ISAS - INTERNATIONAL SCHOOL FOR ADVANCED STUDIES

"BDNF is regulated by visual experience and controls synaptic plasticity in developing visual cortex"

Thesis submitted for the degree of "Doctor Philosophiae"

CANDIDATE

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SISSA - SCUOLA INTERNAZIONALE SUPERIORE I STUDI AVANZATI

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ABSTRACT

The visual system is the most studied sensory system in vertebrates. During development there is an active shaping of the circuitry, mostly studied at the cortical level. The shaping of neuronal connections is particularly evident during particular time windows of the postnatal development, but some aspects of cortical organization are also modifiable, by experience, in the adult.

There is considerable evidence that neuronal activity influences the organization and function of both developing and adult circuits. These processes can also be studied at cellular and molecular levels. Possible mediators of activity-dependent development of visual cortex are neurotrophins. They were initially identified as proteins able to promote the survival and differentiation of target neurons; later they were discovered to be involved in modulating synaptic plasticity in the CNS.

In the mammalian visual system, neurotrophins are good candidates to act as a molecular link between neuronal activity and both physiological and structural modification of visual cortical circuitry occurring during postnatal development.

Brain Derived Neurotrophic Factor (BDNF), is a neurotrophin that specifically binds to TrkB receptors expressed on the membrane of target neurons.

The first aim of this study was to investigate the role of BDNF in one form of synaptic plasticity, called bidirectional plasticity, which represents the capacity of synapses to reverse the sign of a long-term change, in other words to pass from synaptic potentiation to synaptic depression and *vice versa*.

To address this issue we used electrophysiological recording in *in vitro* rat cortical slices containing primary visual cortex. We chose the ability of thalamocortical synapses to be first depressed and then potentiated (de-depression) as a specific

indicator of bi-directional plasticity. Extracellular field potentials in cortical layers II-III were evoked by stimulation of the white matter in rat primary visual cortical slices prepared at different postnatal ages. Low frequency stimulation (900 pulses at 1 Hz) of the white matter was used to induce long-term depression of field potentials amplitude, whereas long-term potentiation was evoked by high-frequency stimulation consisting of three trains at 100 Hz.

We found that bidirectional plasticity is present soon after eye opening (postnatal day 17, P17), but disappears rapidly over one week (P23). This loss of plasticity could be prevented by rearing animals in the dark or by applying BDNF from the recording micropipette. Moreover, blockade of the signal transduction pathway activated by Trk signalling prevents this form of plasticity being induced at P17, an age at which it is normally present.

Our data show that BDNF is able to modulate synaptic plasticity during the development of rat visual cortex, but do not reveal the sites of BDNF action during postnatal development. To answer this question the second part of our work was focused on studying the pattern of BDNF protein cellular expression during postnatal development of rat visual cortex, comparing it with BDNF mRNA.

To address this issue we analyzed BDNF mRNA and/or BDNF protein cellular distribution at different postnatal ages by using immunohistochemistry and highly sensitive *in situ* hybridization. The total amount of protein was measured by quantitative ELISA. We found that before eye opening (P13), all cortical layers have a large number of visual cortical neurons containing BDNF mRNA but no detectable amount of BDNF protein. At later ages (P23 and P90) the number of BDNF immunostained cells increases and most neurons are double labeled for both BDNF

mRNA and protein. The cellular increase of BDNF immunolabeling is blocked in animals deprived of visual experience from birth (dark rearing). In dark reared animals a large population of neurons contain BDNF mRNA but not BDNF protein, a situation which is similar to that observed before eye opening. Exposure of dark reared rats to a brief period of light restores a good match between BDNF mRNA and BDNF protein cellular content. We propose that visual experience controls the neuronal content of BDNF mRNA and BDNF protein in developing visual cortex.

BDNF exerts its action by binding to TrkB receptors. Therefore, we studied the expression of TrkB receptors, considering both cellular expression and protein level. We used two different antibodies: TrkB+ and TrkB – that recognize, respectively, the full length and truncated form of TrkB receptor. TrkB expression was investigated at different postnatal ages: P13, P23 and adulthood. To detect the protein level, a western blot was performed on homogenate of rat primary visual cortex, taken at the various ages. Our results indicate that the expression of both forms of the receptor increases during postnatal development and that the distribution patterns are similar at different postnatal ages. This suggests that the full length and the truncated form of the TrkB are regulated in similar ways during postnatal development. The next question was whether the developmental regulation of TrkB receptors is dependent on visual experience. We found that visual deprivation does not affect the expression of TrkB. Thus, contrary to what was found for BDNF mRNA and BDNF protein, the development of TrkB does not depend on visual experience.

INTRODUCTION

Structure and development of the visual system

The visual system is one of the most studied sensory systems. We can not see the world as described by a physicist, but our perception of the visual world is strongly related to the physiological organization of the visual areas in our brain. In mammals, the anatomical organization of the visual system varies across species, but some common features exist.

Light stimuli are detected by photoreceptors in the retina, and transferred by retinal circuits to other cells and finally to retinal ganglion cells. Axons of retinal ganglion cells constitute the fibers of the optic nerve. The majority of retinal ganglion cells that lies in the nasal portion send fibers that cross at the optic chiasm while most fibers in the temporal retina project to the same side of the brain. The principal target areas of retinal ganglion cells are neurons in the superior colliculus and the dorsal lateral geniculate nucleus (LGN). Retinal projections to LGN are precisely organized with each neuron in LGN receiving from a retinal ganglion cell, thus forming a point-to-point map providing the representation of the visual field of both eyes. In mature animals the LGN layers are anatomically segregated with each layer receiving from one or the other eye (Srevatan and Shatz, 1986). The precise organization of LGN layers differs between mammalian species. For example, in higher primates LGN is composed of six layers in which contralateral optic axons terminate in layers I, IV and VI with the remaining layers receiving input from the ipsilateral eye. In the cat LGN there are five layers in which afferents from either eye segregate. In the rat and mouse LGN lamination is less evident and LGN, according

to retinal projections, can be divided in two regions; a large region receiving input from the contralateral eye and a small region from the ipsilateral eye.

The relay neurons in LGN send (Le Vay et al., 1978; Le Vay et al., 1980) highly ordered projections to the primary visual cortex. In particular, LGN projections terminate in cortical layer IV, as in the other sensory cortices, and to a lesser extent in layers II-II, I and VI.

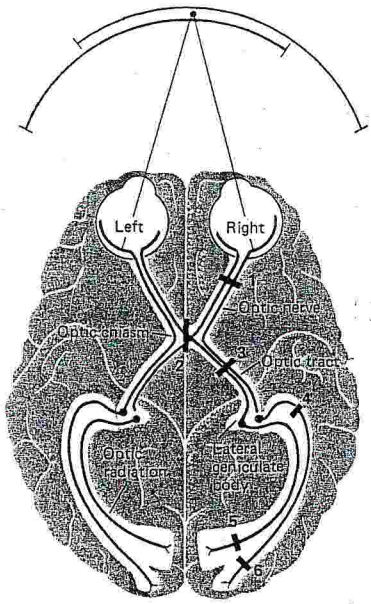
The anatomical organization of the primary visual cortex forms the basis for the functional responses of visual cortical neurons. Indeed, the cortical point-to-point representation of the visual field can be easily detected by recording the response of isolated neurons to stimulation of a portion of visual field (receptive field). Thus, we can speak in terms of a map representation of visual space at the level of visual cortex as well as at previous stations of visual system.

In mature mammals geniculocortical axons are normally segregated into alternating territories or patches in layer IV of the visual cortex receiving input from one or the other eye. These patches are called ocular dominance columns and, when visualized, at the level of layer IV, they appear as regularly alternating stripes (Fig. 1).

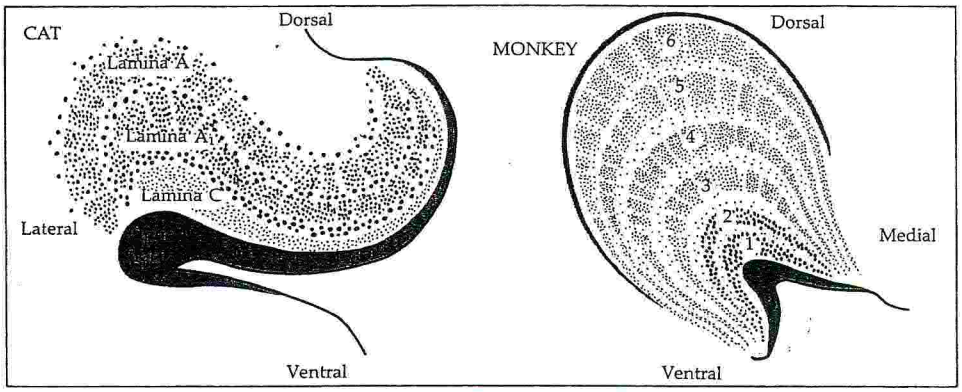
Ocular dominance columns are the anatomical substrate of ocular dominance preference of neuronal responses to contralateral and ipsilateral eye. At the level of layer IV, inputs from each eye are monocularly and functionally segregated. In supragranular and infragranular layers monocular inputs converge, so that neurons respond to both eyes although the preference for the eye sending a monocular input to layer IV spans all layers (physiological ocular dominance columns). To some extent the primary visual cortex of rat and mouse represents an exception, in terms of

Fig. 1

A


Left
Right
Optic nerve
Optic chiasm
Optic tract
Optic radiation
Lateral geniculate body
1
2
3
4
5
6

B


CAT
Dorsal
Lamina A
Lamina A1
Lamina C
Lateral
Ventral
MONKEY
Dorsal
6
5
4
3
2
1
Medial
Ventral

C

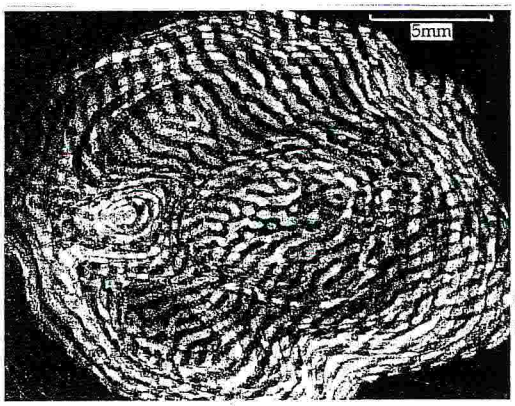

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Fig. 1: Schematic representation of visual pathway, thalamic lamination and cortical ocular dominance columns.

