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Dendritic plasticity in the barrel cortex of postnatal rat.

par

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Dendritic plasticity in the barrel cortex of postnatal rat.

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ABSTRACT

The rat barrel cortex (SI) offers an excellent opportunity to study mechanisms related to experience-dependant plasticity, due to the discrete groups of neurons (termed "barrels") in layer IV which correspond on a one-to-one basis to the vibrissae of the contralateral facial pad. When we removed whisker rows B, C, and D from the right facial pad of rats during the initial two months of life, we were able to analyze the impact that peripheral deprivation had on the total number of dendritic spines and as well the morphological changes (i.e. volume and surface area of spine head, surface area of post-synaptic density, and length of spine neck) that occured as a result. Finally, in an attempt to better understand the role that the GABA system represents, the morphometrics for the population of spines innervated by GABA synapses were collected and compared to the overall population of spines. The method used to determine spine number was Sterio's unbiased disector method, and the material used was from electron micrographs of serial thin sections which were processed for post-embedding GABA immunocytochemistry. A computer and software reconstruction system was utilized to produce three-dimensional models, for the calibration of the different spine constituents. From this data we found that the spine head dimensions were significantly greater in the ipsilateral compared to the contralateral cortex of experimental animals for the both the overall spine population and the population of spines innervated by GABA synapses. Furthermore, in view of a previously documented increase in the volume of layer IV in the ipsilateral cortex, we were able to conclude that the total number of dendritic spines in the ipsilateral barrels had increased by 67% compared to contralateral cortex. The proposed explanation for these anatomical alterations in layer IV of the ipsilateral barrel cortex is a resultant

possible mechanisms for this may be early activation of thalamic afferents, resulting in increased production of trophic factors, such as BDNF.

SOMMAIRE

Un nombre croissant de données expérimentales, acquises chez des mammifères adultes ou en croissance, suggère que le nombre, la forme et les relations synaptiques des épines dendritiques des neurones du cerveau puissent subir des changements considérables sous l'influence de facteurs environnementaux. Le cortex somatosensoriel des rongeurs s'avère un modèle de choix pour l'investigation des mécanismes moléculaires et cellulaires qui sous-tendent cette plasticité neuronale. En effet, la couche IV de cette région du cortex cérébral est constituée de groupes de neurones bien définis (les barillets), qui correspondent un-à-un aux vibrisses de la moitié contralatérale du museau de l'animal. Au cours de leurs travaux récents sur le développement des neurones gabaergiques dans le cortex à barillets du rat, Micheva et Beaulieu ont mis en évidence une diminution importante du nombre des synapses axo-épineuses immunoréactives pour ce transmetteur dans les barillets correspondant à trois rangées de vibrisses épilées depuis la naissance jusqu'à l'âge de deux mois. En conséquence, ces auteurs ont supposé que les synapses axo-épineuses à GABA puissent jouer un rôle particulier comme déterminants fonctionnel des phénomènes de plasticité neuronale dépendante de l'activité observés lors de la corticogénèse. Dans le même matériel expérimental, nous avons voulu examiner les effets de la désafférentation périphérique sur le nombre total et la morphologie des épines dendritiques des barillets ipsi- et contralatéraux, comparativement à du cortex témoin. Les épines ont été dénombrées par la méthode sans biais du dissecteur, appliquée à des micrographies en microscopie électronique de coupes fines sériées préparées pour l'immunocytochimie du GABA avec un marquage à l'or en post-enrobage. Le volume et la surface de la tête des épines, la longueur de leur cou et la

dimension de leurs densités postsynaptiques ont été mesurées à partir de reconstitutions tridimensionnelles obtenues à l'aide d'une station informatisée de reconstruction des volumes. Cet échantillon a été comparé à un nombre plus restreint d'épines dendritiques afférentées par des terminaisons GABA. L'hypothèse de travail voulait que la diminution de stimulation sensorielle secondaire à l'élimination des vibrisses s'accompagne d'une réduction de taille de la tête des épines dendritiques ainsi que de leurs spécialisations membranaires postsynaptiques, avec allongement de leur cou, dans les barillets correspondants (contralatéraux). De tels changements avaient en effet été précédemment décrits dans diverses régions du cerveau par suite de déprivation sensorielle expérimentale ou pathologique. On pouvait également supposer que la densité des épines diminuerait dans le cortex désafférenté par rapport au côté ipsilatéral ou au cortex des rats témoins. Mais contrairement à nos attentes, tous les changements statistiquement significatifs sont survenus dans le cortex ipsilatéral à la désafférentation. De plus, même s'il n'y avait pas de différence de densité numérique des épines (nombre par unité de volume) du côté ipsi par rapport au côté contra chez les animaux épilés, une augmentation précédemment documentée de l'épaisseur (et donc du volume) de la couche IV de ce côté nous a permis de conclure que le nombre total des épines dans les barillets ipsilatéraux était de 67% supérieur à celui du côté contralateral. En outre, la taille de la tête des épines et la surface de leurs densités membranaires postsynaptiques était augmentées, et la longueur de leur cou diminuée, du côté ipsi par rapport au contra, alors que les mêmes différences se retrouvaient entre le cortex ipsilatéral et le cortex des rats témoin. Enfin, ces changements semblaient affecter non seulement la population fortement majoritaire des épines dendritiques relativement petites innervées par des terminaisons axonales à synapse asymétrique, mais aussi le petit contingent d'épines plus grandes

innervées par des terminaisons GABA. Pour expliquer de tels changements ipsilatéraux, on ne pouvait que postuler une utilisation accrue par l'animal de ses vibrisses du côté intact du museau au cours d'une période critique du développement du cortex, en accord avec de nombreuses données démontrant que l'activation d'une voie sensorielle périphérique durant la période néonatale peut avoir des effets considérables sur la morphologie et l'organisation structuro-fonctionnelle du cerveau adulte. Un mécanisme possible dans le cas présent pourrait être l'activation précoce et excessive des afférences thalamiques induisant une production accrue de facteurs trophiques comme le BDNF (" *brain-derived neurotrophic factor* "), lequel est exprimé, de même que ses récepteurs, dans le cortex à barillets en développement. Le cortex à barillet du rat soumis à l'épilation unilatérale des vibrisses de la moustache durant quelques semaines après la naissance s'avère donc un modèle animal éminemment favorable à l'identification et la caractérisation moléculaire des facteurs responsables de la plasticité neuronale dépendante de l'activité au cours de la maturation du cortex cérébral.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

DAB.....3,3' - diaminobenzidine tetrahydrochloride

E..... embryonic day

EPSP.....excitatory post-synaptic potential

GABA.....gamma-aminobutric acid

N_V.....number per unit volume

N_T.....total number

LTP.....long-term potentiation

NGS.....normal goat serum

P.....postnatal day

PBS.....phosphate-buffered saline

PMBSF.....posteromedial barrel subfield

PSD.....post-synaptic density

SI..... primary somatosensory cortex

TBS.....Tris-buffered saline

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CHAPTER 1

GENERAL INTRODUCTION

> *One might suppose that cerebral exercise, since it cannot produce new cells, carries further than usual, protoplasmic expansions and neural collaterals, forcing the establishment of new and more extended intercortical connections (Cajal, 1895).*

Contrary to the traditional belief, there is increasing evidence to indicate that the brain is a plastic organ, capable of responding in a sensitive and adaptive manner to the sensory environment. Documented effects of experience on cerebral structure include changes in weight and dimension of whole brain or certain brain regions, thickness of cortex, neuron and glial cell number, and neuronal morphology, whether of cell bodies, dendrites, axon terminals (varicosities) or synapses (e.g., Jarvinen et al., 1998). Our work has dealt primarily with dendritic spines. Hence, the present introduction will focus on dendritic plasticity. Indeed, as stated by Greenough and Bailey (1988): *"The degree of dendritic branching, the length of individual dendritic branches, and the frequency of dendritic spines are all modified by experience and probably represent the growth of new synapses".*

I.1 Dendritic plasticity in the brain

As early as 1895, Cajal had speculated that dendritic arborization ("protoplasmic expansions") might increase after exposure to an enriched environment. Only in the sixties, however, did experimental data begin to substantiate this prediction. Holloway (1966) was the first to demonstrate

increased branching of the dendritic tree of occipital stellate neurons, in adult or newborn rats placed for 80 days in an environment rich in sensory cues. Soon after, Schapiro and Vukovich (1970) were able to show that the number of dendritic spines on pyramidal neurons of the occipital cortex was increased in rat pups exposed for eight days to multimodal stimulation immediately after birth. Confirmatory results on the effect of an enriched environment on dendritic plasticity were subsequently published by Valverde (1971), Volkmar and Greenough (1972), Rutledge (1976), and more recently by Venable et al. (1989). In 1978, Floeter and Greenough demonstrated that Purkinje cells in the cerebellum of monkeys reared for six months in complex colonies had more extensive dendritic arbors than either social or isolated animals, whereas the granule cells did not demonstrate the same plasticity. Evidence for experience-dependent plasticity was also reported in cortical regions other than visual cortex (e.g. Globus et. al., 1973; Greenough et. al., 1973; Uylings, 1978; Fiala, 1978). In addition, numerous reports dealt with dendritic spine plasticity in the hippocampus, following tetanization and long term potentiation (see Discussion of Chapter 2 for references). In area CA1 of the hippocampus, Berman et. al. (1996) were even able to demonstrate suppressive effects of alcohol intake on dendritic plasticity secondary to an enriched environment. Their study showed that, in contrast with non alcohol-exposed rats, enrichment failed to increase spine density in alcohol-exposed rats, while, in isolated animals, there were no significant differences between the control and the alcohol-exposed group. These authors concluded that alcohol exposure decreased postnatal, activity-dependent neuronal plasticity. Although data from human is limited, there have been some studies suggesting effects of the environment and/or of experience on dendritic morphology. For example, Scheibel et. al. (1985) reported that dendrites in the opercular region of the

frontal lobe (Broca's speech area) had a larger proportion of their lengths made up of higher order branches in the dominant hemisphere, whereas this pattern was partially reversed in non-right-handed patients. Another intriguing study from the same laboratory (Jacobs et. al., 1993) reported slightly greater dendritic length (but also variability), in females as compared to males across the adult age range examined. Length of dendritic branches was also claimed to be increased as educational level increased... Although methodological problems may complicate the interpretation of these studies, it is generally agreed that, at least during a critical period, rearing the young in a complex environment will influence dendritic arborization and the number of dendritic spines, at least in primary areas of cerebral cortex (for others reviews, see Rosenzweig et. al., 1972; Rosenzweig et. al., 1972; Walsh et. al., 1980).

