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Neurobiologia. — *A confocal laser scanning microscope approach to dendritic spine plasticity.* Nota di MICHELE PAPA, presentata (*) dal Socio G. Chieffi.

ABSTRACT. — We describe an original method using confocal laser scanning microscopy (CLSM) and the fluorescent marker DiI, to monitor dendritic spine morphology in cultures of rat hippocampal neurons. We performed a developmental analysis from the first to the fourth week in culture, and a functional study conditioning the culture medium with Tetrodotoxin (TTX) or Picrotoxin (PTX). The density of spines increased 2.7 fold between 1 and 3 weeks, while the mean length of the spines decreased from 1.6 to 1.1 μm. The ratio between the diameter of the spine head and the diameter of the spine neck increased, resulting in larger spine heads at 3 weeks. The TTX increased spine length and decreased spine density. PTX had the opposite effect. We hypothesize that spine density is a growth and aging related process, and not related to input. Spine formation seems to preceed and be independent of synaptogenesis which is in turn involved in spine shaping during maturation. Spine density seems to be directly and spine length inversely related to neuronal firing rate.

KEY WORDS: Dendritic spine; Confocal microscopy; DiI; Picrotoxin; Tetrodotoxin.

RIASSUNTO. — *Analisi al microscopio confocale laser della plasticità delle spine dendritiche.* Si descrive un metodo originale con l'uso del microscopio confocale laser a scansione ed il colorante fluorescente DiI, per studiare la morfologia delle spine dendritiche in culture di neuroni ippocampali di ratto. È stato studiato lo sviluppo in cultura dalla prima alla quarta settimana, e la risposta ad uno stimolo funzionale, condizionando il medium con Tetrodotossina (TTX) o Picrotossina (PTX). La densità delle spine aumenta di 2,7 volte tra la prima e la terza settimana, mentre la lunghezza media cala da 1.6 a 1.1 μm. Il rapporto tra il diametro della testa delle spine ed il diametro del collo aumenta, determinando teste maggiori alla terza settimana. La TTX aumenta la lunghezza delle spine, ne diminuisce la densità. La PTX esplica effetto opposto, la forma non cambia. Si ipotizza che la densità delle spine costituisce una funzione dei processi di crescita e di invecchiamento, e non delle afferenze. La formazione delle spine sembrerebbe indipendente e precedente la sinaptogenesi che a sua volta determinerebbe la forma delle spine durante la maturazione. La densità delle spine sarebbe direttamente, e la lunghezza inversamente, correlata all'attività neuronale.

INTRODUCTION

The response to a stimulus changes during a lifetime, inducing what is described as conditioning or learning. Since the end of the last century, it has been believed that learning and memory require morphological changes in the central nervous system (Ramon y Cajal, 1893; Tanzi, 1893), and considering that the signal runs through fibers and synapses, it seems logical to conclude that these elements form the framework within which morphological changes can occur. The structural transformations linked to the learning and memory processes known as engrams, have been demonstrated at the microscopic and molecular level (Ginty *et al.*,1992; Papa *et al.*,1993). Even if at the moment no proof can be considered definitive, memory traces seem to be stored in synaptic network, therefore the mechanisms underlying synaptic changes remain the main research focus. In many areas across the brain that are thought to be involved in the learning and memory process (Zola-Morgan and Squire, 1993), electron microscopy

(*) Nella seduta del 14 maggio 1994.