A: Schematic representation of the visual pathway (From "Principles of Neuroscience" ER Kandel, JH Schwartz, second edition, Elsevier SciencePublishing Co. Inc. New York, 1985, pp. 362). B: Lateral Geniculate Nucleus (LGN). On the left the cat LGN contains only three layers of cells: A, A1 and C. On the right the monkey LGN, where there are six layers of cells. In both animals, each layer is supplied by only one eye and contains cells with specialized properties. C: Ocular dominance columns in monkey visual cortex demonstrated by injection of radioactive proline into one eye. (Figures 1B and 1C are from: "From neuron to brain", JG Nicholls, AR Martin, BG Wallace, third edition 1992, Sinauer associates, Inc. Massachusetts, USA).

ocular dominance columns. Indeed, at the level of primary visual cortex there is not a clear and regular segregation of geniculocortical axons. Despite lack of an evident ocular dominance columnar organization, neurons respond preferentially to one or other eye, similarly to other mammalian species. Thus, we can conclude that the representation of the periphery and to some extent also the segregation of connections subserving vision is a common feature of primary visual cortex of different mammals.

The development of the visual system is dependent on genetic, maturational, and environmental factors. All connections in the visual pathways are established during early development and are extremely ordered. During embryonic life axons grow along the correct pathway and establish connections with their appropriate targets. The final goal of several intrinsic and extrinsic factors is to control the development of an ordered and highly stereotyped pattern at all the stations of the visual pathway, from retina to primary visual cortex.

Beyond differences in the developmental time courses between the different mammalian species, it is commonly accepted that at birth gross progressive processes such as active cellular division, neuronal migration and growth, target finding and synapse formation as well as regressive processes like cell death, synapse and axonal branch elimination are nearly over. These processes are responsible for formation of neural connections at the level of the different stations of the visual system. For example in LGN, segregation of inputs occurs during prenatal life and precedes the onset of ocular dominance column formation within the cortex. (Rakic, 1977; Shatz, 1983; LeVay et al., 1978; LeVay et al., 1980). Also ocular dominance formation is a

process which occurs largely or entirely pre-natally; at birth, higher primates, which are born with eyes already open, have ocular dominance columns with characteristics similar to mature animals (Le Vay et al., 1980).

Some developmental processes are controlled by extrinsic factors and in particular by neuronal activity. For example, the refinement of retino-geniculate projections leading to segregation of retinal fibers in different geniculate layers is a process under the control of nerve impulses. Indeed, cat fetuses treated with intraocular injections of tetrodotoxin (TTX), a toxin blocking spiking activity, develop an abnormal segregation of retinal afferents. These data indicate that there is a role for neuronal activity in the segregation of geniculate laminae during pre-natal life. Also in frog developing visual system, topographic refinement of retinotectal projections depends on electrical activity (Zhang et al., 1998). A candidate source for neuronal activity at an embryonic stage is represented by spontaneous activity of retinal ganglion cells (Galli and Maffei, 1988), organized as synchronous retinal waves (Meister et al., 1990).

Concerning the segregation of geniculo-cortical projections the situation is much more controversial. It is known that injection of TTX in the retina at an early stage of postnatal development (P14) results in disruption of ocular dominance columns (Stryker and Harris, 1986). The authors of this work suggested that retinal spiking activity was important for formation of ocular dominance columns. A recent experiment by Crowley and Katz (1999) does not support this conclusion; they showed that the total removal of retina in both eyes, early in visual development, does not prevent the formation of ocular dominance columns in the ferret. These results suggest that neuronal activity is not a factor controlling the formation of ocular

dominance columns. However, whether neuronal activity plays a permissive role during postnatal development, allowing geniculo-cortical neurons to respond to other cues is still a matter of discussion.

What are the effects of blocking the spiking activity of retinal ganglion cells on the development of structural characteristics of geniculo-cortical axons? Stryker and co-workers demonstrated that in the absence of retinal input there is an abnormal development of geniculo-cortical terminal axons, causing non specific sprouting of the axonal arbors, thus inducing a new pattern of connections. According to this idea the absence of neuronal activity in the retino-geniculate pathway might induce an abnormal development of ocular dominance columns. Successive experiments showed that it is not only the level of afferent retinal activity that is important for column formation and/or development. For example, the relative timing of activity in the retinal afferents from the two eyes is used to distinguish inputs at a cortical level. In particular, Stryker and Strickland in 1984, first blocked retinal activity by intraocular injections of TTX, and then controlled neural transmission by stimulating optic nerves synchronously or asynchronously. Synchronous stimulation of the two nerves prevented the formation of ocular dominance columns, whereas asynchronous stimulation permitted them to form. So the patterning of afferent neural activity provides sufficient information for segregation to occur at the level of primary visual cortex.

Apart from the controversy on the formation of ocular dominance columns it seems that retinal neuronal activity plays an important role in the normal development of the geniculo-cortical system and possibly in the process of map formation at the level of primary visual cortex.

Neuronal activity triggered by vision is necessary for the normal development of visual cortical receptive fields. Studies performed on cats, monkeys and rats, have shown that during development there is a progressive maturation of the properties of visual cortical neurons: cells become more responsive to visual stimuli, receptive field size decreases and visual acuity increases; moreover, initially binocular cells respond preferentially to one eye and neurons became more selective to orientation and direction (Hubel and Wiesel, 1962; Boothe et al., 1985; Teller et al., 1978; Albus and Wolf, 1984). In animals deprived of visual experience (dark reared from birth) almost all the properties of visual cortical neurons remain immature. From these results some authors (Timney et al.,1978; Fagiolini et al., 1994) concluded that dark rearing prevents normal maturation of all functional properties of visual cortical neurons. More recent data, obtained by Chapman et al. (1999), show that visual experience is not needed for early development of orientation, but is crucial for maintaining orientation selectivity. Infusion of TTX into visual cortex to silence spiking activity at both pre-synaptic and post-synaptic level, completely blocked the maturation of orientation selectivity, while visual deprivation (by bilateral lid suture) impaired but did not completely block the normal development selectivity. These data show that maturation of orientation selective responses in visual cortex requires afferent retinal activity and visual experience.

Thus, it appears evident that afferent retinal activity and to some degree visual experience is important for the normal development and maintenance of cortical circuitry subserving different visual functions. For this reason, the term activity-dependent development of visual cortex has been created to indicate the instructive role exerted by neuronal activity.

Developmental cortical plasticity

Since the pioneering studies of Hubel and Wiesel, it is well-known in mammals, that if during early postnatal development, one eye is deprived of vision by closing the eyelid for several days or weeks, the ocular dominance distribution of neurons in visual cortex is drastically shifted: most of the neurons became monocularly driven, only by stimulation of the open eye. This is particularly evident in animals which present the pattern of ocular dominance columns. The physiological shift in ocular dominance distribution within visual cortex is coupled with changes in the anatomical organization of LGN axons within layer 4: LGN axons representing the open eye now occupy most of layer 4, whereas those representing the closed eye are relegated to very small patches. Moreover neuronal somata in the deprived laminae of LGN are anatomically modified by monocular deprivation; the shrinkage of LGN cell bodies is a retrograde effect, originating in the striate cortex, and it is probably due to the lack of a factor released by cortical neurons and necessary for LGN to maintain the correct cellular shape.

The results of monocular deprivation are effective and irreversible if performed during a "critical period" during the first postnatal weeks. Rat and mouse have the shortest critical periods of mammalian species (Fagiolini et al., 1994) and therefore represent good animal models to test the effects of molecules on plasticity and postnatal development of the visual cortex.

Sensory experience is a strong determinant of the duration of critical periods: lack of visual experience (dark rearing) usually prolongs critical periods for monocular deprivation. Thus, dark rearing induces two clear effects: i) it delays the

end of the critical period for monocular deprivation; ii) it delays the postnatal development of functional properties of visual cortical neurons, as reported in the previous paragraph.

The most reasonable explanation for the effects induced by monocular deprivation is that geniculo-cortical axons driven by the two eyes compete for layer 4 neurons. This has been strongly suggested by an experiment performed in 1972 by Guillery, who sutured one eye of a kitten and simultaneously made a small lesion in the open eye, destroying a localized group of ganglion cells. In this case the effects of monocular deprivation were present in all visual cortex with the exception of a small region receiving LGN axons representing the lesioned area of the open eye and the corresponding region of the closed eye. The use of the two eyes during the critical period subserves competitive interactions whose outcome is manifested in the distribution of ocular dominance.