I.2 Neuronal plasticity in barrel cortex

Many of the studies which have provided neuroscientists with some understanding of the morphological changes that take place during development or adaptive plasticity of the cortex were based on observations in the visual system. Another group of investigations has taken advantage of the discovery, made by Woolsey in 1967, of barrels in the rodent somatosensory cortex to seek information on developmental cortical plasticity, and as well, activity dependent plasticity. Since layer IV of the primary sensory cortex of rodents contains well defined neuronal aggregates (barrels) which correspond in a one-to-one fashion to the vibrissae, it has been possible to observe the morphological and physiological events that take place when whiskers are stimulated or deprived of sensory information (for review see Simons et. al., 1989; Simons and Land, 1987; Squire, 1987; Fox, 1992). One study conducted by Van der Loos (1973) demonstrated that if the whiskers of mice

are lesioned during a `critical period` (1 to 5 days after birth), dramatic alterations in the cytoarchitecture of the barrels occur. It was shown that barrels corresponding to lesioned whiskers failed to develop while adjacent barrels extended into the deprived cortical areas. Similar results were reported for the barrel cortex of the rats (Van der Loos and Woolsey, 1973; Killackey et. al., 1976; Woolsey and Wann, 1976; Jeanmonod et al., 1977). A number of subsequent studies dealt with other structural changes observed after peripheral denervation. The morphological reorganization of neurons observed in the barrel cortex of rat is accompanied by a reorientation of the dendrites, from the affected zones, into active barrel rows (Harris and Woolsey, 1981), or, as reported by Steffen and Van der Loos (1980), by a loss of the tendency of the barrel dendrites to be oriented toward the center of the barrel.

In order to avoid any confounding effects of nerve degeneration, cell death and primary afferent regeneration resulting from whisker lesioning, subsequent studies proceeded by simply trimming the whiskers of new born rat pups. When the whiskers are trimmed, no gross morphological changes appear in the barrels, yet functional plasticity follows (Weller and Johnson, 1975; Hand, 1982). Thus, Simons and Land (1987) as well as Fox (1992) went on to demonstrate that neurons in the barrel cortex of neonatally sensory deprived animals have higher spontaneous activity and altered receptive-field properties, such as enlarged receptive fields, reduced angular tuning and modified temporal patterns of stimulus-evoked discharges.

I.3 Quantitative morphometric studies in barrel cortex

The GABA neurotransmitter system has been suspected to play a crucial role in functional plasticity. In an attempt to document this role, Micheva and Beaulieu (1995a,b) used postembedding GABA immunoelectronmicroscopy in serial sections, combined with an unbiased stereological method, in the barrel field cortex of adult rats following unilateral removal, from birth, of three rows of whiskers. In a first study, the numerical density, proportion and size of GABA immunoreactive neurons was investigated (Micheva and Beaulieu, 1995a). The results showed a significant decrease in density and proportion of GABA-immunoreactive cell bodies in layer IV, contralateral to the deprivation. Moreover, the remaining GABA-immunoreactive neurons were significantly larger when compared to the ipsilateral barrel field cortex. Besides, the barrel cortex ipsilateral to deprivation had a greater number and proportion of GABA-immunoreactive cell bodies, when compared to both sides of the controls. The conclusions drawn from these initial results were that unilateral sensory deprivation induces highly selective changes in the intracortical GABA inhibitory circuitry of both hemispheres. The other study conducted by Micheva and Beaulieu (1995b) examined the "overall synaptic" and as well the "GABA synapse" population in each layer of the somatosensory barrel field cortex of the rats deprived or not from birth. The results demonstrated that there were no statistically significant quantitative changes in the overall synaptic population for all layers, but a decreased number and proportion of GABA synapses in layer IV. GABA synapses contacting dendritic spines were reported to show as much as a two-thirds reduction. These changes imputable to changes in expression of the GABA phenotype were viewed to be a possible explanation for the altered electrophysiology observed in the barrel field cortex after sensory deprivation.

I.4 Purpose of the present study

The aim of the present study was to further investigate the anatomical alterations that occur at the ultrastructural level in layer IV of the rat somatosensory cortex, and specifically those that might affect dendritic spines within the barrel cortex, as a result of varying sensory experience. Using the same material examined for GABA cell bodies and synapses by Micheva and Beaulieu (1995), we determined the influence of the peripheral postnatal deprivation on the number and shape of dendritic spines contacted or not by GABA synapses. The numerical density (N_V) of dendritic spines (number per unit volume) from the deprived hemisphere and non-deprived hemisphere of sensory deprived rats was measured and compared to that in control rats. In a second step, the dimensions of dendritic spines (i.e. volume, surface area of the spine head, surface area of synapse, and length of spine neck), were obtained from computer reconstructed three-dimensional images and comparisons were made between hemispheres and as well to control animals. It was of considerable advantage that the cortical thickness and the surface area of the barrel cortex had been previously measured by Micheva and Beaulieu (1995). Thus, the numerical data could be expressed not only as density but also as "true" number per volumetric unit of tissue. It was expected that the peripheral deprivation might entail some decrease in the number of dendritic spines in the corresponding cortex, and perhaps a compensatory increase in spine head size. Unexpectedly the most striking changes took place in the ipsilateral ("non-deprived") cortex.

CHAPTER 2

Increased Number and Size of Dendritic Spines in Ipsilateral Barrel Field Cortex Following Unilateral Whisker Trimming in Postnatal Rat

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INCREASED NUMBER AND SIZE OF DENDRITIC SPINES IN IPSILATERAL BARREL FIELD CORTEX FOLLOWING UNILATERAL WHISKER TRIMMING IN POSTNATAL RAT

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ABSTRACT

The barrel field area of the primary somatosensory cortex of rodents is a fertile ground for investigating experience-dependent plasticity and its mechanisms, because the neurons in its layer IV are distributed in groups (barrels) which correspond somatotopically to the vibrissae of the contralateral facial pad. After removal of three rows of whiskers from the right facial pad of young rats during the first two postnatal months, we looked for eventual changes in dendritic spine number and morphology in the corresponding barrels ipsi- and contralateral to the deprivation. Intact littermate controls were also examined. Spine number was determined by means of the unbiased disector method in electron micrographs from serial thin sections processed for post-embedding gamma-aminobutyric acid (GABA) immunocytochemistry. The volume and surface area of spine head, surface area of postsynaptic density and length of spine neck were measured from computerized three-dimensional reconstructions. Even though there was no significant side-to-side difference in the numerical density of dendritic spines in the experimental animals, the total number of spines in the ipsilateral barrels had increased by 67%, in view of the greater thickness of layer IV on this side. Moreover, spine head volume and surface area of postsynaptic densities were increased, and the length of spine neck was reduced in the ipsilateral compared to the contralateral cortex, and similar differences were noticeable between ipsilateral and control cortex. These changes apparently involved not only the predominant population of relatively small, dendritic spines innervated by asymmetrical synaptic terminals, but also the relatively small contingent of larger spines receiving symmetrical synapses formed by GABA terminals. The most likely explanation for such ipsilateral changes was an increased use of the intact (contralateral) facial pad

during postnatal life. This would be in keeping with the notion that activation of a peripheral sensory apparatus during the early postnatal period may have profound effects on the neuronal morphology and structural design of the primary somatosensory cortex. A possible mechanism in this case might be the excessive early activation of thalamic afferents, resulting in increased production of trophic factors, such as brain-derived neurotrophic factor.

INTRODUCTION

First described by Ramon y Cajal (1891) and visualized by electron microscopy by Gray (1959), dendritic spines represent a major site of connection between axonal and dendritic processes in the central nervous system. Biophysical and biochemical properties of dendritic spines are the subject of active investigation. It is currently believed that the shape of dendritic spines provides a favorable (protective) biochemical microenvironment for synapses, by confining to the spine head changes in the concentration of calcium and of second messengers, enzymes and structural proteins implicated in synaptic function (e.g., Gamble and Koch, 1987; Koch and Zador, 1993; Harris and Kater, 1994). Changes in the shape of spine head and spine neck may also influence synaptic strength (e.g., Rall and Rinzel, 1973; Miller et al., 1985; Perkel and Perkel, 1985; Segev and Rall, 1988). Both views account for much of the interest in documenting alterations in dendritic spine morphology (morphological plasticity) associated with animal behavior and experience and/or the electrophysiological activation of specific neuronal circuits.

There is considerable evidence to suggest that dendritic spine morphology is responsive to behavior and experience in mammalian cerebral cortex (e.g., Schapiro and Vukovich, 1970; Globus et al., 1973; Freire, 1978; Green et al., 1983). An interesting animal model for investigating such neuronal plasticity is the rodent barrel field cortex (for review, see Kossut, 1992). It has been known for more than 25 years that the neurons in layer IV of the primary somatosensory cortex (SmI) of some rodents are arranged in clusters which constitute the central representation of the vibrissae on the contralateral whisker pad (Woolsey and Van der Loos, 1970; Welker, 1976). The system is entirely crossed (Durham and Woolsey, 1984). The barrel neurons show distinct

receptive field properties and respond to specific displacements of the corresponding whiskers (Simons, 1978; Simons and Land, 1987). During the first few days after birth (critical period), destruction of the vibrissae follicles has marked effects on the organization of the barrels in the contralateral cortex: these fail to develop altogether, whereas neighboring barrels expand toward the deprived area (Van der Loos and Woolsey, 1973; Woolsey and Wann, 1976; Killackey and Belford; Harris and Woolsey, 1981; Jeanmonod et al., 1981; Welker and Van der Loos, 1986). In contrast to these drastic effects of peripheral denervation, sensory deprivation by simply removing the whiskers without damaging their follicles does not cause obvious changes in the cytoarchitectonics of the corresponding barrels (Weller and Johnson, 1975; Hand, 1982; Simons and Land, 1987; Akhtar and Land, 1991; Fox, 1992). It does however result in important physiological changes. Thus, Simons and Land (1987) have reported an increase in spontaneous and stimulus-evoked responsiveness and a concomitant decrease in fine tuning of receptive fields in rats subjected to chronic trimming of the whiskers from birth. Moreover, in adult rats subjected to this procedure until 45 days of age, a persistent inability to master some tactile discrimination tasks has been documented (Carvell and Simons, 1996).

In this same experimental model, Micheva and Beaulieu (1995a,b) have recently demonstrated considerable reductions in the number of neuronal cell bodies and axo-spinous synaptic terminals immunoreactive for gamma-aminobutyric acid (GABA) in layer IV of the contralateral, peripherally-deprived barrel cortex. These changes were taken as an indication that altered local inhibitory mechanisms were part of the electrophysiological adjustments in the deprived barrel cortex. In addition, however, significant changes were noted in the cortex ipsilateral to the peripheral deprivation. Layer IV was

significantly thicker than control on this ipsilateral side, and the number and percentage of GABA immunoreactive neurons were also greater than in controls. In addition, there was a concomitant decrease in the numerical density and proportion of GABA immunoreactive neuronal cell bodies in layer V, and these immunoreactive neurons were larger than control in layers II/III as well as layer V. Thus, a considerable intralaminar redistribution of GABA expression had taken place in the ipsilateral cortex, as well as morphological changes presumably associated with excessive innervation by thalamic fibers, since the thickness of layer IV in different cortical areas appears to be tightly correlated with the amount of their thalamic input (Finlay and Pallas, 1989).

The present electron microscopic investigation was undertaken to determine whether there might also be changes affecting dendritic spines in the barrel cortex ipsi- or contralateral to chronic whisker trimming from birth. In histological material from the same rats previously investigated by Micheva and Beaulieu (1995a,b) and processed for postembedding GABA immunocytochemistry, the disector method (Sterio, 1984) was applied to serial ultrathin sections, in order to obtain unbiased quantitative data on the numerical density of dendritic spines. In a second step, a computerized image analysis system was utilized to reconstruct the spines in three dimensions and obtain accurate measurements of the volume and surface area of their head, length of neck and surface area of postsynaptic densities. Lastly, these same morphometric parameters were assessed in a subpopulation of spines innervated by GABA terminals, to know whether these spines might be differentially affected compared to their much more frequent counterparts receiving single asymmetrical synapses only.