has revealed that dendritic spines constitute the main postsynaptic target of excitatory synapses in the CNS. These dendritic protrusions were first described by Ramon y Cajal (Ramon y Cajal, 1893, 1909). The neck of a dendritic spine sprouts from the dendritic shaft, becoming larger distally to form a terminal bulb known as the head. Electron microscopy has revealed that the dendritic shaft microtubules and neurofilaments do not penetrate into the dendritic spine, and most polyribosomes lie at the base of the spine. A fluffy material is displayed inside these structures and consists of different filaments, characterizing the spines: some are about 10 nm in diameter and are probably made of actin filaments, others are 5 to 7 nm in diameter and are probably composed of α and β tubulin (Peters *et al.*, 1991). An extension of smooth endoplasmic reticulum enters the spine from the cytoplasm of the dendritic shaft (Peters and Kaiserman-Abramof, 1970), producing a small tubular cisterna ending in the basic spine structure known as the spine apparatus. It consists of two or three flat smooth membrane-bound sacs alternating with bands of electron-dense material. Spines are present in many shapes: from the most prevalent «mushroom-shaped» to short and stubby or long and thiny, producing a continuous variation in the diameter of spine head and neck, and spine length (Wilson *et al.*, 1983). Neighboring spines on a neuron may be considerably different in size and shape. The difference in the morphology of neighboring spines raises the question of whether postsynaptic changes could account for changes in synaptic efficacy. In fact, larger spine heads are associated with larger synapses, as measured by the size of the associated postsynaptic density (PSD). Glutamate receptors/ion channels are involved in mediating synaptic transmission, and their number is directly correlated to the PSD. Several evidences demonstrate that pre- and postsynaptic changes are strictly associated. The presynaptic site volume is closely correlated to vesicle number and to the size of postsynaptic structures, including spines. At the moment the most widely accepted hypotheses on spine function include: spines functioning as connecting tissue, spines playing an electrical role, and spines playing a biochemical role (Amaral, 1987; Koch and Zador, 1993). Many data and hypotheses (Lisman and Harris, 1993) are supported by *in vivo* studies and serial electron microscopy reconstruction (Spacek, 1985; Stevens and Trogadis, 1984). The problem with the *in vivo* model is that there are too many unpredictable stimuli. The problem with the serial EM model is that it is very time consuming (Harris *et al.*, 1992) and only small sample sizes can be utilized which could be statistically non significant considering the high variability of spine shape (Braitenberg and Schuz, 1991*a*). These shortcomings leave many basic biological questions unanswered such as the changes in number, size and morphology in dendritic spines. We choose to approach a dendritic spine study with a controlled system of hippocampal neuronal culture (Segal and Manor, 1992) a confocal laser scanning microscope (CLSM) (Carlsson and Aslund, 1987; Cox and Sheppard, 1983) and a high yield fluorescent marker (Hosokawa *et al.*, 1992) with the scope of studying a statistically significant number of dendritic spines. Distinguishing morphological changes induced by normal development from those induced by learning processes compelled us to carry on two experiments: developmental and functional. In the developmental analysis, we observed spine parameters from the first to the fourth week. Afterwards,

we began the functional study, investigating spine behavior in two different conditions. We grew a group of cells in a medium conditioned by Tetrodotoxin, a toxin acting as selective blocker of Voltage gated Na channels which inhibits the action potential. Another group of cells were grown in a medium treated with Picrotoxin, a gamma-aminobutyric acid (GABA) chloride channel blocker capable of inducing seizure (Muller *et al.*, 1993).

Materials and methods

Tissue Culture.

Primary cultures of hippocampus were prepared from 19 day old, Wistar rat embryos. The hippocampus was dissected in chilled Leibovitz L15 medium enriched with 0.6% glucose and in the presence of 15 μg/ml gentamycin. The medium was oxygenated and the dissection carried out at 4 °C. The tissue was mechanically dissociated in small volumes of L15 using a fire polished pasteur pipette. Tissue was suspended in plating medium consisting of 5% fetal calf serum and 5% heat inactivated horse serum prepared in Eagle's minimal essential medium enriched with 0.6% glucose, 2mM glutamine and 15 μg/ml gentamycin. Neurons were plated on poly-L-lysine-coated (15 μg/ml) 13 mm round glass cover slips (# 1, Chance Propper, Ltd., UK), in 24-well culture plates, at a density of 500,000 cells per well. Three to four days after plating, the medium was changed to one containing 10% dialyzed horse serum. In addition, the first change of medium also contained a mixture of 50 μg/ml uridine, and 20 μg/ml 5'-fluoro-2-deoxyuridine to block the proliferation of glial cells. Thereafter, the growth medium was changed once a week, if necessary.