Competitive interactions between the two eyes are clearly based on the level and/or pattern of activity driven by the two eyes. Signaling by neurons is via spiking activity and, thus the effects of visual experience of the two eyes on the organization of primary visual cortex would be the result of changes in the level and/or pattern neuronal activity. Indeed, intraocular application of tetrodoxin (TTX), a sodium channel blocker which abolishes the spiking activity of retinal ganglion cells, reproduces the effects of monocular deprivation. Also the relative timing of activity in the two eyes is used to distinguish inputs at a cortical level as was reported in the previous paragraph for formation of ocular dominance columns (Stryker and Strikland, 1984). Synchronous afferent retinal activity favors the strengthening of geniculate synapses driven by both eyes, thus resulting in more binocular neurons

and a reduction in the segregation of geniculo-cortical inputs. Asynchronous activity favors synapses from either eye and therefore cortical cells become predominantly monocular and geniculo-cortical axons segregated. A more direct support of this hypothesis is represented by strabismus. In mammals with binocular vision a misalignment of the optical axes of the eyes causes dramatic changes at the level of primary visual cortex. Hubel and Wiesel were the first to report that the proportion of binocular cells in neonatal kittens reared with a surgical strabismus was greatly reduced. Since the experiment of Hubel and Wiesel the effects of strabismus have been interpreted as a lack of congruence between the visual inputs coming from the two eyes: with misalignment of the optical axes, corresponding retinal points see different parts of the visual scene; afferent retinal activity is not synchronous and binocular cells are lost.

The next question is whether postsynaptic target cortical neurons, and in particular the postsynaptic activity, have a role in visual cortical plasticity. This question has been addressed by Reiter and Stryker in 1988. They silenced cortical neurons by local infusion of muscimol, a GABAa receptor agonist, and simultaneously performed monocular deprivation during the critical period. They observed that within the silenced region of the cortex, inputs from the closed eye dominate those of the open eye, and this situation was reversed outside the silenced zone. These data suggest that the same patterning of presynaptic activity produces different effects depending on the state of activation of the postsynaptic cell. Direct evidence for this hypothesis comes from the experiment of Fregnac et al. (1988) By modulating the post-synaptic response to different stimuli, they showed that the

temporal coincidence between pre- and post-synaptic activity is a important to enhance visually driven inputs.

Interesting results have been obtained by blocking the cortical activity, both at the pre-synaptic and post-synaptic site, by infusing TTX locally in the visual cortex (Reiter et al., 1986) Such treatment prevents the shift in ocular dominance distribution produced by monocular deprivation performed during the critical period. This serves to illustrate the point that co-incidence between pre-synaptic and post-synaptic activity (in the Reiter's experiment the activity was blocked at both levels) alters the outcome of competition between the visual input from the two eyes.

All of these experiments are consistent with the hypothesis raisen by the Canadian psychologist Hebb. In 1949 Hebb suggested that when pre-and post-synaptic neurons are co-activated, their synaptic connections are strengthened.

Following this hypothesis LGN axons driven by the deprived eye depress their synapses because their activity is not correlated with that of the cortical target neuron. In contrast, the activity of LGN axons driven by the open eye correlates with that of cortical neurons and their synapse strengthen. This type of plasticity is associative, linking the activity of pre-synaptic neurons with that of post-synaptic neurons. An important question is what is the appropriate cellular model for activity-dependent weakening/enhancement of geniculo-cortical connections.

Synaptic plasticity in the primary visual cortex.

The efficacy of synaptic transmission can be regulated over a wide range of temporal scales, ranging from a temporal modulation of msec, to minutes and hours (pair pulse facilitation, short term plasticity, long term plasticity). Two studied forms of long

term synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD).

LTP is a long-lasting increase in synaptic effectiveness of certain excitatory synapses in the hippocampus. Long-term depression can be elicited by prolonged stimulation at low frequency and produces a long lasting decrease in synaptic effectiveness. Because the two forms of long term change can be elicited by protocols of electrical stimulation that resemble patterns of activity observed in different structures of the CNS, both LTP and LTD have been proposed as models of synaptic plasticity; in the hippocampus it has been proposed that LTP and LTD are the cellular basis of learning and memory.

More recently, LTP and LTD have attracted a great deal of interest also to explain cellular mechanisms involved in developmental plasticity and in general in activity-dependent development during the critical period of the visual cortex. For example, monocular deprivation performed during the critical period induces first weakening, and then loss, of geniculo-cortical synapses driven by the deprived eye; the cortical territory driven by the deprived eye shrinks. On the contrary, cortical synapses driven by the undeprived eye are strengthened and geniculo-cortical connections tend to occupy the territory innervated by the other eye; therefore the territory driven by the undeprived eye enlarges.

It has been proposed that the phenomena of homosynaptic or heterosynaptic LTD are responsible for the formation of ocular dominance columns and monocular deprivation (Dudek, 1996).

Beyond the role of LTP and LTD in the visual cortical plasticity, it is conceivable that both forms of synaptic plasticity might also be involved in the activity-dependent development of visual cortex.

Pre-synaptic activity can be translated into frequency of electrical stimulation; low rates of stimulation normally results in LTD while high frequency stimulation elicits LTP. This theory justifies the use of electrical stimulation protocols to elicit LTP and LTD. Indeed, for LTP induction two different protocols can be used: the first one uses fixed frequency stimulation trains of pathways afferent to the recorded neuron in order to induce activity-dependent changes in the efficacy of the synapse. A second strategy which has been applied both in vitro and in vivo does not require high frequency inputs trains. Typically, conditioning relies primarily on the exogenous control of the post-synaptic activity of the conditioned neuron, and consequently on the temporal correlation between pre- and post-synaptic activity. In this situation, the effects are shorter-lived than those produced by tetanic stimulation of afferent pathways. This last protocol can be considered as experimental evidence for the Hebbian hypotesis.

For induction of LTD the most popular experimental procedure is that proposed by Dudek and Bear (1992)., which consists of low frequency stimulation (900 pulses at 1 Hz) of the afferent pathways.

Visual cortex has the capacity to undergo long-lasting changes of synaptic strength. LTP and LTD can be elicited in slices containing the primary visual cortex by stimulation of white matter containing geniculo-cortical connections.

These two forms of plasticity are expressed differently in the rat visual cortex during postnatal development. While LTD does not change, LTP is maximally

expressed soon after eye opening and progressively diminishes during the following period; in the adult visual cortex it is almost impossible to induce this form of plasticity. Interestingly, in rat cortical slices the period of expression of LTP overlaps with the critical period for monocular deprivation. The same is not true for the mouse, since LTP from stimulation of white matter can also be elicited in adult visual cortex (Pesavento E. and Domenici L., personal observation). Thus, the visual cortex of rat represents a good system to test whether cellular models of plasticity reflect *in vivo* models of neuronal plasticity.

The dependence of LTP on developmental stage is not observed if stimulation is conducted in layer 4 instead of white matter. This different behavior has been explained by postulating the development of inhibitory circuitry at the level of layer 4 during the critical period. In this model, as the GABA circuitry develops, so it becomes more difficult to induce potentiation by stimulation of the white matter. Instead, stimulation of layer 4, by-passes the inhibitory circuitry and hence LTP is not affected by development. This hypothesis is supported by experiments in which bicuculline, a GABAa receptor antagonist, was placed in the recording micropipette during the LTP induction protocol; in this case the electrical stimulation of the white matter was able to evoke LTP in layers II-III even in adult animals (Kirkwood and Bear, 1994a). The result that intracortical connections, maintain the capacity to undergo long-term changes of synaptic efficacy fits with the idea that these intra-cortical connections remain plastic also after the end of the critical period for monocular deprivation.

The expression of LTP and LTD in the visual cortex is regulated by visual experience. In dark reared animals it is still possible to evoke LTP even in adulthood,

although the amplitude is reduced. This behavior can be explained by the Bienenstcok-Cooper-Munro model (Bienenstock et al., 1982); the assumption is that synaptic tendency to undergo potentiation or depression is not fixed, but rather depends on synaptic history. According to this model there is a threshold for synaptic modifications, called θm; variations above this value lead to potentiation, on the contrary, variations below produce depression. In 1996 Kirkwood et al., showed that there are several factors that control the value of θm and one of these in the past sensory experience.

In particular the authors found that in visual cortex of light-deprived rats LTP is more easily induced than LTD: considering the induction of LTP or LTD with respect to stimulation frequency, lower frequencies induce LTP in dark reared but not in normal reared animals. These effects can be reversed by two days of light exposure. The hypothesis is that the threshold for LTD/LTP depends on the history of post-synaptic activity. These results are reported in Fig. 2: after a period of increased activity the threshold slides to the right so promoting synaptic depression and vice versa. The prediction in the case of dark rearing, which corresponds to a period of decreased afferent activity, is that the synaptic threshold slides to the left promoting potentiation. For this reason, blockade of activity prevents the loss of potentiation but simultaneously puts synapses at a depressed level and so the probability of further depression is reduced. Exposure to light for two days after dark rearing reestablishes the normal synaptic behavior. Beyond the cellular mechanisms underlying dark rearing effects on LTP it is important that visual deprivation prolongs both the critical period for monocular deprivation and the period of expression of LTP.

