will not affect LTP in a similar manner.

METHODS

Hippocampal slices, 300-μm thick, were prepared from 8- to 9-day-old Sprague-Dawley rats as described before (Stoppini et al. 1991). Briefly, slices were placed on a transparent porous filter (Millicell CM, Millipore), in a 37°C, 5% CO2 humid incubator, and fed twice a week with a medium containing 50% minimal essential medium, 25% Earle’s balanced salt solution (EBSS), and 25% heat-inactivated horse serum supplemented with 7 mg/ml glucose, penicillin, and streptomycin (100 U/ml each). Throughout their growth period, the slices were kept at an interface between the growth medium and the humid atmosphere. Slices were taken to the recording chamber where they were superfused with the recording medium. Intracellular recording was made with 3-μm micropipettes backfilled with 1 M potassium acetate (KAc). Different slices of the same batch were used for the electrophysiological and morphological experiments so as to minimize the delay between the drug treatment and the fixation of the slices assigned to the morphological study. Signals were amplified with an Axoclamp 2A amplifier, digitized, and stored in an IBM-compatible computer using standard Axon Instruments software.

Drug application

The following drugs were applied in the growth medium for periods ranging from 2 to 21 days: picrotoxin (RBI), 50 μM; 2-aminophosphonvalerate (d-APV), Sigma, 50 μM; tetrodotoxin (TTX, Sigma), 1 μM; 6,7-dinitroquinoxaline-2,3-dione (DNQX,
Development of dendritic spines in slice culture

In the first series of experiments, the density of dendritic spines in neurons grown in slice cultures for periods of 1–4 wk were studied. There was a clear distinction between genuine dendritic spines, consisting of a thin and short (1–2 \( \mu \)m) neck and a distinct head, and filopodia, being very thin and long without a distinct head. The two types of protrusions were seen side by side and were counted separately in some experiments. Significant regional and age variability also was observed. Although in some branches, thin filopodia were predominant (Fig. 1B), at 1 wk in culture, already two-thirds of protrusions were spines (Fig. 2).

Because of the relative abundance of filopodia and the smaller diameter of dendritic branches at this age, proper identification of spines was only accurate when confocal sections were analyzed individually. The filopodia were not counted in later drug experiments (see below). A marked age-related increase in spine density during the 1 mo of growth in culture was seen and a concomitant decrease in the relative abundance of filopodia. The majority of spines in the 4-wk-old slices were shorter, had a distinctly larger spine head, and were about three times more numerous per length of dendrite compared with those in the younger cultures (Fig. 2). The mean spine density of 0.399 ± 0.031 spines (mean ± SE) per 1 \( \mu \)m length of dendrite (out of a total of 0.621 ± 0.025 protrusions (spines + filopodia) per micrometer of dendrite) in 1-wk-old cultures, increased to 1.159 ± 0.063 spines per 1 \( \mu \)m dendrite out of a total of 1.176 ± 0.061 spines (spines + filopodia) in 4-wk-old cultures (ANOVA for comparison of spine density across four age groups, \( F = 84.5, P < 0.01 \)). Thus although spine density increased by about threefold across 3 wk in culture, the density of filopodia decreased from 0.222 to 0.017 per 1 \( \mu \)m in this time period. The increase in spine density was associated with an increase in dendritic arborization and cell volume, but these parameters were not studied systematically.

Sufficient to say that the increase in both spine density and dendritic length, contributed to an actual larger increase in total number of spines per neuron in the slice. We analyzed also changes in shape and size of the spine head during development, but the variability within cells and cultures was too large in this sample. An electron microscopy (EM) analysis indicated that young filopodia like structures often contained synaptic junctions and smooth endoplasmic reticulum (SER) (Fig. 3, A and B), indicating that these filopodia eventually might become genuine spines. Mature spines with large heads and necks, as shown in Fig. 3C, became dominant about 2 wk in culture. Concave-shaped spine synapses (Miyaguchi 1994) in which cytoplasmic extensions from the postsynaptic density region embraced the presynaptic terminals also became prominent after 3 wk in culture (Fig. 3C).