Confocal microscopy.

The cover slips containing the cultures were taken out of the 24-well plates, washed with phosphate buffer (PB) and fixed with 4% paraformaldehyde in PB for 30 min. Fixed cultures, in PB, were placed on the stage of an inverted phase microscope, and individual cells were stained with 1,1' dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI, Molecular Probes, Inc., Eugene, Oregon). The DiI was dissolved in halibut liver oil at saturating concentration, loaded in a 2-5 μm tip micropipette and applied by pressure ejection onto medium-size (10-20 μm) neurons (Hosokawa *et al.*, 1992). The coverslips were placed at 4 °C in small Petri dishes containing PB and 6-24 hr were allowed for transport of the dye before examination with confocal microscopy. A Leica, upright confocal laser scanning microscope (CLSM) equipped with a 488 nm argon ion laser and a 40×, 1.30 NA, oil immersion objective was used to visualize individual cells and dendrites. Optical serial sections of 0.3-0.6 μm were taken through the cells and reconstructed to yield complete «three-dimensional» images of individual cells in focus in a single plane.

Individual cells were randomly selected for analysis, and three or four images were taken from proximal, middle and distal regions of secondary dendrites. Only those DiI-

filled cells considered to have «spiny» dendrites were retained for quantitative analyses. For confocal microscopic analysis, dendritic spines were defined as singular dendritic appendages that protruded for relatively short distances ($< 7\,\mu m$) at roughly right angles from the dendrite. Such spine were clearly distinct from dendritic branches and bore no resemblance to growth cones. (Note: No large, multi-branched spines of the mossy fiber-CA3, «thorny excrescences» variety were seen in these cultures). From a selected image, $31 \times 31\,\mu m$, the length and the total number of spines on that dendritic length were measured using the CLSM Image Documentation and Analysis software (Leica Lasertechnik GmbH). In addition, the length, the diameter (long axis) of the head (Dh) and of the neck (Dn) were measured for each spine. For each sample, values were obtained for the number of spines per 10 μm of dendritic length and means ± ± S.E.M. for the length of spines, and diameters of the spine heads and necks. Comparison between groups were made using one-way analysis of variance (ANOVA).

Developmental study.

At one to four weeks following plating, cover slips containing the cultures were taken out of the 24-well plates, washed with PB and fixed with 4% paraformaldehyde in PB for 30 min, then processed as described in the previous section.

Functional study.

Two and half week old cultures, were divided into three groups. In group 1 the medium was changed to a new one containing 0.5 μM Tetrodotoxin (TTX, Sigma Chemical Co.), in group 2, 10 μM of Picrotoxin (PTX, Sigma Chemical Co.) was added to the medium, the cell culture of the third group received unmodified new medium as described previously. Cover slips containing the cultures were washed with PB and processed as previously described after 3 hr, 6 hr, 24 hr, 3 day and 6 day intervals following medium change.

Results

Rat hippocampal cultures consisted primarily of pyramidal-like, medium sized neurons (10-20 μm in diameter) with two to four long (over 100 μm) dendrites extending from the somata. Some cells were large and multipolar, with many dendrites lacking a distinct polarity. Some small round neurons ($\sim$ 10 μm in diameter) with a single primary dendrite, resembled dentate gyrus, granule cells. Most of the labeled cells belonged to the first category.

Developmental study.

The following measurements were made from a single series of cultures grown under identical conditions for 1 to 4 weeks. Preliminary experiments were conducted with 3 to 4 series of cultures and yielded similar values for spine dimensions and density . A total of 45,583 μm of dendritic length were analyzed and a total of 11,063 spines were

measured in cultures from one to four weeks of age. The totals were composed of 509 separate dendritic segments from the one week cultures and 529, 390 and 292 dendritic segments from the 2, 3, 4 week cultures, respectively. In each case the dendritic segments were taken from thirty to forty neurons. The analysis of spine morphology was obtained from 1529 spines in the one week cultures and 3087, 3829 and 2618 spines in the two, three and four week cultures, respectively.