Fig. 2

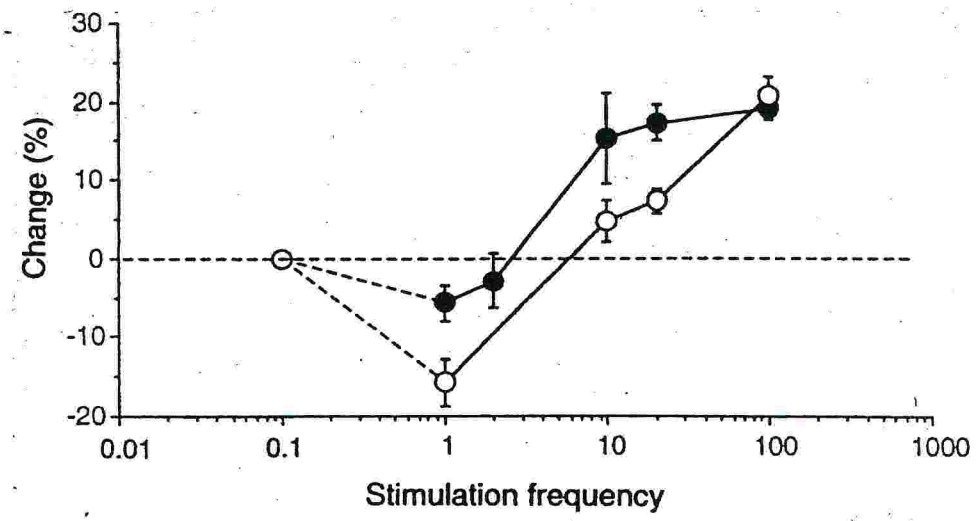


Fig. 2: Frequency-response functions derived from visual cortex of light-deprived (filled symbols) and normal (open symbols) rats.

Data points for stimulation frequencies ≥ 10 Hz represent the average change (± s.e.m.) 20 min. after the delivery of 120 pulses of conditioning stimulation. Data points for 1- and 2-Hz stimulation represent the average change (± s.e.m.) 30 min after delivery of 900 pulses of conditioning stimulation. The data point for 0.07 Hz inferred as baseline stimulation once every 15s does not appear to induce synaptic modification in light-deprived or normal cortex. All data are from slices maintained in an interface chamber. (Kirkwood et al., 1996, Nature 381, p: 528).

Thus, activity-dependent properties of visual cortical synapses during the critical period are reminiscent of some of well known forms of long-term changes of synaptic efficacy, such as LTP and LTD, illustrated in this chapter. However, until now, work on visual cortex synaptic plasticity has dealt with either LTP or LTD but not the two forms together. The question is whether synapses that are depressed can be de-depressed and synapses that are potentiated can de-potentiated. This property would confer a high degree of plasticity on the visual cortex, mainly at a postnatal age when this structure is still immature and there is the necessity to revert a previous synaptic assessment: potentiation or depression. In the hippocampus the maintenance of the capacity to reverse a given long term change in synaptic efficacy correlates well with role of the hippocampus in learning and plasticity.

Factors controlling visual cortical plasticity

Several factors have been proposed to control visual cortical plasticity and the critical period for monocular deprivation.

The first serious attempt to individuate the factors controlling visual cortical plasticity was advanced by Kasamatzu and Pettigrew (1979). By depleting cortical noradrenaline with pharmacological agents delivered through osmotic minipumps it was possible to prevent the effects of monocular deprivation. Successively, Bear and Singer (1986) showed that to prevent monocular deprivation effects it was necessary to deplete and/or destroy both the noradrenergic and cholinergic systems during an early stage of postnatal development.

Mammalian visual cortex receives extensive cholinergic and noradrenergic projections. These projections are established around the time of birth and develop

during the first postnatal month in the rat visual cortex. Acetylcholine facilitates neuronal plasticity through muscarinic M1 receptors, while NA action is mediated by beta-receptors. Ach and NA enhance the depolarizing response to tetanic stimulation; this has been shown in kitten visual cortical slices by Brocher et al., (1992) who found that both cholinegic and noradrenergic agonists raised the probability that tetanic stimulation could induce LTP. More recently, it has been reported (Kirkwood et al., 1999) that homosynaptic LTD in the visual cortex is facilitated by activation of muscarinic (M1 type) and noradrenergic receptors (α1 type).

Further studies have shown that co-activation of serotonin receptor subtypes of the 5-HT1 and 5-HT2 families also have a permissive function in OD plasticity (Gu and Singer, 1995).

Subsequently, other plausible candidates have been indicated. In particular, a class of glutamate receptors, NMDA has been proposed to be responsible for the plastic signal. NMDAr is a receptor composed of two or more different subunits. Three classes of NMDAr subunits have now been identified. The NR1 class is essential for receptor function and comprises several splicing variants whose function is not clear. NR2 class comprises four different subtypes (A-D) conferring functional specificity to NMDAr and responsible for functional heterogeneity of NMDAr. Many experiments point to NMDARs as molecular determinants for neuronal plasticity in the visual cortex. First, the composition of NMDAr subunits varies in the visual cortex during the critical period. The subunit 2B is progressively substituted by 2A and this is related to the progressive shortening of NMDA currents in cortical layer IV. Thus as postnatal development proceeds NMDAr current shorten and as a consequence visual inputs are less integrated at the level of cortical target neurons of

layer IV. The second consequence is that at the end of the critical period less calcium enters into the cell through NMDAr with respect to early postnatal development. The developmental shortening of the currents is delayed in dark reared animals where the level of 2A subunits is lower than in normal-reared animals (Carmignoto and Vicini, 1992).

These results indicate that the regulation of NMDAR subunits is dependent on visual experience: dark rearing reduces the expression of 2A subunits, but re-exposure to a brief period of light restores normal levels (Quinlan et al., 1999a). Moreover, the reduction of 2A expression occurs also if animals are put into the dark at P23 after a period of normal rearing (Quinlan et al., 1999b).

There is much evidence on the role of NMDA receptors in synaptic plasticity. It has been reported that NMDAR control LTP expression Infact, in experiments where APV, a NMDAr receptor antagonist, was applied, it was not possible to induce LTP (Artola and Singer, 1987).

It was initially thought that the LTD form of synaptic plasticity was controlled by receptors different from NMDAr, but this hypothesis has not been confirmed by recent experimental evidence. Indeed, experiments conducted in slices of visual cortex show that application of the NMDAr antagonist AP5 prevents induction of LTD. (Chistianova et al., 1999) If the same receptors control both LTP and LTD, what determines which form of plasticity is to occur? It is known that activation of NMDA receptors leads to an increase of intracellular Calcium. How can the same signal represented by the calcium entry trigger both LTP and LTD? Use of calcium chelating agents e.g. rhod-2 (Yasuda end Tsumoto, 1996) has shown that during LTP and LTD there is an intracellular increase of Calcium, but the amount of Calcium

influx during potentiation is higher and more sustained. Thus, modest elevation of Calcium causes LTD while a more robust elevation triggers LTP (reviwed by Linden, 1999). One hypothesis is that the threshold θm is also controlled by Calcium concentration.

The discovery of NMDAr and their role in the induction of LTP and LTD prompted several researchers to investigate whether similar mechanisms are also involved in experience-dependent processes occurring in developing visual cortex. Kleinschmidt et al. (1987) showed that NMDAr blockade prevents the shift in ocular dominance toward the open eye occurring when monocular deprivation is performed during the critical period. More recently, it has been shown that blockade of NMDA receptors with antisense oligodeoxynucleotides in monocularly deprived ferrets produces ocular dominance histograms resembling those of monocularly deprived animals (Roberts et al., 1998). These studies suggest that NMDAr contribute to the mechanisms underlying synaptic rearrangement occurring during the critical period and revealed by monocular deprivation; however, this matter remains controversial. Physiological studies of cortical neurons demonstrate that NMDA blockers decrease the spontaneous and evoked firing of visual cortical neurons, particularly during postnatal development (Kato, 1993).

Thus, two alternative explanations have been proposed. The first hypothesis is that the plasticity signal passes through NMDAr. The second possibility is that the effects produced by NMDAr blockers on monocular deprivation are aspecific, and are due to the reduction of synaptic activity. This second idea is supported by the fact that NMDAr blockers not only prevent the effects of monocular deprivation but also

affect some basic functional properties of visual cortical neurons such as orientation selectivity, thus producing similar effects to those induced in dark reared animals. On the contrary, orientation and direction selectivity of cortical cells were not affected by dark rearing (Roberts et al., 1998).

Recent data suggest a role of other subclasses for glutamate receptors: AMPA receptors and metabotropic receptors in the modulation of these forms of plasticity. There are different classes of metabotropic glutamate receptors. They are differentially expressed in different cortical layers of cat primary visual cortex and their expression is regulated during development. Recent results have shown that metabotropic receptors play an important role in LTP expression (Cammarota et al., 1998; Nayak et al., 1998; Leonard et al., 1999; Zamanillo et al., 1999)

Although NMDA receptors are essential for the initiation of LTP, expression of LTP is mediated primarily by AMPA receptors. The model proposed suggests that LTP activates some internal kinases that act on AMPA receptors subunits, causing an increase in current through these channels. One of the kinases involved in these processes has been identified as CaMKII. On the other hand, phosphatases actions would lead to LTD. In fact calcineurin is essential for LTD expression (Zhuo et al., 1999).

Among neurotransmitters, GABA appears to influence activity-dependent development and plasticity of visual cortex. The maturation of GABAergic cortical circuitry occurs during early postnatal development; in the rat visual cortex most neurons respond to GABA already before eye opening (Luhman and Prince, 1991). The application of the GABA antagonist bicuculline in the kitten visual cortex induces a loss of orientation selective neurons and an alteration of receptive field

organization (Wolf et al., 1986). As reported in previous paragraphs GABAergic cortical circuitry influences developmental visual cortical plasticity (Huang et al., 1999). By infusing muscimol in the visual cortex, Reiter and Stryker (1988) were able to prevent the ocular dominance distribution shifting towards the open eye, with a conspicuous number of neurons driven by the closed eye (paradoxical shift of ocular dominance distribution).