Development of electrophysiological properties of CA1 neurons in slice cultures

Input/output relationships of excitatory postsynaptic potentials (EPSPs) were recorded in stratum radiatum (Fig. 4A, top) and stratum pyramidale (SP; Fig. 4A, bottom) at 1 \(( n = 10)\), 2 \(( n = 6)\), 3 \(( n = 10)\), and 4 \(( n = 8)\) wk in

Electrophysiology

Extracellular recordings were made from strata pyramidale and radiatum of the CA1 region of the slices. Stable responses to stimulation of stratum radiatum or the adjacent CA3 area was recorded, an input/output curve was constructed, and the intensity of stimulation that evoked two maximal responses was chosen for later experiments. Stimulation was applied at a rate of 0.1 Hz, and the responses to all stimulations were stored for later analysis.

Electron microscopy

Slices of different ages or drug treatments were fixed in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer (PBS) for 1 h. After a rinse in PBS, they were postfixed in 1% osmium in PBS for 1 h. The slices were then dehydrated, embedded in plastic, thin sectioned and contrasted. Alternatively, slices were frozen rapidly on a copper block (Life Cell) and freeze substituted as described elsewhere (Bridgman and Reese 1984; Pozzo Miller and Landis 1993).
FIG. 1. Dendritic spines in slice cultured hippocampal neurons stained with Lucifer yellow. A: low power image (×20) of a 3-dimensional (3-D) reconstructed CA1 pyramidal neuron in a 4-wk culture. B: high power (×100) image of a 3-D reconstructed apical dendrite at 1 wk in culture showing abundant long and thin, filopodia-like projections. C: within 4 wk in culture, dendrites are populated by mature spines only. D: 3 days after addition of 50 μM picrotoxin to a mature (3-wk-old) culture, spine density and morphology were not significantly changed. E: maturation of dendritic spines was retarded in cultures raised for 2 wk in presence of 1 μM tetrodotoxin (TTX). F: 2-amino-phosphonovalerate (D-APV) treatment caused a reduction in spine density but no major alteration in spine morphology. Scale bar in A = 75 μm, B–F = 5 μm.
in EPSP size 30 min after tetanic stimulation (including slices with failures of LTP, but not including slices that did not express short-term potentiation) amounted to 20.3 ± 8.7% at 1 wk, 35.1 ± 8.8% at 2 wk, 51.4 ± 4.1% at 3 wk, and 68.9 ± 5.3% at 4 wk in culture (Fig. 4B). This difference across age of culture was highly significant when tested by ANOVA (F = 196.57, P < 0.0001).

Activity-dependent changes in dendritic spine density

Slices were exposed for 3–21 days to drugs known to affect spike and synaptic activity. These include blockade of action potential discharges with TTX, selective blockade of the N-methyl-D-aspartate (NMDA) receptor with d-APV, blockade of both types of ionotropic glutamate receptors with d-APV/DNQX, and activation of the hippocampal circuit with the γ-aminobutyric acid (GABA) antagonist picrotoxin. Chronic exposure to d-APV for 2 wk (Figs. 1F and 5, left), or d-APV/DNQX for 3 wk reduced spine density by ~25–30%. (For the APV treatment, 0.55 ± 0.018 compared with control, 0.71 ± 0.031 spines per 1 μm, and for the APV/DNQX treatment, 0.817 ± 0.019 in the drug treated slices, compared with 1.176 ± 0.061 spines per 1 μm in the control group, F = 78.2, P < 0.01.) There was no apparent change in the shape of spines after exposure to d-APV or d-APV/DNQX. TTX, treated for 2 wk (Fig. 1E), prevented development of the spines into a mature form, as indicated by the presence of long, filopodia-like structures. The spine density was reduced by about the same magnitude, 30%, as seen with d-APV (0.65 ± 0.057 of TTX-treated slices compared with 0.915 ± 0.041 of age-matched controls, F = 11.7, P < 0.01, Fig. 5, right).