MATERIALS AND METHODS

Experimental and control animals

All experiments were performed according to the policies and guidelines of the Canadian Council on Animal Care and the regulations of the Animal Care Committee at the Université de Montréal. The histological material came from six male rat pups (Long-Evans, Charles River Co., St-Constant, QC), born in our laboratory, left with their mother until 4 weeks of age, and then housed together in a standard cage, with food and water ad libitum, under a 12/12 hour light-dark cycle. Two of these littermates were kept as intact controls and four were subjected to unilateral whisker trimming, as previously described by Micheva and Beaulieu (1995a,b). From postnatal day 1, whiskers from rows B, C, and D of the right face whisker pad were carefully plucked, in accordance with the procedure described by Fox (1992). Since the whiskers quickly regrew, the procedure was repeated three times a week. The whiskers in outer rows A and E were left intact, for the purpose of reducing inadvertent activation of the follicles in the three middle rows of removed whiskers. All animals were sacrificed at postnatal day 60.

Tissue processing

After deep anesthesia with sodium pentobarbital (Somnotol, 65 mg/kg i.p.), the animals were perfused through the ascending aorta, first with 0.1M cacodylate buffer (pH 7.4) for 1-2 minutes, and then with 600 ml of freshly prepared fixative which contained 1% paraformaldehyde (Fisher, Nepean, ON), 2.5% glutaraldehyde (Mecalab, Montreal, QC) and 3 mM calcium chloride (Sigma, Oakville, ON) in the same buffer. The brain was then removed from the skull, the two hemispheres separated, and the gross location of the parietal

barrel field cortex determined by using the template published by Strominger and Woolsey (1987). Cuts were made perpendicular to the brain surface at the anterior and posterior limits of the barrel field, and vibratome sections of alternating thickness (50 μm and 100 μm) were obtained from each block, to be respectively processed for cytochrome oxidase histochemistry and electron microscopy.

The 50-μm-thick sections were stained for cytochrome oxidase as described by Silverman and Tootell (1987). After rinses (4 x 10 minutes) in 0.1M phosphate buffer (PB, pH 7.6) containing 10% sucrose, the sections were transferred for 10 minutes in 0.05 M Tris buffer (TB; pH 7.6) containing 275 mg/l of cobalt chloride and 10% sucrose, washed in PB (2 x 5 minutes), and incubated for 4-6 hours at 40°C in 0.1M PB containing 50 mg of 3,3`-diaminobenzidine tetrahydrochloride (Sigma), 5 g of sucrose, 7.5 mg of cytochrome C (type III; Sigma) and 2 mg of catalase per 100 ml. The stained sections were then washed in PB, dehydrated and mounted on gelatin-coated slides.

The 100-μm-thick sections intended for electron microscopy were post-fixed for 30 minutes in 1% osmium tetroxide (J.B. EM, St-Laurent, QC) in cacodylate buffer (0.1 M, pH 7.4), followed by 10 minutes in the same solution containing 1.5% potassium ferrocyanide (Sigma). The sections were then washed in the buffer, dehydrated in a graded series of alcohols and propylene oxide, and flat embedded on glass slides in Durcupan ACM resin (Fluka, Oakville, ON). No shrinkage was detected as a result of these procedures, as assessed by comparing the contours of selected sections before and after the post-fixation. Using the adjacent cytochrome oxidase sections as a guide, large pieces of sections corresponding to the three middle rows of vibrissae were removed from the slides and re-embedded in resin. Series of semithin sections

(1-μm) were then cut from each block (Ultracut S, Reichert), stained with azure-methylene blue, and used to determine the boundaries of cortical layer IV. After retrimming of the blocks so as to include only layer IV, series of 20-36 ultrathin sections of gray interference color were cut and placed on single slot nickel grids coated with Pioloform (Bio-Rad, Cooksville, ON). These sections spanned across the entire thickness of layer IV and came from the inside of barrels as verified in the adjacent cytochrome oxidase-stained light microscopic sections.

Post-embedding GABA immunocytochemistry

The thin sections were processed for GABA post-embedding immuno-cytochemistry on grids, according to a modified version of the protocol by Somogyi et al. (1985). In brief, the grids were successively placed on droplets of 1% periodic acid for 7 minutes, 1% sodium periodate for 3 minutes, 1% sodium borohydride in Tris-buffered saline (TBS, 0.05 M Tris buffer and 0.9% NaCl, pH 7.4) for 5 minutes and 1% ovalbumin in TBS for 30 minutes. This was followed by immunostaining with rabbit anti-GABA serum (Beaulieu et al., 1994) diluted 1:3 000 in TBS containing 1% normal goat serum for 90 minutes; incubation in 1% bovine serum albumin and 0.5% Tween in TB (pH 7.6) for 2 x 5 minutes and then incubation for 2 hours in the same solution containing a 1:25 dilution of colloidal gold (15 nm, Janssen, Hornby, ON) coated with goat anti-rabbit IgG. After each step, the grids were rinsed in TBS. Lastly, the grids were placed on drops of 1% glutaraldehyde for 1 minute, washed in distilled water and then stained with uranyl acetate and lead citrate.

Numerical estimates of dendritic spine density

The number of dendritic spines (numerical density per unit volume) in layer IV of the barrel field cortex was determined with Sterio's disector method (Sterio, 1984) as used in previous studies (Beaulieu et al., 1992, 1994). Two blocks were analyzed on each side in each animal. In each block, an area measuring approximately 80 μm^2 was selected on the basis of its technical quality and photographed at a working magnification of X 8 900 (print magnification: X 24 000) across the entire series of thin sections from the corresponding ribbon (20 to 36 sections per ribbon). Each series was then subdivided into sets of four consecutive micrographs (5-9 sets per series), in which the first and fourth were referred to as the "reference" and the "look-up" sections, respectively. The intervening pictures were used to trace structures of interest or to confirm their disappearance from view. In order to further increase sample size, the counting procedure was applied in both directions, i.e. from reference to look-up sections or in reverse. The dendritic spines were counted in a picture area equivalent to 8.75 µm by 6.75 µm (59 μm^2) of tissue. Spines intersected by two of the border lines were excluded. Counts were obtained for all spines that were present in the reference but not in the look-up section. The numerical density per unit volume (N_V) was given by the formula:$N_V = \Sigma Q^- / (a \times h)$, where " ΣQ^- " stood for the number of dendritic spines that were present in the reference section and had disappeared in the look-up section, " a " was the area of sampling (52 μm^2) and " h " was the height of the tissue volume examined, i.e., the thickness of the four sections, known from measurements obtained by the small fold method (Weibel, 1979).

Three-dimensional analysis

Sterio's procedure was also used to select spines for morphometric analysis. Starting from pictures in the middle of the series of thin sections, and working in both directions, spines were selected in each set of 4 sections according to the disector method, i.e. those spines which were present in the reference sections and absent from the look-up sections. A total of 122 spines were thus selected: 84 from experimental animals (42 in the left, deprived, and 42 in the right, intact hemisphere) and 38 from control animals (24 in the left and 14 in the right hemisphere). Only 4 of these spines were innervated by a GABA terminal. Since a major objective of the study was to compare spines receiving or not a GABA terminal, the sample size for GABA innervated spines was increased by including all GABA-innervated spines entirely visible in the same micrographs. This resulted in a total of 43 GABA-innervated spines, 29 from experimental and 14 from control animals.

Three-dimensional models of fully visualized spines were produced with a computer-assisted reconstruction system (high resolution video camera, Hitachi KP-116, linked to a Powermate microcomputer, NEC Information Systems, San Jose, CA) running the AMICUS program (Toronto Western Hospital). In a first step, the negatives showing a given spine were digitized according to the procedure of Harris and Stevens (1988). The "Toggle" feature of the program, which rapidly switches between live and previously stored images, was used to visually align the negatives. Numerous neuropil structures were always used as landmarks in this process. Digitized images, alignment coordinates, size calibration, section thickness, and identification entries were sent to the reconstruction workstation by an Ethernet link. In a second step, the reconstruction proper was made with the Icar 80.8 program (ISG Technologies Inc., Mississauga, ON) ran on a Silicon Graphic workstation (IRIS 4D / 85GT).

The outlines of individual spines were traced on the monitor screen with the aid of a mouse driven cursor, care being taken to trace separately spine head, spine neck, and post-synaptic density. Integration of the separate spine profiles by means of the software program made it possible to visualize the three-dimensional structure of individual spines within the surrounding neuropil rendered as background. Surface texture, light orientation, and shadowing functions were set to provide a clear-cut rendition of spine surface. This powerful system also allowed for rotation in the vertical and horizontal planes and for three-dimensional reconstruction of the surrounding neuropil.

Perimeter and sectional area of spine head, and length of post-synaptic densities were measured from the stored outlines in each section. They were recorded for subsequent calculation of surface area of spine head, surface area of post-synaptic densities and volume of spine heads, based on summation of the values in each section and section thickness. To achieve accurate measurement of spine necks, the three-dimensional images were rotated so as to obtain four different views at 90 degree angles, starting from any full view of the spine and its neck. Spine neck was measured as the distance from the closed outline of the spine head to attachment of the neck to the parent dendrite. The four lengths were averaged for each spine.

Statistics

Macintosh Excel and Statview programs were used to accumulate and analyze the data for numerical density, as well as the various morphometric parameters. Differences between left and right hemisphere, experimental and control animals and GABA-innervated spines versus spines receiving asymmetrical synapses only were assessed by two-way analysis of variance, followed by post-hoc Fisher test. Null hypotheses were rejected at the alpha

level of 0.05. Pearson's product test was used to assess the correlation between volume of spine head, surface area of spine head and surface area of postsynaptic density on the two groups of spines from experimental animals: the 84 spines randomly selected by Sterio's method and the 29 GABA-innervated spines entirely visible in the same micrographs.

RESULTS

Numerical density and total number of dendritic spines

As shown in Fig. 1A, there were no statistically significant differences in numerical density (N_V) of spines between experimental and control animals nor between sides in either group. The N_V value was 34% higher on the right (ipsilateral) than left (contralateral) hemisphere in experimental animals (364 x 10^6 versus 271 x 10^6), but this difference did not reach statistical significance ($p< 0.07$). The average N_V of dendritic spines in layer IV of the control barrel cortex was 358 x 10^6 per mm^3.

In an earlier study of GABA neurons in these same rats (Micheva and Beaulieu, 1995a), the thickness of layer IV was shown to be significantly greater in the cortex ipsilateral to whisker trimming than in the contralateral cortex or the cortex of control animals (264 μm versus 219 μm, and mean of 228 μm for both sides in the controls). Consequently, it could be estimated that the cortical volume occupied by the barrels (surface area of the barrel cortex multiplied by the thickness of layer IV) was 20% greater in the cortex ipsilateral to the whisker trimming (1.84 mm^3) than in the contralateral (1.48 mm^3) or control cortex (mean of 1.51 mm^3 for both sides). From these data, it could be inferred that the total number of spines (N_T) in the barrel cortex of experimental

animals was 670 x 10^6 ipsilateral to whisker trimming and 401 x 10^6 contralaterally, versus 540 x 10^6 in the control cortex (Fig. 1B).