As shown in fig. 1, there was a large, steady increase in the density of dendritic spines between week one and week three in culture resulting in 2.7 times as many spines per 10 μm of dendritic length. However, at four weeks in culture the density of spines declined. Spine densities increased from 1.3 spines/10 μm at one week to 2.2, 3.5, and 2.9 spines/10 μm of dendritic length at two, three and four weeks, respectively. The percentage of DiI-filled neurons that had «aspiny» dendrites was fairly constant from one to three weeks (8-5%). However, in the four week culture, 30% of the cells had «aspiny» dendrites. Included in this latter population were some neurons that had only 20-30 spines scattered over the entire dendritic arbor. The density of spines on dendrites did not appear to vary as a function of the density of cells in the culture dish; as dendrites with a high density of spines were sometimes found on cells that were fairly isolated in the culture.

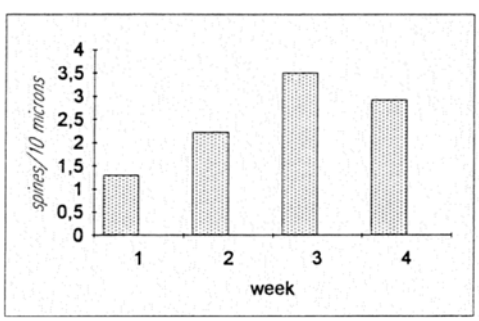


Fig. 1. – Change in spine density over time. Each column represents the total number of spines divided by the total dendritic length at each time point.

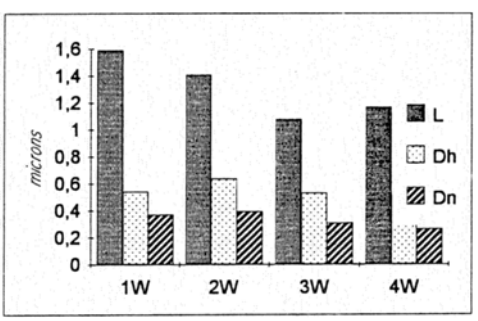


Fig. 2. – The change in dendritic spine length (L), in the diameter of the spine head (Dh) and in the diameter of the spine neck (Dn) from 1 to 4 weeks.

The analysis of spine shape revealed significant changes in morphology from one week to three week old cultures (fig. 2). Over the course of one to three weeks in culture, spines generally became shorter and developed narrower necks and, thus, more prominent spine heads. Little change occurred in the morphology of spines from three to four weeks. The most apparent change was in the length of spines. The average length of dendritic spine decreased significantly ($p < 0.001$) from 1.6 ± 0.03 μm at one week, to 1.4 ± 0.01 at two weeks, 1.1 ± 0.01 μm at three weeks and 1.21 ± 0.01 μm at 4 weeks in culture.