Picrotoxin changed physiological properties of cells in the slice (see below), but did not affect spine density in these cells: six cells exposed to picrotoxin for 2 wk in four experiments, mean spine density in control 0.90 ± 0.055 and in picrotoxin treated cells 0.88 ± 0.10 spines per 1 μm dendrite). Picrotoxin also failed to cause significant changes in spine density when applied for short periods (3 days) in already-mature 3-wk slices (Fig. 1D).

Activity-dependent changes in ability to express LTP

D-APV treatment for 2 wk (n = 15 slices) caused both a larger STP and an apparently larger LTP compared with controls (Fig. 6A). Exposure of the slices to d-APV/DNQX caused a large enhancement of spontaneous synaptic activity when slice cultures were transferred to the recording chamber and the drugs were washed away. In these cases, tetanic stimulation triggered massive epileptiform discharges. Picrotoxin-treated slices (n = 15) expressed a marked reduction in the magnitude of STP and a complete elimination of LTP. Blockade of both types of ionotropic glutamate receptors (NMDA) with D-APV, toxin-treated slices (0.55 ± 0.061 spines per 1 μm, and for the APV/DNQX treatment, 0.817 ± 0.019 in the drug treated slices, compared with 1.176 ± 0.061 spines per 1 μm in the control group, F = 78.2, P < 0.01.) There was no apparent change in the shape of spines after exposure to d-APV or d-APV/DNQX. TTX, treated for 2 wk (Fig. 1E), prevented development of the spines into a mature form, as indicated by the presence of long, filopodia-like structures. The spine density was reduced by about the same magnitude, 30%, as seen with d-APV (0.65 ± 0.057 of TTX-treated slices compared with 0.915 ± 0.041 of age-matched controls, F = 11.7, P < 0.01, Fig. 5, right).

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**FIG. 3.** Age- and drug-induced changes in spines as seen at ultrastructural level. A: CA1 region of chemically fixed slice culture after 1 wk in vitro. Dendrites have filopodia-like protrusions (→). Bar, 500 nm. B: higher magnification of a filopodia seen in A, having a synaptic junction. C: rapidly frozen and freeze-substituted CA1 area of a slice culture after 4 wk in vitro, showing a dendrite with a mature spine, which typically had a concave-shaped synapse, in which cytoplasmic extensions from the postsynaptic density region embraced the presynaptic terminals. D: CA1 region of a chemically fixed slice culture, 4 wk in vitro, to which TTX was added between 1 and 4 wk. Dendrites often have immature-like filopodia with synaptic junctions. B–D: synapses (long arrows), smooth endoplasmic reticulum (SER; arrowheads), microtubules (short arrows), bar = 200 nm.

**DISCUSSION**

The present results confirm and extend earlier observations that demonstrated the potential use of slices of developing hippocampus maintained in culture for the study of long-term regulation of spine morphology and for the study of physiological functions of the hippocampus (Debanne et al. 1995; Emptage et al. 1995; D. Muller et al. 1993; Pozzo Miller et al. 1993; Stoppini et al. 1993). Thus we found similar developmental changes in physiological properties of the slices, measured in both the percentage of slices expressing LTP and the magnitude of the sustained change. In addition, the slice/culture allows a detailed analysis of factors that regulate the formation of dendritic spines. As has been shown before, the slice develops in vitro in a surprisingly ordered manner and maintains the morphology of the cells as well as the connectivity among regions of the slice. In the absence of extrinsic afferents, e.g., those originating in the contralateral hippocampus and the entorhinal cortex, the local afferents to CA1, coming from CA3 area, proliferate and extensively innervate the basal dendritic regions of cells in CA1 (Sacaguchi et al. 1994). Thus the cells in CA1 area are innervated sufficiently to allow extracellular recording of population EPSPs generated by stimulation of CA3 neurons. Incidentally, the fact that the basal dendritic area (stratum oriens) is innervated heavily by fibers of CA3 origin may underlie the lack of difference seen here between apical and basal dendritic spines. In the absence of afferent...
FIG. 4. Development of plastic properties of hippocampal neurons in slice cultures. A: population excitatory postsynaptic potential (EPSP)'s amplitude measured in stratum radiatum (top) and pyramidale (bottom) of 1-wk (left) and 4-wk-old (right) cultures. Responses were elicited after exploration of stimulation sites in CA3 and recording sites in CA1. Superimposed traces were elicited by increasing intensity of CA3 stimulation. B: development of ability to produce long-term potentiation (LTP). EPSPs were recorded in stratum pyramidale every 60 s and, after 10 min of baseline recordings, a single, 100-Hz, 1-s tetanus was applied. EPSPs were recorded and plotted for 30 additional minutes. Data points are averages ± SE of 1-wk- (n = 10), 2-wk- (n = 8), 3-wk- (n = 8), and 4-wk- (n = 10) old cultures.
innervation of basal dendrites, they are expected to shrink and reduce density of spines.