From work in the cortex it is well-known that lowering the level of inhibition reduces the threshold for eliciting LTP. For example, reducing the level of inhibition in cortical slices enables LTP to be elicited by stimulation of white matter also in adulthood. This led to the notion of the inhibitory gate hypothesis; at the level of cortical layer IV inhibitory circuitry acts as a gate in adult animals. If there is a link between GABA action on LTP and the critical period, when GABA inhibition is reduced LTP must be enhanced, it must be expressed also in adult animals and the critical period for monocular deprivation should be prolonged. To clarify this question, recently, experiments have been performed in mice lacking the gene for glutamic acid decarboxylase (GAD) GAD65, the isoform of GAD localized in synaptic terminals. In these mice inhibition exerted by GABA is clearly reduced but LTP is normal and the effects of monocular deprivation are completely prevented indicating the presence of a deficit in plasticity; this deficit disappeared when a GABA agonist was supplied in the visual cortex. These data suggest that cortical inhibitory circuitry controls visual cortical plasticity during the critical period.

From the experiments reported above, it seems that the development and plasticity of the visual system is the outcome of the action of many factors: NMDA receptors, noradrenergic and cholinergic systems and GABA circuitry. These systems

cooperate in the formation of cortical circuitry and in the acquisition of the properties of mature visual cortical neurons. Anyway, the definitive proof that they act in a specific way and represent an instructive signals for visual cortical plasticity is still missing.

However, one important question remains unanswered: what are the factors which communicate to pre-synaptic cells that they have to weaken/suppress or to strength/stabilize their synapses? All the factors illustrated until now act at the post-synaptic level through different sets of receptors. Since experience-dependent modifications of visual cortical circuitry associate the level and/or patterning of pre-synaptic activity with that of post-synaptic neurons it is essential to find a molecule whose expression is developmentally regulated, whose production and/or secretion is activity-dependent; in addition this molecule must be able to act on specific pre-synaptic receptors by activating an intracellular cascade of events responsible for cell response.

Neurotrophins and their receptors in the CNS.

During the last ten years neurotrophins have emerged as attractive candidates for playing a central role in synaptic plasticity and activity-dependent development.

Neurotrophins are a family of related protein factors which were initially found to be involved in survival and differentiation of neuronal cultures. Later it was shown that these molecules were also present in the Central Nervous System (CNS) where they exerted their action at many levels of neuronal life. At least four neurotrophins have been identified and studied: Nerve Growth Factor (NGF) (Levi-Montalcini, 1951; Cohen, 1960; Levi -Montalcini, 1987), Brain-Derived

Neurotrophic Factor (BDNF) (Barde et a., 1982; Leibrock et al., 1989; Ernfors et al., 1990), neurotrophin 3 (NT-3) (Ernfors et al., 1990; Hohn et al., 1990; Jones and Reichardt, 1990; Kaisho et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990) and neurotrophin-4/5 (NT-4/5) (Berkemeier et al., 1991; Hallböök et al., 1991).

Neurotrophins are small homodimeric polypeptides (they have about 120 aminoacid residues) with similar structure, as they share approximately 50% sequence identity (Hallböök et al., 1991). All neurotrophins have a similar dimer arrangement (Robinson et a., 1995), and are synthesized as precursor proteins as it has been shown for NGF and BDNF.

The BDNF gene has four different short 5' exons (I-IV), each of which associate with a separate promoter, and one 3' exon (V) that encodes the mature BDNF protein (Timmusk et al., 1993; Nakayama et al., 1994).

The four different promoters produce eight transcripts, each containing one of the 5' exons with or without exon V. Studies using probes specific for the exons I-IV have shown that transcripts containing these exons are differentially expressed in brain regions (Timmusk et al., 1993; Bishop et al., 1994), and are differentially regulated by a variety of stimuli (Metsis et al., 1993; Timmusk et al., 1993; 1995; Nakayama et a., 1994; Lautereborn et al., 1996). These studies suggest that the alternative usage of four promoters within the BDNF gene and differential splicing may be used for tissue-specific and stimulus-induced expression of BDNF mRNA species. The distinct functions of these different mRNA species remain unknown.

Neurotrophins exert their action by binding to appropriate receptors on the cell membrane. The first identified receptor was p75. It was first thought to be the

NGF receptor, but was then shown to bind all neurotrophins with low affinity. Neurotrophins have specific "high affinity receptors" which belong to the Trk family: NGF selectively recognizes the TrkA receptor, BDNF and NT-4/5 the TrkB receptor and NT-3 the TrkC receptor.

Some limited cross-reactivity has also been reported, whereas interaction of neurotrophins with p75 may be important for increasing the specific activation of Trk receptors (Benedetti et al., 1993; Clary and Reichardt, 1994; Chao and Hempstead, 1995). Trk receptors have an extracellular domain and a cytoplasmic portion. Binding of neurotrophin causes autophosphorylation of the receptor and an increase of the phosphorylation of some cellular substrates, with subsequent activation of specific intracellular pathways. In general, the tyrosine phosphorylation events result in downstream stimulation of the activity of cellular proteins involved in the regulation of cell shape and the activation of gene transcription machinery that controls growth, migration, morphology and survival of neurons.

TrkB and TrkC also exist in a truncated form, lacking the intracellular domain. Although identical to its full-length counterpart in its extracellular portion, the truncated form of TrkB replaces the intracellular domain with a short peptide of unknown function (Klein et al., 1990a, b; Middlenass et al., 1991). The biological significance of the truncated receptors is still unclear, but it is believed that they could compete, behaving as negative dominants, with the full length catalytic receptors; this could represent an independent mechanism to modulate the availability and uptake of neurotrophins (Biffo et al., 1995). Different splicing forms of the truncated and full-length receptors have been described. At least seven different transcripts encoding either full-length or truncated TrkB receptor have been identified in the CNS (Klein

et al., 1990a; 1990b; Middlemas et al., 1991). Full length forms are expressed in neuronal cells. In contrast, truncated forms are found in both neurons and glial cells (Barbacid 1994, Linsday et al., 1994). Co-expression of the two forms has been reported to occur in several brain regions.

Recently, some studies have been performed to localize the intracellular distribution of the TrkB receptor (Drake et al., 1999). This paper studied the expression of full length TrkB in hippocampal neurons. It was found that TrkB is present in glutamatergic pyramidal and granule cells. Labeling in these neurons appeared as discrete clusters. It was primarily in axons, excitatory-type axon terminals and dendritic spines, and to a lesser extent in somata and dendritic shafts; in other subcellular regions TrkB was often intracellular. TrkB was found in interneuron axon initial segments, a few axon terminals forming inhibitory-type synapses. This suggests that TrkB is contained in GABAergic interneurons.

The sub-cellular compartmentalization of TrkB receptors has been studied in CNS neurons. In peripheral neurons TrkB is highly expressed both on the cell membrane and intracellularly. In contrast, central neurons display only low levels of surface TrkB, though high levels are present intracellularly. When these neurons are activated, the cellular expression of TrkB increases, and this occurs also in presence of the translation inhibitor cycloheximide; these results indicate that neuronal activation rapidly recruit TrkB to the plasma membrane by translocation from intracellular stores (Meyer-Franke et al., 1998). These authors propose that a fundamental difference between Peripheral Nervous System and Central Nervous System neurons is the activity-dependence of CNS neurons, responsiveness to

neurotrophins. Membrane compartmentalization of the neurotrophin receptors may underlie this difference.

Neurotrophins and their receptors are distributed specifically in several brain areas. For example, BDNF and its receptors are particularly highly and widely expressed in the hippocampus and to a lesser degree in the cortex; therefore in two well-studied sites for neuronal plasticity. In addition to their specific patterns of expression, BDNF, as well as other neurotrophins, changes during postnatal development. In particular, BDNF expression increases during the first weeks of postnatal life. The developmental pattern of TrkB largely overlaps that of BDNF in several brain regions. Thus, there is much evidence that the increase of neurotrophins and their receptors is temporally coincident with progressive processes such as neuronal growth, synapse formation and phenotype differentiation (Ernfors et al., 1990; Dugich-Djordjevic et al., 1992; Ringsedt et al., 1993; Altar et al., 1994; Knusel et al., 1994).

Experiments both in culture and in vivo have shown that modulation of neuronal activity influences the expression of neurotrophins. The different studies have been conducted primarily at the mRNA level by in situ hybridization. NGF and BDNF mRNA levels are rapidly upregulated by epileptiform activity in the hippocampus and cerebral cortex (Gall and Isackson, 1989; Zafra et al., 1990; Ernfors et al., 1991; Isackson et al., 1991; Dugich-Djordjevic et al., 1992). While after induction of seizures in the hippocampus the level of BDNF mRNA is increased (Ernfors et al., 1991), a similar stimulation reduces NT-3 expression (Castren et al., 1993; Elmer et al., 1996; Mudo et al., 1996). In vitro depolarization of hippocampal neurons and cerebellar granule cells by glutamate receptor agonists or high potassium increases

BDNF and NGF mRNAs (Zafra et al., 1990; Lu et al., 1991; Zafra et al., 1992; Bessho et al., 1993; Lindholm et al., 1994; Berninger et al., 1995). Conversely, activation of GABAa receptors lowers BDNF and NGF levels (Zafra et al., 1992; Berninger et al., 1995). Moreover, the expression of BDNF mRNA requires high intracellular calcium and involves activation of the transcription factor CREB (Shieh et al., 1998; Tao et al., 1998).

Studies on hippocampal cultures have shown that BDNF mRNA expression is not mediated by protein synthesis; as a consequence BDNF appears to be regulated as an immediate-early gene (Lauterborn at el., 1996).