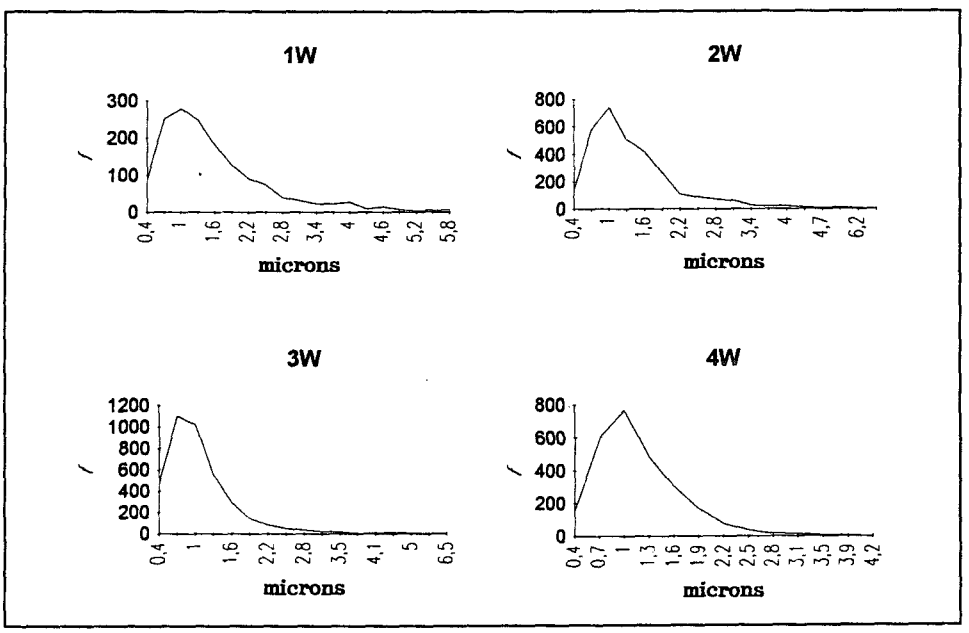


Fig. 3. – Frequency distributions for the spine length data presented in fig. 2. Bin widths are 0.3 μm and the number of spines in each category is expressed as the percent of the total spines at the various time points. The *x*-axes (spine length) have been truncated at 5 μm.

In addition to the changes in average spine length across the various ages, there were also differences in the variability in spine length within the age groups. In the younger cultures, the spine population was highly variable. As fig. 3 demonstrates, this was evident in the shape of the spine frequency distribution profiles. Most of the variability existed in spines among different neurons and not in spines within the same neuron. This was most apparent in the two week cultures, where there were sometimes cells that looked like those in one week old cultures, as well as cells that looked like those in three week cultures in proximity to one another. While there did not appear to be any obvious correlation between the sizes or shapes of neurons and the length of their dendritic spines, there did appear to be a correlation between density and spine length. That is, in the two week old cultures, the cells with long spines had low spine densities and the cells with shorter, three week-like spines, had a high density of spines. At three and four weeks the length of spines was far more homogeneous.

While there was a significant but small change in the average diameter of spine heads, the diameters of spine necks decreased from 0.37 μm at one week, to 0.31 μm at three weeks and 0.26 μm at four weeks. This resulted in spine head to spine neck diameter ratios of 1.7 at one week and ratios of 2.1, 2.4, and 2.5 at two, three and four weeks, respectively (fig. 4).

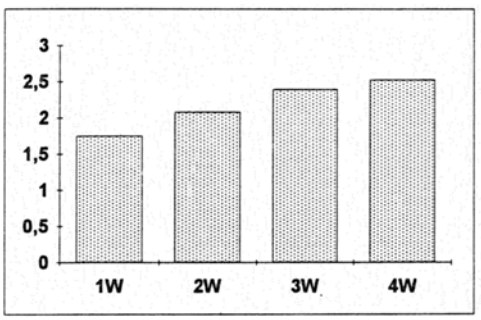


Fig. 4. – Each column represents the ratio of mean value of the diameters of spine heads (Dh) and necks (Dn). The increase in Dh/Dn leads to more prominent spine heads at 3 and 4 weeks.

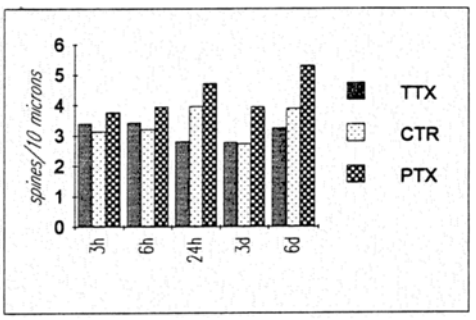


Fig. 5. – Columns represent change in spine density over time. Each point represents the total number of spines divided by the total dendritic length at each time point.

Functional study.

A total of 36345 μm of dendritic length was analyzed consisting of 13414.9 μm of the TTX treated cells; 10611.4 μm of the PTX conditioned medium; and 12318.7 μm of the control group. The morphology of 12819 spines was obtained from 4097 spines of the TTX group, 4618 spines of the PTX group and 4104 spines of the control group. As shown in fig. 5, the behavior of spine number/10 μm is very distinct in the three different group: PTX induced a noteworthy effect on the number of spines which rose from 3.7 to 5.3 spines/10 μm after 6 days, being at every time larger than the control and TTX groups. The control group values range in the mean for the developmental study: 3.1 to 3.8 spines/10 μm. The TTX group fell at 24 hrs to a value of 2.8, remaining at this value for 3 days and reaching 3.2 spine/10 μm at 6 days, a very low value compared to control and PTX group.