Our results indicate that dendrites of young cells are endowed with filopodia-like structures that coexist on the same dendrites with the genuine spines. The filopodia may have synaptic junctions with presynaptic boutons, and they contain SER, a typical organelle present in mature spines. During the second week in culture, the filopodia disappear rapidly and the dendritic spines become prominent. During this transition period, one single neuron sometimes contains two different domains: one occupied by filopodia and the other by spines, consistent with a developmental progression from filopodia to spines. The difference between synapses on spines and filopodia may contribute to the understanding of the functional roles of spines in synaptic integration. The present results are also in agreement with a developmental progression observed in time-lapse confocal imaging by Cooper and Smith (1992) and Dailey and Smith (1996) but not with studies in dissociated hippocampal cultures, where filopodia were not associated with synapsin-stained particles (Papa et al. 1995).

The ability of CA1 neurons in slice cultures to express LTP in the normal growth conditions has a developmental pattern akin to that found in vivo, with the exception that in acute slices taken from the developing brain, the maximal LTP is obtained with ~2-wk-old rats, whereas here the ability to express LTP continues to develop at least until 3 wk in vitro (equivalent to a 4-wk in vivo hippocampus), when the response stabilizes. This difference may indicate the presence of additional growth factors in vivo, which will accelerate the ability to express LTP or the additional time it takes CA3 fibers to proliferate and reinnervate the vacated CA1 in slice cultures.

**Drug effects on spine density and LTP**

Drugs that block or enhance ability to express LTP produce different effects on spine density. The NMDA antagonist d-APV enhanced LTP magnitude, either because it prevents degeneration of neurons (Pozzo-Miller et al. 1994) or because it prevented saturation of LTP-generating mechanism by ongoing activity in the slice. Hyperexcitability and epileptiform activity observed in slices exposed to a combination of d-APV and DNQX could be explained by the washout of excitatory antagonists when cultures were transferred to the normal recording medium and also may be similar to epileptiform activity induced by the removal of the glutamate antagonist kynurenic acid and elevated magnesium shown previously in neuronal microcultures (Furshpan and Potter 1989; Segal and Furshpan 1990). The enhanced activation resulting from removal of the glutamate antagonists may indicate that the chronic blockade causes an upregulation of the glutamate receptor. This needs to be further examined using more direct methods of estimating glutamate receptor functions.