Recent evidence suggests that central neurons may release neurotrophins in an activity-dependent manner (Bloch and Thoenen, 1995; Wang and Poo, 1997). For example, high potassium, glutamate and acetylcholine all evoke NGF secretion from hippocampal neurons overexpressing NGF (Block and Thoenen, 1995, 1996). BDNF secretion is also induced by depolarization of hippocampal neurons overexpressing BDNF (Goodman et al., 1996). Interestingly, BDNF has been localized to dense-core vesicles in dendrites of hippocampal neurons: these vesicles are potentially capable of undergoing regulated release (Fawcet et al., 1997; Smith et al., 1997; Moller et al., 1998).

Thus, in the CNS neurotrophins can be secreted and possibly released through the activity-regulated pathway, similarly to classical neurotransmitters.

Role of neurotrophins in the CNS.

Neurotrophins exert many actions on neuronal survival, phenotype differentiation and regulation of synaptic transmission and plasticity. Peripheral

neurons have been shown to depend on a specific class of neurotrophin, while in the CNS, despite the broad distribution of Trk receptors, no obvious abnormalities are observed after elimination of the neurotrophin genes (Ernfors et al., 1994a,b; Jones et al., 1994; Farinas et al., 1994; Crowley et al., 1994). To explain this discrepancy it has been suggested that neuronal elimination is controlled in the CNS by multiple neurotrophic factors and none of them is individually essential for neuronal survival (Davies 1994; Snider, 1994; Lewin and Barde, 1996; Cellerino et al, 1995).

There is evidence that NGF regulates phenotype differentiation and maintenance in the CNS. A classical example is the cholinergic neurons of the basal forebrain. These neurons present NGF receptors and respond to NGF by increasing the intracellular expression of choline acetyl transferase (ChAT). In the absence of NGF, or when the NGF is functionally blocked by antibodies, basal forebrain cholinergic neurons develop abnormally; in particular, ChAT expression is strongly reduced and thus these cells do not acquire their normal cholinergic phenotype. An endogenous normal level of NGF is also required in adults to maintain the normal phenotype. Indeed, when NGF is functionally deprived ChAT is down-regulated and therefore basal forebrain cholinergic neurons are reduced (Li et al., 1995).

Initially, NGF was isolated for its ability to stimulate neurite growth, subsequently, each neurotrophin has been demonstrated to stimulate neurite outgrowth of specific neuronal populations in the PNS, both in vitro and in vivo (Thoenen, 1991). Neurotrophins exert this effect also in CNS neurons. In cell culture of cortical neurons, BDNF promotes the neurite growth of interneurons. In addition, BDNF can influence the complexity of axonal arbors (Inoue & Sanes, 1997), and control dendritic form (McAllister et al., 1995; Baker et al., 1998) of pyramidal

neurons. In organotypic coltures it has been observed that the effects of neurotrophins are layer-specific and restricted to particular dendritic patterns (McAllister et al., 1996). This indicates that these molecules do not simply act to enhance neuronal growth, but rather act instructively to modulate patterns of dendritic arborization. Not only the acute administration of neurotrophins, but also their removal has dramatic consequences for dendritic growth (MaAllister et al., 1997). A recent experiment in which TrkB receptor was eliminated only in pyramidal neocortical neurons, shows that lack of TrkB produces progressive atrophy of dendritic arborization and finally cell death (Xu et al., 2000).

Neurotrophins appear to control several aspects of synaptic transmission. For example they influence neurotransmitter phenotype of neurons in the CNS (Gnahn et al., 1983; Li et al., 1995; Nawa et al., 1994; Jones et al., 1994), and modulate the release of neurotransmitters (Carmignoto et al., 1997). In particular, NGF and BDNF increase neurotransmitter release in rat visual cortex (Sala et al., 1998)

Using biochemical tools, it has been shown that NGF and BDNF enhance high potassium-induced release of acetylcoline and glutamate from hippocampal synaptosomes (Knipper et al., 1994) and that BDNF increases the expression levels of several synaptic vescicle proteins (Takei et al., 1997). Moreover, BDNF increases the phosphorylation of NMDA receptors (Suen et al., 1997) and alters the functional properties of NMDA receptor channels (Jarvis et al., 1997; Levine et al., 1998).

BDNF also acts on AMPA receptors: it regulates the expression of AMPA receptor proteins, in particular GluR1 and GluR2/3 in neocortical neurons (Narisawa et al., 1999) and plays a role in the maintenance of the balance between cortical excitation and inhibition by modulating AMPA and GABA currents (Rutherford et

al., 1998; Desai et al., 1999).

In studies conducted on neuronal cell lines, it has been shown that neurotrophins modulate the expression of voltage-gated channels (Sherwood et al., 1997; Blowlby et al., 1997).

In addition to controlling synaptic transmission, neurotrophins are capable of modulating the strength/depression of synapses within minutes. In hippocampal slices, acute application of BDNF or NT-3 potentiates synaptic transmission at Schaffer collateral/CA1 synapses (Kang & Shuman, 1995). In the visual cortex BDNF and NGF both acutely potentiate excitatory transmission (Carmignoto et al., 1997).

LTP and LTD have been shown to be modulated by neurotrophins. LTP induction at the hippocampal Schaffer collateral-CA1 synapse was found to be affected in BDNF knock-out mice (Korte et al., 1995; Patterson et al., 1996). These alterations could be rescued either by transfection of hippocampal slices with a BDNF expressing adenovirus (Korte et al., 1996) or by application of exogenous BDNF (Patterson et al., 1996). Exogenous BDNF promotes LTP in young hippocampal slices at ages when LTP is normally not inducible (Figurov et al., 1996). Also depression, not only potentiation, is regulated by BDNF: in the visual cortex, the induction of LTD by low frequency stimulation is prevented by pretreatment with BDNF (Akaneya et al., 1996; Huber et al., 1998).

Neurotrophins and visual cortical plasticity.

Segregation of visual inputs at the level of primary visual cortex and development of visual cortical circuitry are both dependent on visual experience. Thus, the visual

cortex can be considered a model system for studies of molecules involved in sensory-experience dependent processes. During the last ten years many studies have been conducted in the visual system with the aim of demonstrating that neurotrophins control visual cortical plasticity and activity-dependent development of visual cortex. The first experiments were done in the visual cortex of rat: the authors showed that an exogenous supply of Nerve Growth Factor (NGF) is able to prevent the effects induced by monocular deprivation and strabismus during the critical period (Domenici et al., 1991; Domenici et al., 1992; Maffei et al., 1992; Domenici et al., 1993). In particular, adding NGF prevents the shift of ocular dominance distribution, the reduction of visual acuity in the deprived eye, and the shrinkage of LGN neurons driven by the deprived eye. Later, it was demonstrated that endogenous NGF is necessary for the normal development of the geniculo-cortical system and for the temporal definition of the critical period for monocular deprivation. Indeed, NGF blockade by antibodies affects the functional properties of visual cortical neurons and the morphological characteristics of geniculo-cortical neurons, with the critical period longer than in normal animals. These findings led to the hypothesis that LGN fibers compete for NGF released by cortical target neurons. A hypotesis related to this is that NGF expression and/or release is neuronal activity-dependent and that TrkA, the specific receptor for NGF is expressed in LGN and/or cortical neurons. TrkA is not expressed in LGN neurons. In the visual cortex, TrkA has been found in cholinergic neurons of basal forebrain sending a well-defined projection to several areas including the visual cortex. Recently, it has also been shown that intrinsic cortical neurons express TrkA receptors during postnatal development. Thus, one possible explanation for NGF results is that NGF acts on cholinergic terminals present in the

visual cortex and on intrinsic neurons to regulate developmental cortical plasticity.

More recently, it has been reported that brain derived neurotrophic factor (BDNF) and neurotrophin 4 (NT-4) are also able to modulate visual cortex development and plasticity. An exogenous supply of BDNF/NT-4 prevents the formation of ocular dominance columns (Cabelli et al., 1995). A possible interpretation is that LGN fibers compete for BDNF instead of NGF. Following this hypothesis, adding an excess of BDNF may attenuate competition between the LGN fibers in layer IV, in this way preventing the segregation of LGN inputs leading to the formation and/or maintenance of ocular dominance columns. BDNF mRNA is expressed in the visual cortex and its receptor TrkB has been found in both the visual cortex and LGN. However, to validate such hypothesis one must demonstrate that blockade of TrkB signaling increases the competition between LGN fibers and therefore induces the formation of ocular dominance columns even sharper than in the normal case. In striking contrast, infusion of TrkB fusion proteins blocking TrkB ligands prevents the segregation of LGN inputs, similarly to what is found by supplying BDNF. These results suggest that BDNF has a role in the processes underlying maturation of visual cortical circuitry instead of controlling segregation of visual inputs and in more general visual cortical plasticity; adding or removing BDNF affects the maturation of the visual cortex, including ocular dominance columns. In agreement with this hypothesis it has been reported that BDNF, as well as NT-3 and to lesser degree NGF, regulates the dendritic growth of pyramidal neurons in organotypic cultures of the visual cortex (McAllister et al., 1997). The effects of BDNF are layer-specific and indicate that BDNF, and more generally neurotrophins, act to modulate the development of particular dendritic patterns. Interestingly, recent

data suggest that TrkB signaling is necessary to maintain the normal pattern of dendritic arborization also in adults, as shown in CRE Knock-outs lacking TrkB at a late stage of postnatal development.

BDNF also controls the maturation of inhibitory neurons in mouse visual cortex. The maturation of cortical inhibition is accelerated and the length of the critical period for visual cortical plasticity is shortened in mice overexpressing BDNF (Hanover et al., 1999; Huang et al., 1999).