The analysis of spine length revealed dramatic changes with a maximum peak at 24 hrs. These changes are still evident up to the sixth day (fig. 6). The TTX group showed the most significant increase of spine length, ranging from 1.5 μm at 3 hrs, to 1.6 μm at 6 hrs to an impressive 2.3 μm at 24 hrs, resembling only for the extreme length the filopodial-like structure described in the developmental study in the one week old culture. The length reached 2.1 μm at day 3 and 1.76 μm at day 6. Even though the control group was slightly larger than the related group in the developmental study, the length remained at an almost constant value of 1.5 μm. The PTX group showed a time-related behavior comparable to TTX: it descended to a value of 1.23 μm at 24 hrs remaining at a value (1.25 μm) lower than the control group until the sixth day. The maximum peak (24 hrs) can be demarcated most clearly by analyzing the percentage length variation (PLV) at that time. This value was about 58% more for the TTX group and 17% less for the PTX group than the control group (fig. 7). After 24 hrs the effect of TTX decreased and the PLV reached a value of 16% more than the control at the 6th

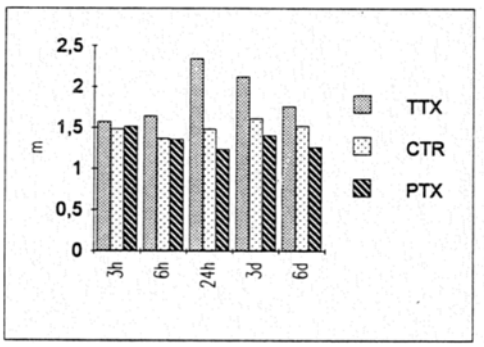


Fig. 6. – Histogram showing dendritic spine length of control (CTR), Tetrodotoxin (TTX) and Picrotoxin (PTX) conditioned cell culture, at different time point.

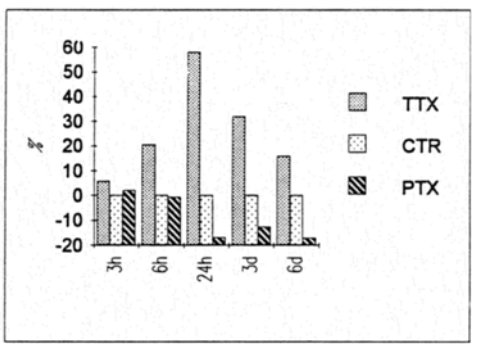


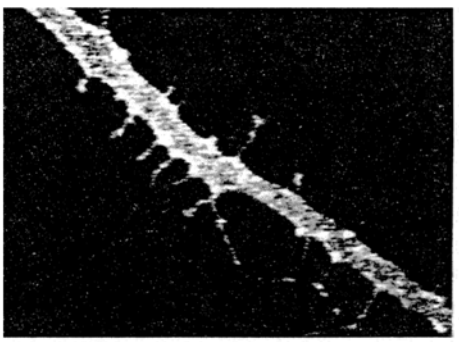
Fig. 7. – Histogram shows percentage length variation between Tetrodotoxin (TTX) and Picrotoxin (PTX) conditioned cultures, compared to control (CTR) at different time point.

day. The PTX group values remained constant during the experiment. No estimable change can be perceived analyzing the Dh/Dn ratios, the values for all three groups range between the minimum mean of 1.63 μm of TTX group, the 1.69 μm of the PTX group and the maximum mean of 1.76 μm of the control group.