Morphometrics

All spines. The data for the 122 spines selected from experimental and control barrel cortex by Sterio's method is presented in Fig. 2. There were no statistically significant differences for any of the parameters examined between left and right hemisphere in control animals. On the contrary, each of the parameters differed between right (ipsilateral) and left (contralateral) cortex in experimental animals. Volume of spine head was 66% bigger in ipsilateral compared to contralateral cortex (0.070 μm^3 vs. 0.042 μm^3; $p< 0.05$); surface area of spine head was 39% greater (0.605 μm^2 vs. 0.435 μm^2; $p< 0.01$); surface area of post-synaptic density was 40% greater (0.066 μm^2 vs. 0.047 μm^2; $p< 0.05$), and length of spine neck was 35% smaller (0.373 μm vs. 0.577 μm; $p< 0.05$). There were no such statistically significant differences in the comparison of ipsilateral nor of contralateral hemisphere to control. However, there was a definite tendency for bigger volume of spine head, greater surface area of spine head and of post-synaptic density, and shorter length of spine neck on the ipsilateral side of experimental animals. For example, 60% of the spine heads in the ipsilateral cortex were larger than 0.04 μm compared to only 45% in the controls. In experimental as well as control cortex, there was a tight linear relationship between volume of spine head, surface area of spine head and surface area of postsynaptic density ($r = 0.91$ and $r = 0.99$ for volume vs. surface area of spine heads, and $r = 0.87$and $r = 0.95$ for surface area of spine heads vs. surface area of postsynaptic densities in 84 and 38 spines from experimental and control animals, respectively; $p < 0.01$).

Comparison of spines with or without GABA innervation. Fig. 3 represents spines from experimental animals only. It compares 29 of these spines that were GABA innervated, i.e. received a synapse from a GABA-labeled terminal, to the 80 spines included in the previous analysis and which were not GABA innervated (called asymmetrical in the graph). Sixteen of these GABA-innervated spines were from the ipsilateral and 13 from the contralateral hemisphere. Perhaps due to their small number, these GABA-innervated spines showed no significant differences between sides for any of the parameters examined. However, as in the overall population of spines, the volume and surface area of spine head, and the surface area of postsynaptic density showed a tendency toward bigger size in the ipsilateral compared to contralateral cortex. As expected, the spines without GABA innervation, which represented the vast majority of all spines in the present material showed the same differences between hemispheres as already reported in Fig. 2. There were also differences between the spines with and without GABA innervation: volume of spine head, surface area of spine head and area of postsynaptic density were significantly greater for GABA-innervated spines pooled as one group than for the non GABA-innervated spines on either the ipsilateral or contralateral side. The volume of the GABA-innervated spines ranged from 0.009 μm^3 to 0.230 μm^3, averaging 0.120 μm^3. Fourteen percent of these spines were larger than the largest non GABA-innervated spines from the ipsilateral hemisphere of experimental rats. However, the size of these 29 GABA-innervated spines from experimental rats was not significantly different from that measured for the 14 GABA-innervated spines from control rats. As was the case for "all spines", the GABA-innervated spines showed a strong correlation between volume and surface area of spine head ($r = 0.94$ with $p < 0.001$) or surface area of spine head and surface area of postsynaptic density ($r = 0.80$ with $p < 0.01$).

Synaptic innervation of dendritic spines

As already described in previous studies (Jones and Powell, 1969; Micheva and Beaulieu, 1995b), the vast majority of spines in layer IV of the barrel field cortex receive a single afferent terminal making an asymmetrical synapse on their head (Fig. 4 and Fig. 6). This was confirmed for more than 95% of the 122 dendritic spines randomly selected from both sides of the cortex in experimental as well as control animals. It is also known that most of the remaining spines are dually innervated by one asymmetrical synapse on their head plus one symmetrical (GABA) synapse on their head or their neck (Micheva and Beaulieu, 1995b; Fig. 5). Among all spines which were entirely examined in the present study, ten (< 1%) displayed unusual patterns of innervation which, to our knowledge, have never been illustrated: three received two asymmetrical synapses on the head (Fig. 6), five a single GABA (symmetrical) synapse on the head (Fig. 7), and two either two GABA synapses or two GABA synapses plus one asymmetrical synapse on the head (Fig. 8). It was beyond the scope of this study to compare the frequency of these configurations in the cortex ipsi- and contralateral to whisker trimming.

DISCUSSION

The present study demonstrated that, in addition to a considerable difference in the number of dendritic spines, prolonged unilateral sensory deprivation by whisker trimming from birth entailed significant differences in spine head dimensions, size of postsynaptic densities and length of spine necks in the ipsilateral compared to contralateral or control barrel field cortex (layer IV) of young adult rats.

Methodological considerations

The initial objective of our study was to compare the number of dendritic spines between sides in the barrel cortex deprived (contralateral) or not (ipsilateral) of peripheral sensory input during the postnatal period. The use of Sterio's method was particularly appropriate for this purpose, since it allowed for obtaining precise quantitative estimates unbiased by the size and shape of the examined objects (Sterio, 1984). The data could then be confidently compared not only between sides but also between experimental and control animals, regardless of changes in the size and shape of spines which might take place in the experimental group. The use of Sterio's method as a sampling technique for the random selection of the general populations of spines to be subjected to morphometric analysis similarly avoided biases due to size that might have been expected with other methods of so-called random selection in single thin sections.

The thickness of the various layers of the barrel cortex had already been measured by two of us in the present experimental and control material, and the surface area of the barrel cortex estimated in horizontal sections from similarly treated rats of the same age (Micheva and Beaulieu, 1995a) . The data on numerical density of dendritic spines, i.e. number per mm^3, could therefore be transformed to true number of spines in the barrel cortex. They could also be expressed as average number of spines per neuronal cell body, since the total number of neurons had also been determined for both the ipsilateral and contralateral cortex of experimental animals as well as control cortex (Micheva and Beaulieu, 1995a).

For the purpose of comparing dendritic spines innervated or not by GABA terminals, the availability of material labeled for GABA with a postembedding immunocytochemical technique was uniquely suitable. It

overcame penetration problems which often complicate the global detection of a given species of transmitter-defined neuronal element by pre-embedding immunocytochemistry. Even then, however, the small number of GABA- as opposed to non GABA-innervated spines precluded relying only on Sterio's method for obtaining a representative sample. Therefore, every GABA-innervated spine present in our material had to be considered. This did not introduce any size or shape bias, however, since the whole volume of the sampled tissue was in fact being examined in serial thin sections.

Ipsilateral increase in dendritic spine number

The number of dendritic spines per unit volume of tissue (N_V) in the barrel cortex of rat had not been previously determined by means of an unbiased stereological technique. It was here estimated to be in the order of 358×10^6 per mm^3 in control rat, a value somewhat lower than the earlier estimate of $502 \pm 31 \times 10^6$ per mm^3 for synaptic junctions on spines in the same cortical area of the same rats (Micheva and Beaulieu, 1995b). As demonstrated in previous studies and indeed verified in our material, less than 10% of dendritic spines in this part of the barrel cortex receive more than one synaptic contact (Jones and Powell, 1969; Micheva and Beaulieu, 1995b). The earlier study of Micheva and Beaulieu (1995b) has shown that 78% of all synaptic junctions in the control barrel cortex are made on spines, 20% on dendritic branches and 2% on neuronal somata, and that these proportions remain the same in the cortex contraleral to whisker trimming.

In the present study, the lack of statistical differences in numerical density between left and right barrel cortex of experimental animals did not imply an equal number of dendritic spines on both sides. Indeed, as already measured in these same rats, layer IV was significantly thicker and the volume

of the barrel cortex 20% greater on the side ipsilateral to whisker trimming compared to the contralateral side or control cortex (Micheva and Beaulieu, 1995a). In view of the present averages of numerical density, this meant a 67% greater number of spines in the ipsilateral compared to contralateral barrels of experimental animals, whereas the contralateral number was not significantly different from control. It was thus obvious that the difference in number of spines between the ipsi- and contralateral cortex of the experimental animals represented an ipsilateral increase rather than a contralateral decrease. Also noteworthy was that this change took place in the absence of significant side-to-side difference in the overall number of neuronal cell bodies (Micheva and Beaulieu, 1995a), and hence presumably corresponded to an increase in the average number of spines per barrel field neuron.

The predominant neuronal type in layer IV of rat barrel field cortex consists of spiny stellate (Class I) neurons considered to be excitatory interneurons, accounting for approximately 85% of the total population, and monosynaptically contacted by afferents from the thalamic ventrobasal complex, mostly on their spines (Woolsey et al., 1975; White, 1978; Simons et al., 1989). The remaining 15% of the barrel neurons are aspinous or sparsely spined interneurons of Class II, presumed to be GABAergic, and which are also contacted monosynaptically by thalamic afferents but mostly on their dendritic branches (Simons et al., 1989). Thalamocortical afferents account for approximately 20% of the asymmetrical synapses in the barrels (White, 1979; Keller et al., 1985), where a majority of synaptic contacts on spines are likely to be formed by local excitatory connections as in other parts of the cortex (Gruner et al., 1974; Dehay et al., 1991). Future experiments will be needed to determine to what extent the considerable increase in number of dendritic spines

detected in the ipsilateral barrel cortex is associated with an increased number of axo-spinous synapses of thalamocortical versus intracortical origin.

Ipsilateral changes in dendritic spine morphology

The measurements of the volume and surface area of spine head, surface area of postsynaptic densities and length of spine neck in experimental as well as control animals were consistent with earlier data from a number of studies carried out at electron microscopic level in rat hippocampus (Harris and Stevens, 1988, 1989; Trommald and Hulleberg, 1997). In normal mouse cerebral and cerebellar cortex, rat neostriatum and rat hippocampus, the surface area of spine heads and size of post-synaptic densities has repeatedly been shown to be linearly related to spine head volume (see Spacek and Miroslav, 1983; Wilson et al., 1983; Harris and Stevens, 1988; 1989; Harris et al., 1992; Trommald and Hulleberg, 1997). A similar relationship was observed in the present material from experimental animals.

More importantly, however, the statistical analysis of our morphometric data confirmed the inference that the most significant changes in our experimental animals had taken place on the side ipsilateral to whisker trimming. Indeed, there was a strong, almost significant tendency toward bigger size of spine heads and larger postsynaptic densities in the ipsilateral compared to control cortex ($p < 0.07$), and no such differences in the contralateral compared to control cortex. The statistical analysis was not conclusive as regards the length of spine necks. However, there was a tendency toward shorter spine necks on the ipsilateral side with the larger spines. This was consistent with earlier morphometric data on Purkinje cell dendritic spines having shown that spines with larger heads and synapses tend to have shorter necks than spines with smaller heads and synapses (Harris and Stevens, 1988).

Surface area of postsynaptic densities was also significantly greater in the ipsi- compared to contralateral barrel cortex of the experimental animals. Larger synaptic junctions on bigger spine heads have usually been interpreted as an indication of greater synaptic efficacy, particularly in hippocampus, where such changes have been associated with long term potentiation (for review, see Harris and Stevens, 1989; see also Desmond and Levy, 1986, 1988). In the present context, they may be viewed as an indication of activity-dependent plasticity induced by the increased flow of sensory information from the intact, contralateral side of the animal.