Particularly interesting results were recently obtained by Katz and collaborators (Horch et al., 1999). They showed that local modulation of BDNF levels is capable of changing in a short time scale the site of dendritic spines, suggesting that this neurotrophin contributes to remodeling the spatial distribution of synapses along neuronal processes.

Thus, there is increasing evidence that BDNF plays a central role in various processes sculpting the neuronal circuitry in the visual cortex during postnatal development. These processes, such as branching pattern formation and dendritic specialization (spine formation) are modified by neuronal activity and in the visual cortex are dependent on visual experience. The idea is emerging that BDNF plays a fundamental role in activity-dependent development of visual cortex. To confirm this hypothesis the following criteria must be satisfied:

a) BDNF and its receptors must be expressed in appropriate sites of the visual cortex and at the right time;

b) BDNF and/or its receptors must be modulated by visual experience;

c) supply of BDNF must regulate neuronal function and, in particular, potentiation/depression of synapses in the visual cortex during postnatal development;

d) functional deprivation of BDNF must affect synaptic plasticity.

In the visual cortex, BDNF mRNA expression changes during postnatal development. Before eye opening, the total level of BDNF mRNA is low (Bozzi et al., 1995) and neurons expressing BDNF mRNA are evenly distributed throughout the cortical layers (Capsoni et al., 1999a). After eye opening, BDNF mRNA levels increase (Bozzi et al., 1995) while the number of neurons expressing BDNF mRNA in layer IV and V decreases (Capsoni et al., 1999a). Alteration of normal visual experience influences the endogenous level of BDNF mRNA in the visual cortex (Castren et al., 1992; Bozzi et al., 1995; Schoups et al., 1995; Capsoni et al., 1999a, 1999b). In particular, deprivation of visual experience from birth (dark rearing) induces a reduction in the endogenous level of BDNF mRNA: in neurons of layer IV and V, BDNF mRNA is reduced but distributed in the great majority of neurons (Capsoni et al., 1999a). These studies indicate that BDNF mRNA cortical expression undergoes developmental changes and is regulated by visual experience. However, whether developmental changes of BDNF mRNA result in actual changes of the protein is an unanswered question. This is a crucial question because it is possible that mRNAs and proteins are differently regulated by neuronal activity. Indeed, BDNF mRNA and protein levels have been shown to be regulated differently after kainate-induced seizures in the hippocampus (Wetmore et al., 1994).

To understand the role BDNF in visual cortex development, it is important to consider also its specific receptor TrkB. As we have reported in previous paragraphs,

BDNF selectively binds TrkB, but this receptor is recognized also by NT-4 and, with low affinity, by NT-3. Recent studies show that, although NT-4 and BDNF bind the same receptor, they activate different intracellular pathways (Minichiello et al., 1998). In the visual system as well as in other areas of the brain there are different forms of TrkB. As we have reported in previous paragraphs there is a full length form and a truncated form. In adult visual cortex the full length form is expressed in pyramidal neurons and inhibitory interneurons with a pattern of distribution similar to that of BDNF mRNA. Indeed, pyramidal neurons co-express BDNF mRNA and its receptor TrkB (Cellerino et al., 1996).

During postnatal development the full length form of TrkB changes its pattern of cellular expression at the level of visual cortex. In particular, TrkB is at first localized in fiber tracts and later it appears in neuronal cell bodies and dendrites. Concerning the laminar distribution, TrkB expressing neurons are mostly concentrated in cortical layer V and II-III. Thus, the pattern of TrkB immunoreactivity suggests that in different cortical layers specific subsets of neurons respond to TrkB ligands. A study by Allendoeffer et al. (1994) is particularly interesting. They showed that at an early stage of postnatal development the full length TrkB receptor is predominant while during the late phase of the critical period the relative proportions of full length and truncated receptor is reversed.

These results prompted the authors to suggest that the switch between the two forms of TrkB receptors may be important for segregation of visual inputs and for the length of the critical period. However, the cellular expression of the two forms of TrkB receptors and their laminar distribution is unknown. In addition, it is essential to know if the developmental regulation of TrkB receptors is experience-dependent.

Very few studies have attempted to clarify the role of neurotrophins in forms of synaptic plasticity expressed in the primary visual cortex during postnatal development. Most work deals with LTP and LTD alone but no study has been tried to put the two forms together.

AIM OF THE WORK

The first aim of this thesis was to investigate the role of BDNF in synaptic plasticity of the developing visual cortex. In particular, we wanted to address the question of whether LTP and LTD are the only forms of long term synaptic plasticity expressed in the primary visual cortex. Work in the visual cortex has dealt with either LTP or LTD but not the two forms together. We addressed the question of whether synapses can be first depressed and then potentiated and considered this as a parameter of synaptic plasticity. The expression of this form of plasticity was studied at different postnatal ages by using extracellular electrophysiological recordings in slices containing the primary visual cortex. In addition we studied the effects of visual deprivation (dark rearing) on the temporal expression of this form of synaptic plasticity. The role of BDNF on synaptic plasticity was studied by adding or removing the neurotrophin.

As a second point we wanted to investigate whether i) the cellular expression of BDNF protein changes during postnatal development, ii) developmental changes are visual experience-dependent. To date, studies on visual cortex have been almost completely focused on the expression of BDNF. The question of whether changes in BDNF mRNA correspond to actual change of proteins is still unanswered. In this investigation we compared BDNF protein and mRNA expression both qualitatively and quantitatively. We performed our experiments using immunohistochemistry and high-sensitivity non-isotopic in situ hybridization in rat sections containing primary visual cortex.

To understand BDNF action it is important to consider the site of expression and to clarify if BDNF expression is developmentally regulated. We studied the

expression of TrkB receptors during the postnatal development of rat visual cortex. Finally, we evaluated the effects of visual experience deprivation on TrkB cellular expression and endogenous level. To address these issues we performed immunohistochemistry for the full length and the truncated form of TrkB receptors in sections of the rat visual cortex.

MATERIALS AND METHODS

Visual cortical slices preparation and electrophysiological recordings.

Wistar rats were deeply anaesthetized with urethane (20% urethane, 100 µl/ 100 g body weight) and then decapitated. All efforts were made to minimize animal suffering and to reduce the number of animals used. The brain was rapidly removed and coronal slices (400 µm) containing the visual cortex were prepared using a Vibratome (Pelco, U.S.A.); they were superfused in a submerged recording chamber at 33 °C with artificial cerebrospinal fluid (aCSF) at a rate of 4 ml/min. An average of two or three slices per animal (the second and the third slices from the occipital pole of each emisphere) was used for experimental purposes. The aCSF was gassed with 95% O_2 and 5% CO_2 and had the following composition in mM: NaCl, 126; KCl, 3.5; NaH_2PO_4, 1.2; MgCl, 1.3; $CaCl_2$, 2; $NaHCO_3$, 25; glucose, 11. Extracellular field potentials in the inferior half of cortical layers 2/3 were recorded with an electrode filled with a 2M NaCl solution and evoked, by stimulation of the white matter containing geniculocortical fibers, using a bipolar concentric stimulating electrode. Baseline responses were acquired at a stimulation frequency of 0.07 Hz. LTD was induced by low frequency stimulation (LFS, 900 pulses at 1 Hz) of the white matter. The protocol for inducing LTP consisted of three trains of high frequency stimulation (HFS, 100 Hz, 1s). Slices containing the primary visual cortex were prepared from either postnatal day 16-17 (P17) or 23-24 (P23) or 35 (P35) rats. In dark rearing experiments, slices were prepared from animals light deprived from P17 to P29, that is for a period considered sufficient to mantain P17 synaptic characteristics. BDNF (Alamone Labs, Israel) was applied by filling the recording electrode with a 100

ng/ml in 1M NaCl solution. K252a (Alamone Labs, Israel) was first aliquoted in dimethylsulfoxide (DMSO), then diluited in aCSF to a final concentration of 200 mM and administered to slices by superfusion.

Data analysis

The amplitude of the maximum negative field potential in layer 2/3 was used as a measure of the evoked population excitatory current. Changes in the amplitude of the maximum negative field potential mirror changes in the magnitude of a monosynaptic current sink and correlate with changes in the initial slope of the negative potentials. In order to avoid polysynaptic sinks before starting the recording we ensured that field potentials were monophasic and that the shape was not changed by frequency of stimulation higher than 1 Hz. Following this recording protocol the latency of negative field potentials ranged between 5 and 7 ms. To minimize the possibility of widely activated fibers in the white matter, the stimulating electrode was placed at the border between the white matter and layer 6 in a way that, according to Kenan-Vaknin and Teyler (1994), and Woodward et al.(1990), would facilitate the selective stimulation of vertical pathways projecting to cortical layers II-III and IV. Baseline responses were obtained every 15 s and averaged every four responses with a stimulation intensity that yelded a half-maximal response. The magnitude of both LTD and potentiation after LTD (dedepression) was measured starting 20 minutes after the end of the respective conditioning protocol. The mean value of 20 consecutive averaged responses was calculated relative to the baseline averaged control values for each experiment. In the case of failure of potentiation after LTD at P23, baseline responses were followed for at least 20 minutes unless otherwise stated.

Data were then pooled together for each experimental group and expressed as percentage change from control baseline (PCCB) ± S.E.M. Statistical comparison was done by applying a Mann-Whitney rank sum test among LTD and dedepression PCCB values. Difference was considered significant with $P< 0.05$.

Immunohistochemistry for BDNF.