Discussion

As previously outlined, many *in vivo* studies have revealed spine plasticity. Spines have been shown to differentiate naturally during development (Braitenberg and Schuz, 1991*b*), to undergo a cyclic fluctuation across the estrous cycle (Woolley *et al.*, 1990), to be modified by hibernation (Popov and Bocharova, 1992) and even by protein malnutrition (Brock and Prasad, 1992). The spines have been demonstrated to change in response to a stimulus (Fifkova and Anderson, 1981; Benes and Vincent, 1991), to maintain long term potentiation (LTP), and to play a role in the Hebbian synapse which has been postulated to underlie learning (Zador *et al.*, 1990). In spite of many evidences, the basic mechanisms underlying plastic structural changes remain completely unclear. At this end, our model seems adequate to approach this biological

Fig. 8. – Confocal laser scanning images of DiI-filled hippocampal neurons in culture. 1-4. Examples of dendrites from one to four week cultures, respectively, demonstrating the differences in the density and morphology of dendritic spines. The dendritic protrusions seen at one week (1) are very long without prominent spine heads. At two and three weeks (2, 3), the spines are shorter and have narrower necks, giving the appearence of larger spine heads. The density of spines increases from one to three weeks. At four weeks (4) spine heads are not as prominent and spine density has decreased. In TTX (5) treated culture, dendrites show sparsely very long spines, in PTX (6) the spines become shorter and the density of spine decreased. (7) Low-power image of a typical medium-sized, pyramidal-like neuron. (8) Image showing many finger-like protrusions (arrow heads), which come out from spine head. Perhaps they were left over after synaptic contact and head formation. ▶



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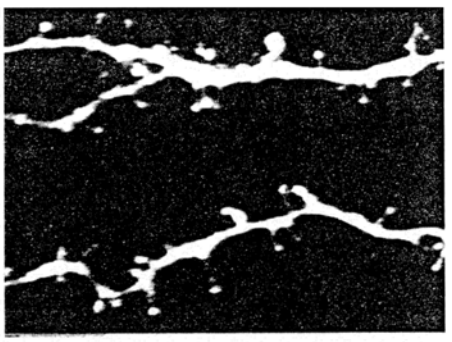

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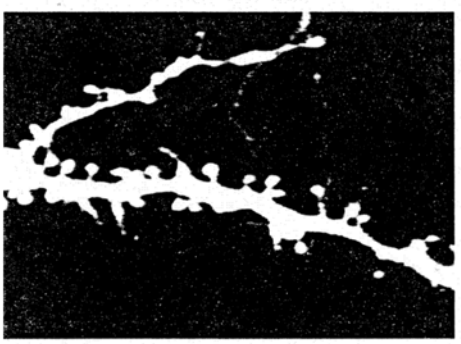

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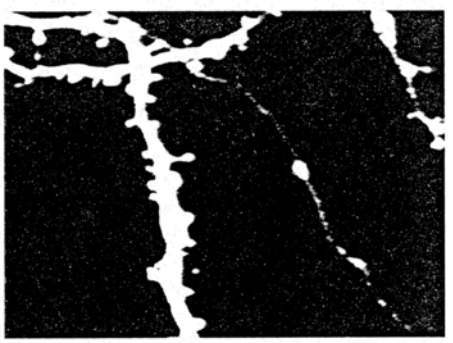

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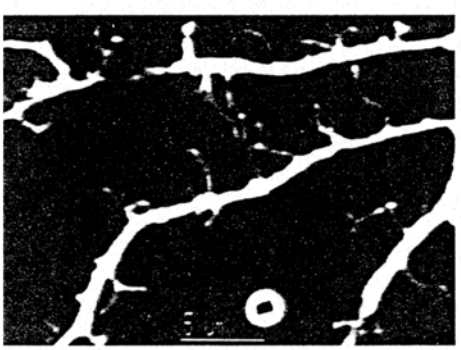

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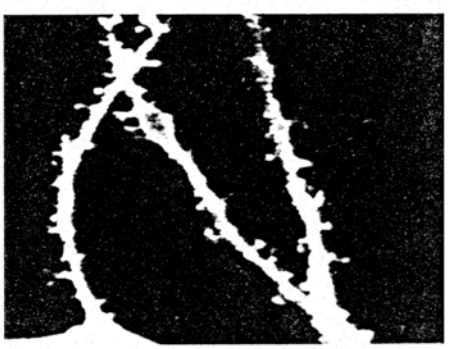