Synaptic innervation of the supernumerary dendritic spines

It was noteworthy that the increases in the size of spine heads and of postsynaptic densities in the ipsilateral hemisphere seemed to involve not only the largely predominant population of relatively small, dendritic spines innervated by asymmetrical synaptic terminals, but also the smaller population of generally larger spines receiving symmetrical synapses formed by GABA terminals. This did not come as a surprise since all but one of the GABA-innervated spines in the present material were found to also receive an asymmetrical, presumably excitatory, synaptic terminal. The larger size of spines innervated by GABA compared to those without GABA innervation was consistent with the generally larger size of GABA compared to other species of transmitter-defined axon terminals in rat brain. For example, GABA terminals in the hippocampus (stratum radiatum of CA1) of adult rat have already been shown to be larger than acetylcholine, noradrenaline and serotonin terminals (Umbriaco et al., 1995). It could not be determined from the present material whether the rare atypical patterns of double or triple innervation of the same spine were more frequent in experimental animals.

The present material gave no hint of an increased number of synaptic junctions per dendritic spine, at least as judged from the low proportion of all spines contacted by more than one synaptic terminal in the ipsi- as well as contralateral and control cortex. The small number of spines with multiple innervation precluded significant comparison of the observed patterns between sides. However, as most of the spines irrespective of side received at least one asymmetrical synapse, it may be assumed that the number of such synaptic contacts in the ipsilateral cortex was increased in parallel with the number of spines. In the earlier studies of Micheva and Beaulieu (1995b), a 66% decrease in GABA immunoreactive synapses on spines in the barrel cortex contralateral to the deprivation was interpreted as indicating a decreased expression of GABA, rather than actual loss of these axon terminals, since the total number of synapses remained similar to control. Micheva and Beaulieu (1995a) insisted on the possibility of changes in the ipsilateral cortex in view of the increased thickness of its layer IV, but did not actually determine the number of synapses in this cortex. It must be pointed out, however, that a subsequent study ruled out an overproduction of synapses in normal rat barrel cortex during the first two months after birth (Micheva and Beaulieu, 1996). This is noteworthy in the present context, since it excludes the possibility that the increased number of dendritic spines (and possibly of synapses) on the ipsilateral side was somehow due to a failure in some cropping process following a period of overproduction.

Activity-dependent plasticity as an explanation

In view of the increased number and size of dendritic spines in the barrel cortex ipsilateral to whisker trimming, it seemed reasonable to postulate activity-dependent plasticity arising from the intact side as the explanation for these changes. Indeed, in this fully crossed system, our data was hardly

compatible with the results of a peripheral deprivation entailing some breakdown in the vertical transfer of information from layer IV to other layers and secondary, transsynaptic changes on the opposite side. This was all the more unlikely since callosal transfer of information does not seem to be a prominent feature in the barrel cortex (Yorke and Caviness, 1975; White and De Amicis, 1977; Welker et al., 1988; but see Pidoux and Verley, 1979). On the other hand, several reasons seemed to argue in favor of ipsilateral changes resulting from increased activity of the intact whisker pad. Although there is no reported data to document behavioral changes following unilateral removal of the mystacial vibrissae in newborn rat, increased whisking on the intact side has been measured in adult rats following unilateral vibrissae removal, in the form of thigmotactic scanning (Steiner et al., 1986; Milani et al., 1989). These adult animals recover in several days from the behavioral asymmetry, but increased use of the intact side might be more prominent and/or permanent after a similar procedure initiated soon after birth. Moreover, as in the paradigm of ocular dominance after monocular deprivation or of animals raised from birth in impoverished or enriched environment, the effects of preferential usage of the mystacial apparatus on one side might be particularly strong during the critical early postnatal period of cortical development.

Various experimental procedures in which specific neuronal circuits are being activated in the adult have been shown to be accompanied by morphometric changes in dendritic spines, and notably enlargement of spine heads, shortening and widening of spine stalks and increase in length of synaptic appositions. These include tetanizing stimuli with long-term potentiation (Fifkova and Van Harreveld, 1977; Lee et al., 1980; Fifková and Anderson, 1981; Chang and Greenough, 1984; Desmond and Levy, 1986, 1988; Trommald et al., 1990; reviews in Wallace et al., 1991, and Harris et al., 1992).

Spine head enlargement has also been documented by electron microscopy in the hyperstriatum accessorium of dark-reared chicks as the result of repeated bouts of visual stimulation (Bradley and Horn, 1979). It has also been observed in the case of honeybee calycal interneurons, in relation with cumulative nursing and foraging experiences; spines with significantly larger profile areas and shorter stems were then found in the foragers compared to newly emerged and nurse bees (Coss et al., 1980; see also Brandon and Coss, 1982). Shortening of spine stems has also been described in tectal interneurons of jewel fish after social stimulation (Coss and Globus, 1978).

A number of possible mechanisms have been proposed to account for such experience or activity-dependent changes in dendritic spine morphology. On the basis of the presence of actin filaments in the spine head and their longitudinal organization in the spine stalk, Fifkova (1985) has suggested that upon stimulation and the opening of voltage-dependent calcium channels, the concentration of calcium activates actin-regulatory proteins and myosin in the spine head, triggering a chain of events leading to enlargement of the spine head and contraction of the spine stalk. The increased free cytosolic calcium would also activate protein-producing systems localized at the base of the spine, which under certain conditions would stabilize the morphometric changes of the spine. More recent hypotheses have been based on the demonstration of other molecules thought to be specifically involved in synaptic plasticity through some effect on spine structure, such as the microtubule associated protein MAP2 (Aoki and Siekevitz, 1985), spectrin, a structural component of the spine cytoskeleton (Siman et al., 1990), the actin-binding protein, drebrin (Hayashi et al. 1996), and trophic factors such as NGF, BDNF and NT-4 (e.g. McAllister et al., 1995).

In the barrel field cortex of rodents, BDNF is now the subject of increasing attention. In situ hybridization studies have revealed that the respective mRNAs for both BDNF and its high affinity receptor, tyrosine kinase B (trkB), are expressed in neurons of the neonatal barrel field cortex, and particularly its spiny stellate cells which receive the thalamic afferents (Singh et al., 1997). It has also been shown that BDNF mRNA levels may be up-regulated in the adult barrel field cortex, and particularly its stellate cells, upon stimulation of the mystacial vibrissae (Rocamora et al., 1996). Moreover, a recent study has demonstrated that this effect could be mediated through activation of 5-HT2A serotonin receptors (Vaidya et al., 1997), which indeed peak in density in the barrels (presumably in stellate cells) within the first three weeks after birth in the rat (Mansour-Robaey et al., 1998). It has also been known for some years that trkB mRNA is expressed early during development in the sensory relay thalamic nuclei (Masana et al, 1993), whereas Cabelli et al. (1995) have shown that BDNF could be involved in some reshaping (and presumably excessive development) of thalamocortical connections when locally infused into rat visual cortex. In addition to possible direct effects on dendritic spine growth, overexpressed BDNF released from intracortical neurons might actually promote such an overgrowth of thalamocortical fibers, which might in turn contribute to the increased number as well as size of the dendritic spines receiving these synaptic afferents.

In conclusion, the ipsilateral barrel field cortex of rat after chronic unilateral whisker trimming from birth provides a unique *in vivo* model to identify and characterize molecules that might be involved in activity- and experience-dependent plasticity of the synaptic connectivity in cerebral cortex.

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FIGURE LEGENDS

Figure 1. *Numerical density (N_V) and total number (N_T) of dendritic spines in the barrel cortex (layer IV) of experimental (n=4) and control (n=2) animals.* In both graphs, the hatched and solid columns respectively correspond to mean values (±S.E.M. in A) for left and right cerebral hemisphere, i.e., the hemisphere contra (C) and ipsilateral (I) to whisker trimming in experimental animals. In B, the left and right control values are pooled, since separate values of layer IV thickness for left and right hemisphere were unavailable from Micheva and Beaulieu (1995a). The N_V values in A were not significantly different between left and right side or between experimental versus control (ANOVA and post-hoc Fisher test). In B, the N_T value is considerably greater for ipsilateral than contralateral cortex of experimental animals or control cortex. Statistical analyses could not be performed on these N_T data, because they were derived from estimates of barrel cortex volume themselves based on measurements of surface area necessarily obtained from different animals as those used in the measurements of layer IV thickness and of N_V.

Figure 2. *Morphometrics of dendritic spines in experimental (n=4) and control animals (n=2).* As in Figure 1, the hatched and solid columns in both groups correspond to left and right cerebral hemisphere, i.e. the hemisphere contra (C) and ipsilateral (I) to whisker trimming in experimental animals. Values are means ± S.E.M. There are statistically significant differences between left and right hemisphere in the experimental animals only (A, B: *$p < 0.05$ and **$p < 0.01$ by ANOVA; A, B, C, D: *$p < 0.05$ and ** $p < 0.01$ by post-hoc Fisher test).

Figure 3. *Morphometrics of dendritic spines innervated or not by gamma-aminobutyric acid (GABA) synapses in experimental animals (n=4).* For the present comparative purposes, the designation "asymmetrical" was restricted to spines without GABA synapses, even if the vast majority of GABA-innervated spines (~ 10% of total population) also received asymmetrical synapses. The light and dark columns again correspond to left and right side, i.e. the hemisphere contralateral (C) and ipsilateral (I) to whisker trimming. Values are means ± S.E.M. As in the case of the total population of spines in experimental animals (Fig. 2), there were statistically significant differences between contralateral and ipsilateral hemisphere for all parameters in the large population of non GABA innervated spines (A,B: * $p < 0.05$ and ** $p < 0.01$ by ANOVA; A,B,C,D: * $p < 0.05$ and ** $p < 0.01$ by post-hoc Fisher test). Because of the small size of the GABA sample and the absence of differences between left and right side in this group, the GABA data from both hemispheres were pooled for statistical comparison with the "asymmetrical" data. The hooks in A, B, and C designate statistically significant differences in this comparison (**$p < 0.01$ by ANOVA and post-hoc Fisher test).

Figures 4 and 5. *Three-dimensional reconstructions of dendritic spines from the barrel cortex (layer IV) of experimental animals.* Both spines were visualized in their entirety, in series of 8 and 10 thin sections, respectively. They are presented over the background of one of the thin sections from which their 3D-image was generated, but after rotations of ± 180°. Fig. 4 represents a singly innervated spine from the cortex ipsilateral to whisker trimming. This typical spine head (sp) is linked by a relatively short neck to the dendritic branch (db) from which it arises. It is relatively small (volume: 0.0355 μm^3; surface area: 0.423 μm^2) and the length of its neck, reaching the spine head from

behind, is 0.330 μm. As the vast majority of dendritic spines in the cortex, this one receives a single terminal making an asymmetrical synapse on its head (shaded area; post-synaptic density: 0.045 μm^2). The incoming synaptic terminal itself is not visible, but several gold-labeled, GABA terminals, are noticeable in the background section. Fig. 5 is the reconstitution of a doubly innervated spine from the cortex contralateral to whisker trimming. This relatively small spine is also seen in continuity with its parent dendrite. Its head (sp) measures 0.026 μm^3 in volume and 0.396 μm^2 in surface area; the length of its neck is 0.575 μm. Two synaptic contacts are present on the head of this spine. One, asymmetrical (shaded area on the upper right of the spine head: 0.040 μm^2), is formed by the axon terminal designated by the asterisk in the background section. The other, symmetrical (shaded area on the left of the spine head; 0.0135 μm^2), is formed by a gold-labeled, GABA terminal which is not visible in this section. Scale bar (Fig. 5): 0.5 μm.