The GABAergic antagonist picrotoxin blocked the ability of slices to express LTP. This can be caused by a possible toxic effect of picrotoxin on the slice, resulting in the elimination of dendritic spines, as reported before (M. Muller et al. 1993), or by the possible saturation of the LTP generating mechanism by the blockade of inhibitory activity, which will enhance excitability of the slice. It is not likely that picrotoxin affected viability of the slices, because the labeled cells in the picrotoxin-treated slices appeared normal and had about the same spine density as the control slices. These results contradict those of M. Muller et al. (1993), who found that picrotoxin causes elimination of spines in thin
FIG. 6. Drug effects on ability to produce LTP in slice cultures. A: sample traces recorded in stratum pyramidale in control, top, picrotoxin (PTX)-treated, middle, and TTX-treated slices, bottom, in baseline condition, left, at peak of response after tetanic stimulation at time 0, middle, and 30 min after tetanic stimulation, right. B: percent change in EPSP amplitudes after a single 100-Hz tetanus of 1-s duration was evaluated in 3-wk-old cultures exposed to drugs. D-APV-treated cultures \((n = 15)\) showed larger short-term potentiation (STP) and LTP compared with controls \((n = 15)\). TTX treatment \((n = 12)\) did not affect STP and only caused a small reduction of LTP compared with controls. PTX treatment \((n = 15)\) caused not only a marked reduction of STP but also completely blocked the ability to induce LTP. B: EPSP amplitudes were normalized between controls and STP levels to separate STP from LTP changes. This analysis shows that D-APV did not enhance LTP relative to STP. Data points are means \(\pm\) SE.
hippocampal slice cultures, and those of Papa and Segal (1996), who found that picrotoxin causes an increase in spine density and shortening of spine length in dissociated cultured hippocampal neurons. In the intact brain, Bundman et al. (1994) found that seizure activity produces new spines in the dentate gyrus. In mouse somatosensory cortex organotypic cultures, picrotoxin also was found to increase spine density in pyramidal neurons (Annis et al. 1994). These discrepancies may be due to the type of preparation employed in the different studies. However, it is also likely that cells in our slice culture are more intrinsically active, and the added blockade of inhibition does not make a morphological difference. In support of this are the results of the TTX experiment. TTX blocks action potential-related synaptic activity and could be expected to enhance the ability to express LTP for the same reasons as those of d-APV. In fact, TTX caused a different effect on both spine morphology and ability to express LTP than those of d-APV/DNQX. TTX prevented or markedly retarded morphological development of spines, characterized by abundant filopodia-like structures, akin to those of the immature cells in the slice culture (Annis et al. 1994; Cooper and Smith 1992). This structural difference may explain the difference in ability to produce LTP between the TTX- and d-APV-treated slices. At any rate, after development is completed in our mature cultures, there is no simple correlation between spine density and ability to generate LTP.

Although the present results were obtained in an in vitro slice culture preparation, they have clear implications with respect to the factors affecting the growth and maturation of dendritic spines in vivo. The type of studies conducted here, where cells are exposed chronically to drugs that affect their electrical activity, cannot be conducted easily in vivo. The gross morphological similarity between neurons in slice cultures and the ones in vivo encourages such an approach and adds validity to these results. These results are congruent with the previously discussed results obtained in the intact brain that show marked variations in dendritic spine density.

Roles of dendritic spines in neuronal integration

Numerous models and theories ascribed roles for dendritic spines in long-term plasticity and neuronal integration in mature animals (Bliss and Collingridge 1993; Gold and Bear 1994; Koch and Zador 1993; Wikens 1988). The fact that neurons respond to ambient afferent input by changes in spine density as well as spine morphology indicates that spines are under continuous afferent control. Hormonal factors, such as the estrus cycle in female rats, also have been shown to cause a 30% variation in spine density. Because of the pivotal role that elevation of intracellular calcium concentration plays in synaptic plasticity, dendritic spines can provide the anatomic substrate for temporal and spatial segregation of these signals. In fact, it has been shown recently that the spine is a unique calcium compartment, capable of accumulating high concentrations of calcium, relative to its parent dendrite (Guthrie et al. 1991; Muller and Connor 1991; Petrozzino et al. 1995; Segal 1995). Our results show a strong correspondence of the density of mature looking spines to the magnitude of LTP as it develops between 1 and 3 wk in culture. However, large changes in LTP magnitude also were observed in the absence of any significant change in spine density or morphology after picrotoxin treatment.

Any simple correlation between spine density or shape and LTP also is complicated by the fact that synaptic substrates for LTP do not seem to be restricted to dendritic spines. Malinov (1991) obtained LTP for EPSCs evoked by impulses in single CA3 neurons, where the number of spines involved may be very small. In fact, LTP-like plasticity in nonspiny synapses has been reported recently in immature cells (Durand et al. 1996). However, the ability to produce LTP can be strongly correlated with development and maintenance of neuronal architecture. Thus the slice culture offers an excellent controlled condition for a systematic analysis of factors regulating development and adult plasticity in a central network.

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