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problem in a simple, feasible and statistically significant way. Several reports have characterized hippocampal and cortical neuron behavior in culture, describing dendritic spine morphology by transmission or scanning electron microscope (Ray *et al.*, 1993; Harris and Rosenberg, 1993). In cell culture we identified presynaptic terminals by synaptophysin immunocytochemistry and described dendritic spine ultrastructure (Papa *et al.*, 1994). With DiI and CLSM we developed a simple method which allowed us to measure a huge number of spines and to unequivocally assess spine size and shape changes. Spines and dendrites seem to be electrically coupled (Wickens, 1988) but calcium regulation independent (Guthrie *et al.*, 1991). The highly chemically defined cell culture conditions assure the correct approach in revealing modifying input. The first outcome in the developmental study was in dendritic spine density, which increased as a function of growth. This data trend confirmed the *in situ* EM study, also revealing a peculiar discrepancy in density. *In vitro* there were one third less spines than *in situ*. This difference demonstrates the existence *in vivo* of mechanisms and/or conditions lacking *in vitro*. Although it is possible that cell dissociation could account for the alteration in the normal rate of spine proliferation, other explanations should be considered: *i*) *In vitro*, the neurons do not receive the same input as *in situ*, and this could have induced the reduction of spines. *ii*) The chemically defined medium lacked the increase of some constituent in a very close time window (*e.g.* sexual hormones or growth factors rise during embryonic life). The decrease in spine density and in the number of cells with spiny dendrites at four weeks may be indicative of an aging process in culture. These very sparsely located spiny cells were seen only at four weeks and probably indicate that cells tend to begin losing spines between 3 and 4 weeks in culture. The mechanisms which control the density of spines are probably not related to afferent inputs to the spines because, as mentioned above, some very spiny cells were found quite isolated from other cells in the culture dish. We did not find any relationship between spine density and cell density. A major question, yet unresolved, concerns the origin of new spines. It has been postulated that a synaptic contact which forms on a dendritic shaft could induce the origin of a stubby spine, which in turn may change shape (Harris *et al.*, 1992). In our study, in one week old culture, we found many protrusions with the same internal ultrastructure as spines, that did not show any synaptic contact and that we defined as filopodia-like (Papa *et al.*, 1994). The high number of these protrusions, the change in neck shape during maturation, and many peculiar confocal images (fig. 8, *8*) led us to formulate another hypothesis. The spines originate and grow during cell development, which correspond to low cell activity (consider functional results). Several synaptic contacts will induce cell activity and morphological change in primitive spines. The mature spine can transform into any of the shapes previously described. Time-lapse recordings of hippocampal neurons in culture have demonstrated that once stable contacts are made between axonal growth cones and pre-existing, target cell filopodia, the filopodia shrink (Cooper and Smith, 1992). Concerning the relationship among neuronal plasticity and cell function, we have demonstrated that neuronal firing increases the length of the spine and decreases the spine density. On the contrary, a seizure causes a decrease in spine length and an increase in dendritic spine density. The

action potential has no effect on spine morphology. This phenomenon could be accounted for by two different mechanisms. The first mechanism is one in which the spine length is dependent on neuronal firing. The other is dependent on a developmental process, probably related to synaptogenesis which shapes the dendritic spine neck. The PTX experiment demonstrates that an increase in spine density is possible even in the absence of a change in spine shape. Since the change in spine morphology seems to be strictly related to synaptogenesis we can hypothesize that the variation of spine density could be, as in this case, existent even in absence of synapse formation. This could mean that increases in spine density could precede synaptogenesis. Our new approach, utilizing a confocal microscope and highly fluorescent membrane probe, should help in obtaining more definitive data in future studies. We will continue investigating questions regarding the biochemical and structural properties of spines and the dynamics of interaction with their cellular and chemical environment.

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