Figures 6-8. *Serial electron micrographs from the rat barrel field cortex (layer IV) of control and experimental animals illustrating various patterns of synaptic input onto dendritic spines.* In this material processed for GABA immunocytochemistry with a postembedding immunogold technique, GABA axon terminals were readily identified by dense accumulations of gold particles. The length of the section series allowed to visualize entire spines with their full complement of synaptic innervation. As described in the text and designated by asterisks in surrounding tissue, the most frequent innervation pattern was a single asymmetrical synapse per spine. The pictures were however centered on unusual patterns of innervation. Scale bars: 0.5 μm.

In **Fig. 6**, two asymmetrical synapses are made with the same spine (open arrowheads in A to C and B to F). The continuity between the doubly

innervated dendritic spine and its parent dendrite (d) is visible in F. Left cerebral hemisphere from a control animal. X 28 500.

In **Fig. 7**, a single GABA synapse (symmetrical) is made on the head of a first spine (closed arrowheads in A to G), whereas a second spine (asterisks in B to H) issued from the same dendrite (d in E) receives the typical single asymmetrical synapse on its head (visible in C to E). Left hemisphere from a control animal. X 30 000.

In **Fig. 8**, one asymmetrical synapse (open arrowheads in A to J) and two GABA synapses (closed arrowheads in B to H) are made on the head of the same spine. Contralateral hemisphere from an experimental animal X 24 000.

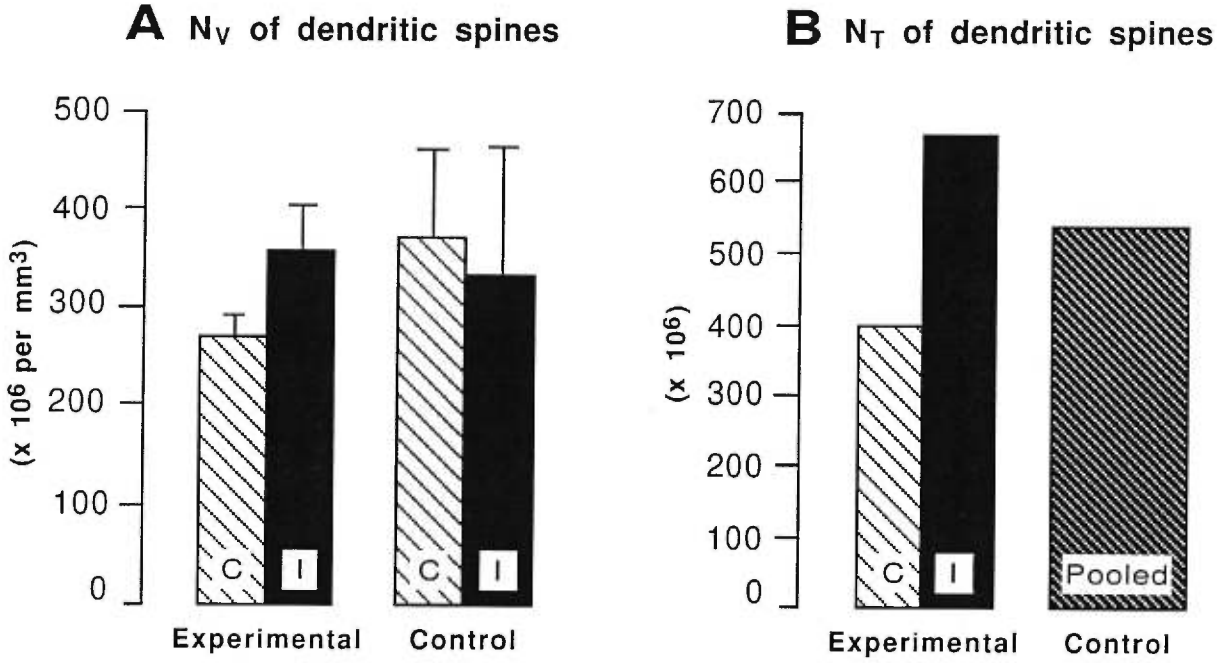
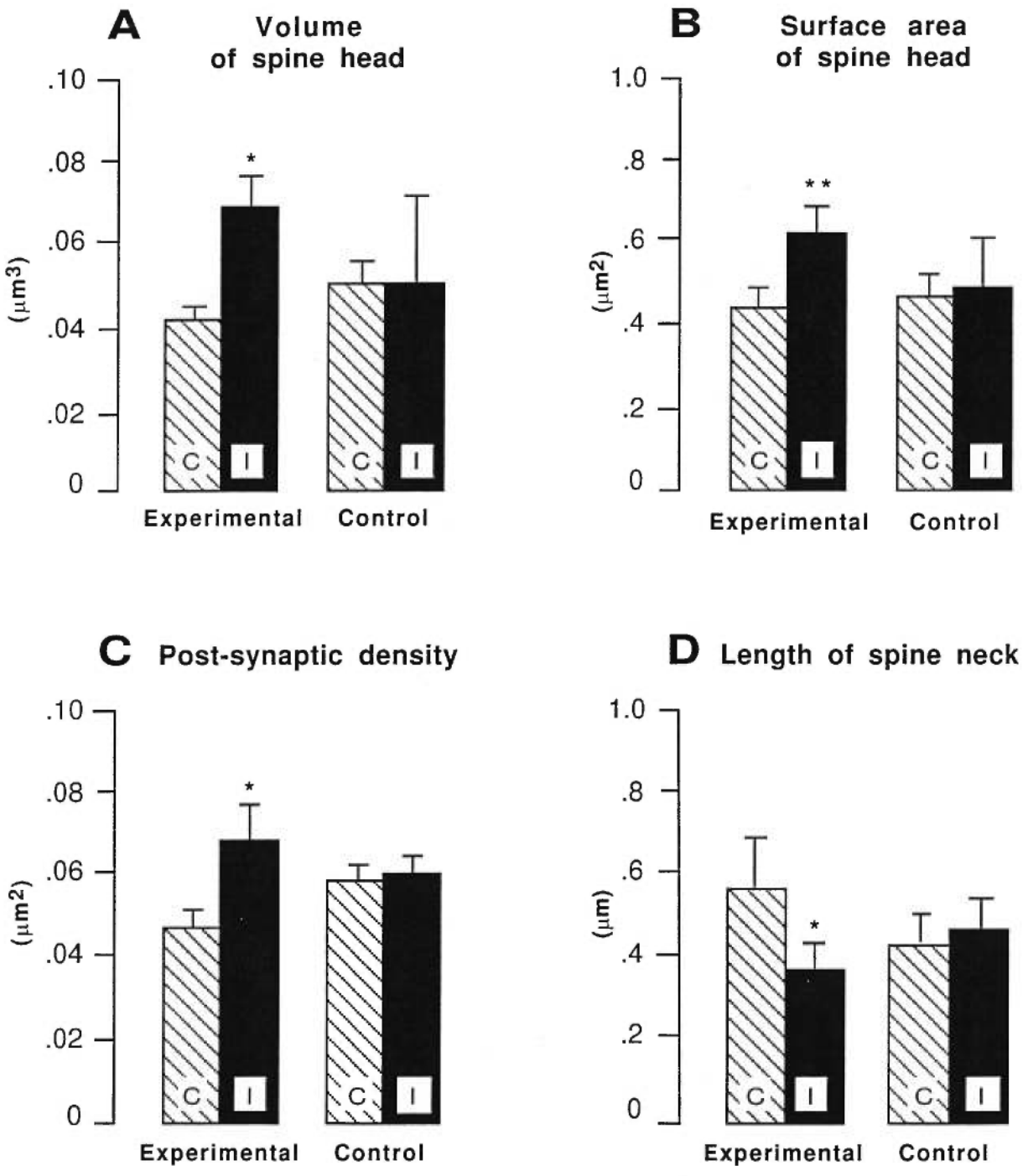

A N_V of dendritic spines
500
400
300
200
100
0
(x 10^6 per mm^3)
C
I
C
I
Experimental
Control
B N_T of dendritic spines
700
600
500
400
300
200
100
0
(x 10^6)
C
I
Pooled
Experimental
Control

Fig 1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(μm3)
*
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I
C
I
Experimental
Control
B
Surface area
of spine head
1.0
.8
.6
.4
.2
0
(μm2)
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C
I
C
I
Experimental
Control
C
Post-synaptic density
.10
.08
.06
.04
.02
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(μm2)
*
C
I
C
I
Experimental
Control
D
Length of spine neck
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(μm)
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C
I
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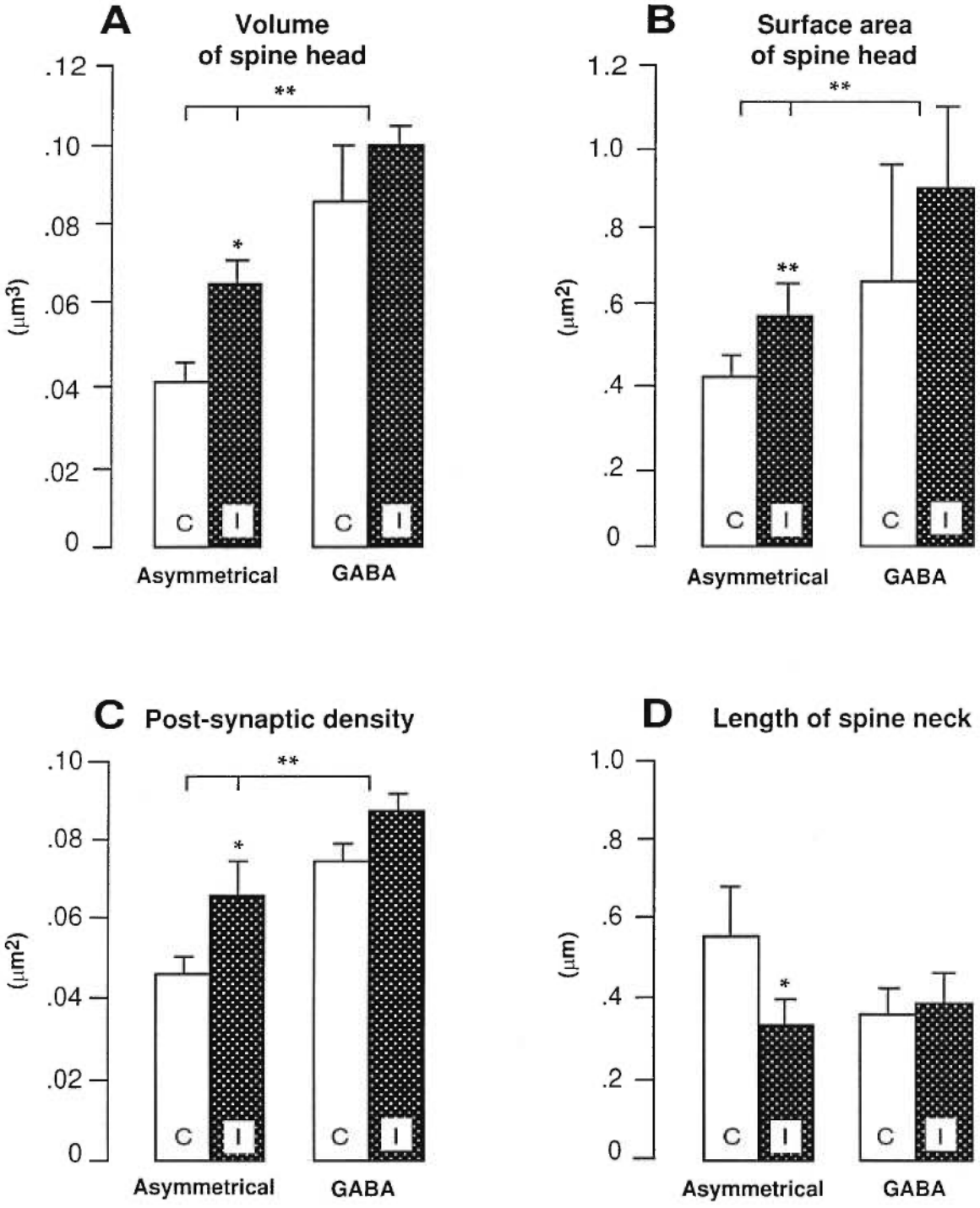
I
Experimental
Control

Fig 2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of spine head
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(μm3)
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Asymmetrical
GABA
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Surface area
of spine head
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1.0
.8
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(μm2)
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Post-synaptic density
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Asymmetrical
GABA
D
Length of spine neck
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C
I
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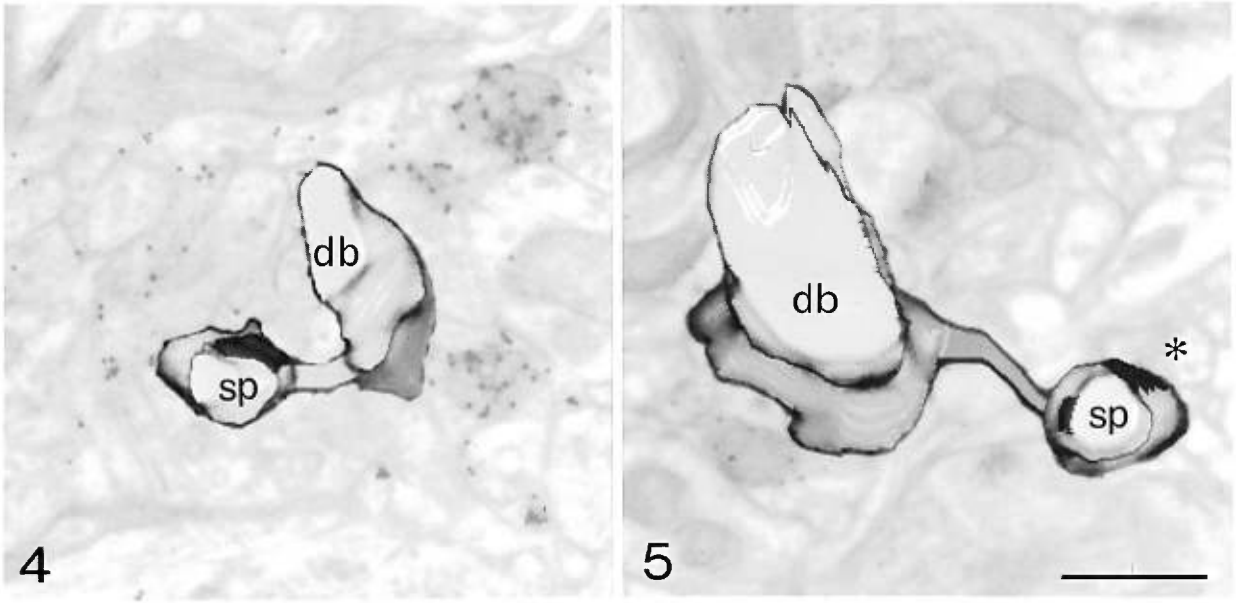
I
Asymmetrical
GABA

Fig 3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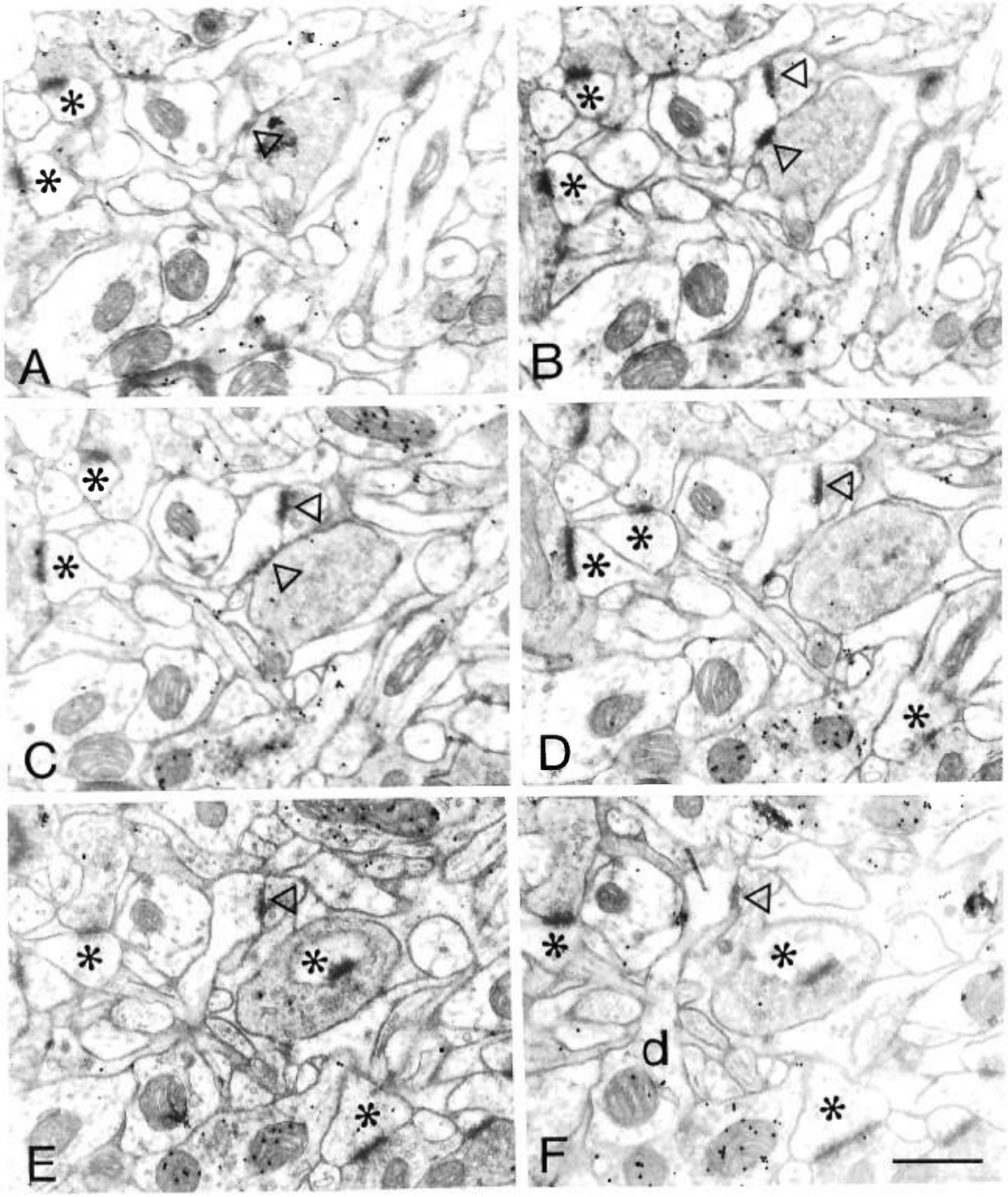
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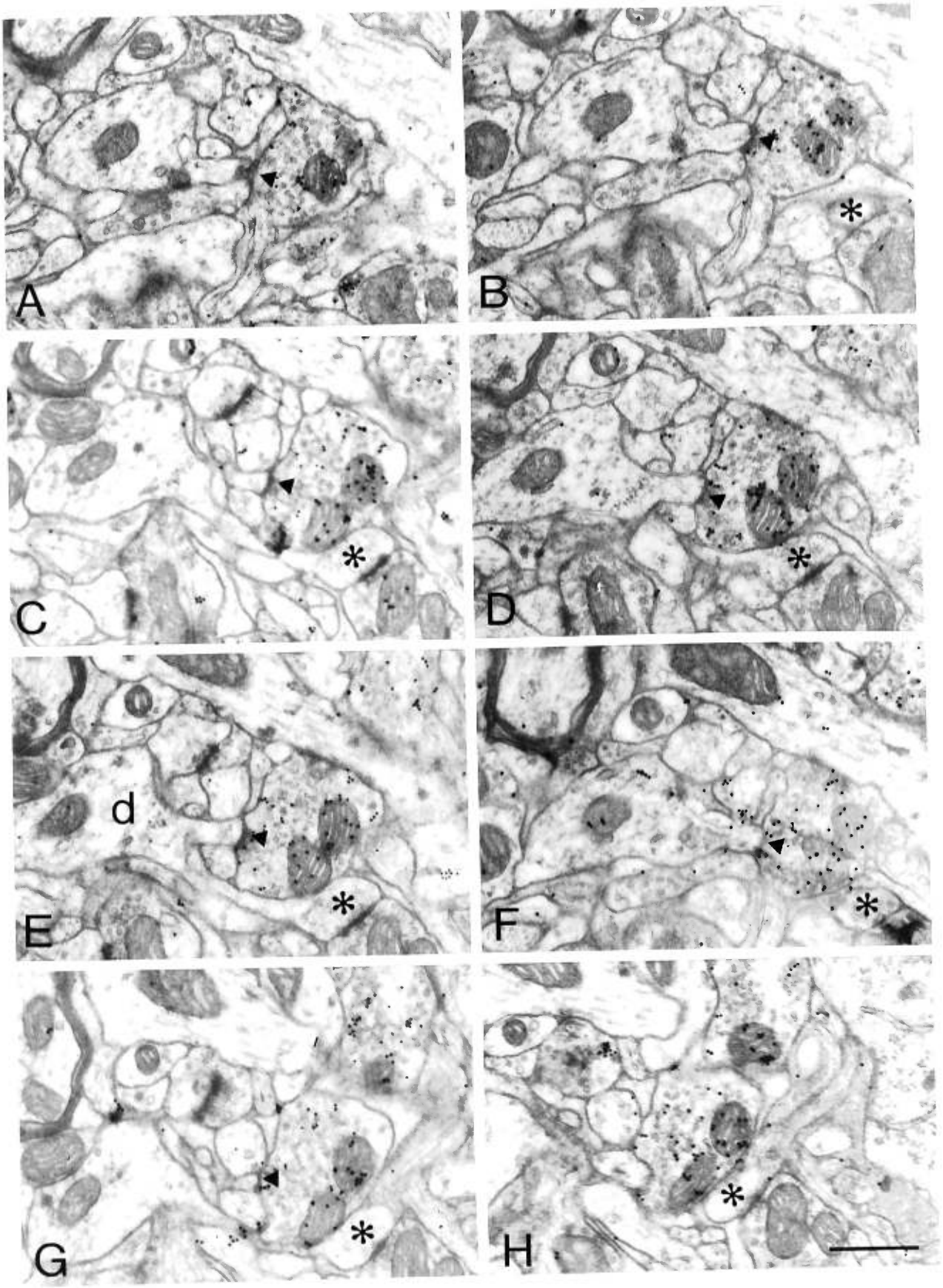
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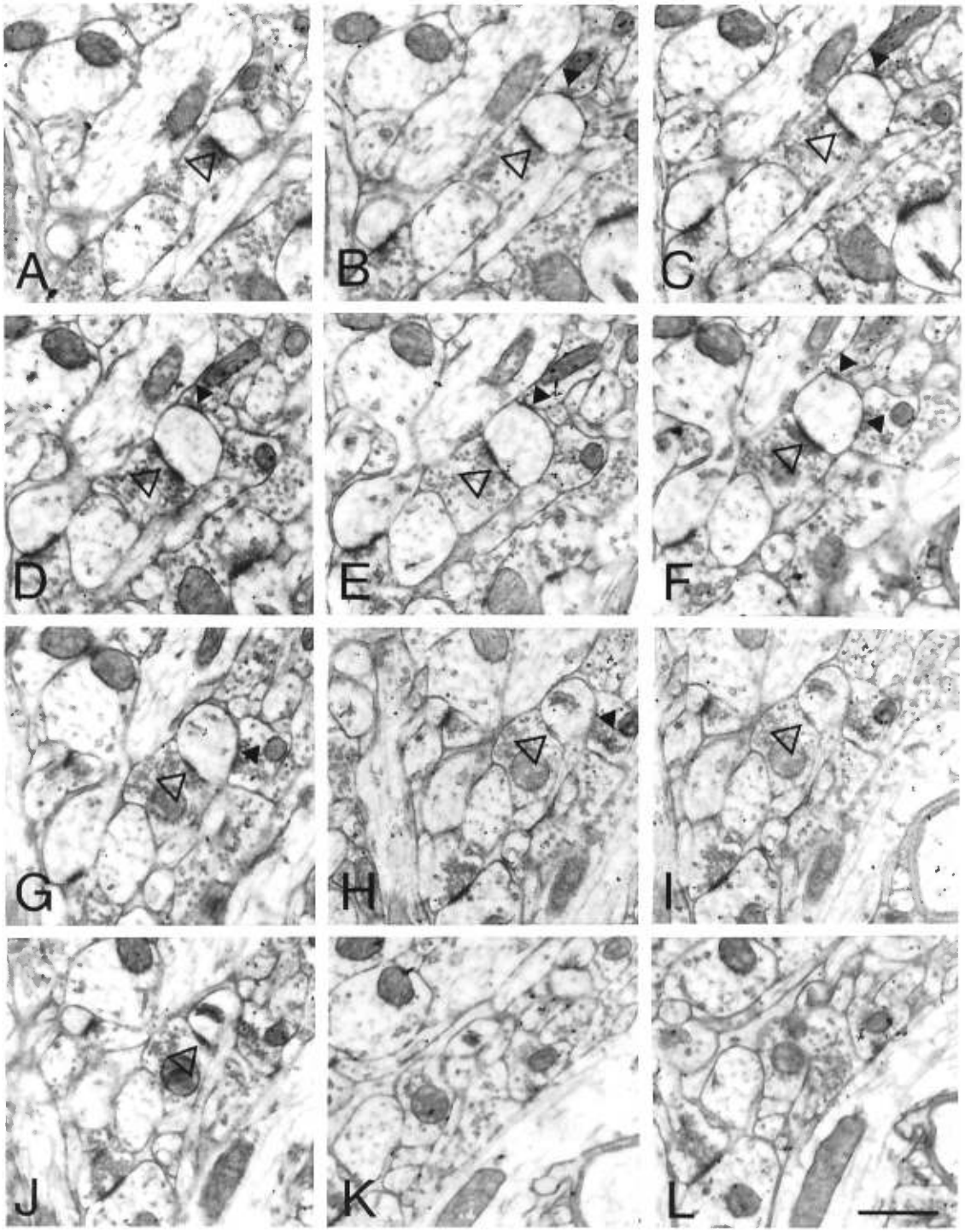
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CHAPTER 3

GENERAL DISCUSSION

III.1 Main findings

The present study leads us to conclude that removal from birth and during two months of three rows of mystacial vibrissae on one side of the face results in an increase in the total number of dendritic spines in the corresponding *ipsilateral* barrels (layer IV) of the rat somatosensory cortex. The results also showed an associated increase in spine head dimensions and size of post-synaptic densities, as well as a reduction in the length of spine necks, in the ipsilateral compared to contralateral or control barrel field cortex. We have discussed in Chapter 2 the reasons for believing that these phenomena are imputable to an activity dependent plasticity, resulting from the early and/or excessive activation of the intact whisker pad as primary inductor of the observed changes. Rather than reiterating these arguments here, we will insist on the general significance of our findings in terms of underlying molecular mechanisms and of their eventual impact from both an experimental and perhaps even clinical point of view.

III.2 Activity-dependent changes in number and morphometrics of spines

There is considerable evidence to suggest that increases in dendritic spine *density* may occur after modification in synaptic input (e.g. Globus et. al., 1973; Shapiro and Vukovich, 1970; Ryugo et. al., 1975; Rutledge, 1976; Horner et. al. 1991; for review see Horner, 1993). More rarely, however, have such changes been proven to imply an actual increase in the *total number* of spines, since this also calls for measurements of the volume of the anatomical compartment in which they are counted. Because dendritic spines are the major synaptic target of excitatory input in the cortex, they presumably play a major role in the processing of information in this part of brain. Therefore, as the

number of dendritic spines increases, it may be assumed that cortical processing is also more elaborated or "improved". In this line of thinking, it is reasonable as well as intuitive to conclude that newborns reared in a rich and stimulating environment will develop more complex and "efficient" cortical function as adults.

Studies in the mouse hippocampus have shown that when the perforant pathway of the dentate molecular layer is stimulated electrically, a single tetanic shock sufficient to induce long term potentiation (LTP) may result in long-lasting increases in the surface area of dendritic spines of pyramidal neurons (Van Harreveld and Fifkova, 1975; see also Fifkova and Van Harreveld, 1977; Lee et. al., 1980; Fifkova and Anderson, 1981; Chang and Grennough, 1984; Desmond and Levy, 1986, 1988; Trommald et. al., 1990; reviews in Wallace et. al., 1991, and, Harris et. al., 1992). This enlargement of the spine heads has been postulated to be the result of an enhanced synthesis of structural proteins, such as actin and/or, actin regulating proteins (Landis and Reese, 1983; Fifkova, 1985; Cohen, 1985). Although various models have been proposed, the exact mechanisms by which such dimensional changes might increase synaptic efficacy remain to be empirically determined.

In the present study, just as the surface area and volume of spine heads were found to be significantly greater, so was the surface area of the post-synaptic densities (PSDs). Such a change is usually interpreted to suggest that, as synaptic junctions are enlarged, the synaptic efficacy increases, leading to the facilitation observed in LTP and hence learning and memory. This particular finding was consistent with several earlier studies having shown that the total area of the PSDs is normally proportional to spine head dimensions prior to any experimental manipulation (Westrum and Blackstad, 1962; Peters and Kaiserman-Abramof, 1970; Wilson et.al., 1983; Harris and Stevens, 1988,

1989; Harris et. al., 1992; Chicurel and Harris, 1992). It provides additional support for Ramon y Cajal's initial contention that changes in "protoplasmic expansions" may be established after "cerebral exercise" (1893).

III.3 Molecular correlates of activity-dependent dendritic spine plasticity

Since dendritic spines are so small, their electrophysiological recording is not yet possible. This may be one of the reasons why researchers have spent so much time and energy trying to determine their structure, and structural variations, during development and/or as a result of environmental changes or enrichment. The basic goal being to better understand the function of dendritic spines.

Thus far, experimental and computational studies have provided evidence that spines may function as biochemical compartments rather than electrical isolators within the neuron, as initially believed (Rall, 1970; Wilson et. al., 1983, 1984; Coss and Perkel, 1985; Koch et. al., 1992; Harris and Kater, 1994). Because the surface area of dendritic spines is so small, it has been determined that very little, if any, charge loss occurs through the membrane of the spine head or neck. Therefore, no changes in spine dimensions would provide a mechanism for synaptic attenuation (Koch et. al., 1992; Harris and Kater, 1994; Yuste and Denk, 1995). Yuste and Denk (1995) went on to conclude that dendritic spines represented individual calcium compartments, capable of detecting temporal coincidences of pre- and postsynaptic activity, therefore serving as basic functional units of neural integration. Additional studies provide further support for this functional concept of the dendritic spine (Holmes, 1990; Muller and Connor, 1991; Guthrie et. al., 1991; Segal, 1995; Denk et. al., 1996). Yuste and Denk (1995) have indeed been able to demonstrate that calcium channels exist on spine heads, and action potentials do

invade dendritic spines. From these findings they concluded that a single spine may use calcium to register the temporal rate of input and output for the neuron. Consequently, the accumulation of calcium within the spine may be viewed as one of the mechanisms allowing for activity dependent synaptic plasticity, as well as for other calcium-dependent forms of synaptic activity (i.e. phosphorylation).

Since it is known that the induction of LTP at some synapses requires a post-synaptic increase in the intracellular calcium concentration (Bliss and Lomo, 1973; also reviewed in Wigstrom and Gustafsson, 1988), then one of the key functions of spines may be to amplify and isolate the synaptically induced calcium increases, and/or second messengers, within individual spines (Koch et. al., 1992; Harris and Kater, 1994). In other words, the dimensional increases in dendritic spines may be important for the facilitation of LTP, which may in turn lead to information storage in the brain (Koch et. al., 1992; Yuste and Denk, 1995).

Several possible mechanisms have been put forth to explain how changes in dendritic spine morphology may actually come about. One interesting area of research is examining the importance of cytoskeleton proteins which may play an important role in neuronal plasticity and development. Brain derived neurotrophic factor (BDNF) is one of the trophic factors which have been implicated. BDNF and its receptor, tyrosine kinase B (trkB), are both known to be expressed in the somatosensory cortex of the rats and its has been shown that BDNF expression is increased subsequent to whisker stimulation in adult rat. Furthermore, it is known that BDNF is modulated by serotonin, via the 5-HT2A receptor, which is strongly expressed in the barrel cortex of newborn rat pups (Mansour-Robaey et al., 1998).

Multiple factors, including neurotransmitters, may therefore be important in the induction of activity dependent dendritic plasticity.

III.4 Conclusions

With this in mind, it may be concluded that the newborn rat barrel cortex represents a unique in vivo model of inducible, activity dependent dendritic spine plasticity, in the search for molecular determinants and correlates of such a basic neuronal property. One can even imagine (or dream) that, if proteins were identified that are exclusive to dendritic spines as they grow or respond to stimulation, specific ligands could eventually be developed to visualize and investigate these phenomena in human living brain by means of functional imaging techniques.

CHAPTER 4

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