Wistar rats were anaesthetized (20% urethane, 100 µl/100 g body weight) and perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). Brains were removed and cryoprotected in 20% sucrose/PBS at 4°C until cutting. Coronal sections (30 µm) containing binocular visual cortex (OC1b; Paxinos, 1986) were cut with a freezing microtome and uniform random series (1 section every 20) were collected for the experiments. Rats of different postnatal ages, P13 (n=6), P23 (n=6), P39 (n=5), P90 (n=6) rats, were used. Effects of light deprivation were investigated in two distinct groups of rats reared in a dark room for two different periods. The first group includes rats reared in darkness for 39 days, from birth to P39 (n=6), while in the second group the period of visual deprivation was shorter (10 days, from P13 to P23 n=6). In a third group P23 dark reared rats (n=6) were exposed to light for two hours.

For immunohistochemistry, two different antibodies were used in adult animal sections. One was a non-blocking rabbit anti-BDNF antibody (Rb anti-BDNF, IgG fraction, Chemicon International Inc., Temecula, CA-USA) and the other was a blocking rabbit anti-BDNF antibody (Yan et al., 1997) (Rb anti-BDNF A.P. IgG. Amgen Inc., Thousand Oaks, CA); the two antibodies were used at a concentration of

10 μg/ml, 2.5 μg/ml, and 1.6 μg/ml and the different pattern of staining compared. The specificity of the primary antibody was assessed by preadsorbing it with saturating concentration of BDNF: 10 times more concentrated than the antibody was used for coating an ELISA plate, and then removed. Antibody was adsorbed twice on the ELISA plate coated with BDNF, for 3 hours at R.T. shacking and over night at 4°C, with no shaking.

The series of sections were washed once in PBS for 5 min R.T., treated with 0.5% H_2O_2 in PBS in order to quench endogenous byotin, and washed 2 times with PBS. Then were treated with 0.01% Triton X-100, 10% Normal Goat Serum, in PBS for one hour at room temperature. Incubation with the primary antibody was performed for 48 hours at 4°C. Experiments were carried out at a concentration of 2.5 μg/ml unless otherwise stated. Then, sections were washed 4 times with PBS and then treated with a secondary antibody (7.5 μg/ml biotinilated goat anti-rabbit; Vector Labs Inc., Burlingame CA) for 3 hr at room temperature. After incubation with anti-rabbit, slices were washed 4 times in PBS for 5 min each and then treated with an avidin-biotin-peroxidase complex (1:100 dilution, ABC Elite, Vector Labs, Burlingame CA) for 1hr at room temperature. After washing in PBS, the labeling was revealed in 3-3' diaminobenzidine HCl (10 mg in 25 ml Tris/HCl, pH 7.5, Sigma, St Louis, MO) for 20 min. at room temperature. Counterstaining of sections (Fig 1g) was obtained by incubating them in a 0.25% solution of cresyl violet acetate (Sigma, St Louis, MO).

For sections processed for both immunohistochemistry and *in situ* hibridization during incubation with Normal Goat serum, 5 mg/ml heparine were added. This to block the RNAse present in the serum.

Double immunohistochemistry for BDNF and parvalbumine was performed in P90 sections containing primary visual cortex. Sections were immunostained for BDNF as previously described and then incubated with anti-parvalbumine mouse monoclonal antibody 0.3 μg/ml (Sigma, MO) according to Cellerino et al. (1996). After washing in PBS, sections were incubated with anti-mouse alkaline phosphatase conjugated for 3 hours at R.T. with shaking. Staining for parvalbumine was revealed by using alkaline-phosphatase conjugated ABC kit (Vector Labs Inc., Burlingame CA).

Immunohistochemistry for TrkB receptors.

Animals were anaesthetized and perfused as previously described. After perfusion brains were removed and cryoprotected in a solution containing 20% sucrose in PBS over night at 4°C. The day after coronal sections 30 μm thick containing primary visual cortex were cut with a freezeng microtome. Immunohistochemistry was performed on animals at different ages: P13 (n=4), P23 (n=4), P40 (n=2) and P90 (n=6). For each animal two different antibodies were used: one directed against the intracellular portion of TrkB receptor then able to recognize the full lenght form of the receptor (TrkB+) (sc794 Santa Cruz, 0.5μg/ml); the other one (TK- Santa Cruz) specifically recognizing both the truncated form of TrkB receptor.

For the detection of TrkBs the following procedure was used: slices were post fixed for 30 min in PFA 4% at R.T. with no shaking, then were treated with 0.5% H_2O_2 in PBS for 10 min in order to quench the endogenous residues of biotin, finally they were washed for 15 min with PBS at R.T., shaking. For detection of TrkB+ slices were preincubated for 1 hr in a solution containing 0.3% triton, 10% Normal Goat

Serum (Vector), and then incubated over night at 4°C in the same solution containing the antibody (0.5μg/ml). Then sections were washed for 20 min in PBS at R.T with shaking, and then incubated with anti rabbit (7.5 μg/ml, Vector) in PBS/10% NGS for 3 hours at R.T. with shaking. After 20 min washing in PBS, slices were incubated with avidin-biotin complex (1:100 dilution, Vector Laboratories) for 1 hr at R.T., and then, again washed in PBS for 10 min. To reveal immunoreactivity we used a solution containing 3-3' diaminobenzidine HCl: a chromatogen, (10 mg in 25 ml Tris/HCl, pH 7.5, Sigma, St Louis, MO) for 20 min. at room temperature.

For detection of TrkB- receptors, the first steps were the same used for revealing the full lenght receptor. The preincubation was done in a solution containing 10% Fetal Calf Serum (FCS), 5% Bovin Serum Albumine (BSA), and 0.01% triton in PBS, for 30 min at R.T. Incubation with primary antibody (0.5μg/ml) was done in 5% FCS, 2% BSA, 0.01 triton, PBS over night at 4°C. The day after sections were washed with PBS for 20 min at R.T., and then incubated with secondary antibody (anti rabbit 7.5μg /ml, Vector) for 3 hours at R.T. in a solution containing 5% FCS, 2% BSA, PBS. After washing for 20 min in PBS at R.T. with shaking, sections were incubated in avidin-biotin complex (1: 100 dilution, Vector Labs), and then washed for 10 min in PBS. The labeling was revealed with 3-3' diaminobenzidine HCl (10 mg in 25 ml Tris/HCl, pH 7.5, Sigma, St Louis, MO) for 20 min. at room temperature.

Western Blot.

Neurotrophins were resolved on a 12% SDS polyacrylamide gel and transferred to nitrocellulose. For each neurotrophin (Alamone Labs, Jerusalem, Israel) two lanes

were loaded: one containing 100 ng and the other one containing 30 ng of the protein. For NGF, only one lane containing 150 ng of the protein was loaded. Membrane was blocked in 5% non-fat dry milk in Tris-buffered saline (TBS)/0.05% Tween 20 (TBST). After incubation with the primary antibody in blocking solution, overnight at 4°C, at the same concentration used in immunohistochemistry (2.5 μg/ml), membrane was washed three times with TBST, incubated with biotinylated secondary antibody (Vector Labs Inc., Burlingame CA) for two hours at R.T. After washing, the membrane was incubated with alkaline-phosphatase conjugated ABC kit (Vector Labs Inc., Burlingame CA) and then washed 2 times with TBST and one time with TBS. Proteins were visualized using p-nitro blue tetrazolium chloride (NBT)(Sigma, Sant Louis, M0, 0.5 mg/ml) and 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt (BCIP) (Sigma, Sant Louis, MO, 0.25 mg/ml) in developing buffer (0.1 M Tris, 0.5 mM $MgCl_2$, pH 9.5).

ELISA bioassay.

Occipital poles of P13, P23 and P90 rats were dissected in order to separate layer IV from supragranular layers and infragranular layers (Bolz et al., 1996). At each age, different cortical layers were dissected from several animals (n=10) and then pooled. Tissue (1mg/10 μl) was homogenized in extraction buffer (25 mM Tris HCl pH 7.5, 1 mM EDTA, 1 mM Spermidin, 1 mM Phenilmethylsulfonilfluoride, 1 mM iodacetamide, 5 μg/ml Aprotinin, 4 μg/ml Soy Bean Trypsin inhibitor, 10 μg/ml Turkey Egg White Inhibitor, 1% Triton X-100). All inhibitors were purchased from Sigma (Sant Louis, MO). The preparation was incubated for 1 hr at 4 °C vortexing 3 min every 7 min, and then centrifuged 5 min at 10,000 rpm at 4 °C in order to

separate pellet and supernatant.

ELISA was performed on supernatant with BDNF E_{max} ™ ImmunoAssay System (Promega, Madison WI-USA).

Cell counts.

At each age at least five animals were analyzed. For each animal about 100 coronal slices were obtained from occipital pole. Slices were divided in 20 series (five slices/series) (Howard & Reed, 1998). A single random series (1/20) of coronal sections/animal was analyzed. The number of total and BDNF mRNA-positive neurons was counted by means of an ocular grid and a 40x objective (Axiophot, Zeiss). The lateral and medial limits of area OC1b were recognized according to Zilles et al. (1984). For each layer, the number of cresyl violet-positive and BDNF immunopositive neurons was counted (Capsoni et al., 1999 a, b). We avoided counting non-neuronal cells by using highly restrictive morphological criteria such as the absence of nucleolus, the darker cresyl violet staining of the nucleus with respect to neurons, and the small dimension of the cells and their localization close to the apical shaft of dendrites or to the basis of pyramidal neurons (Hilman, 1986; Cajal, 1899). The number of BDNF immunopositive neurons/layer was expressed as percentage of the average number of cresyl violet stained neurons (BDNF Cell Index; BCI). BCI was calculated for each cortical layer taking into account all neurons present in Oc1b area in each slice of the random series considered. Statistical analysis was performed by using Kruskal-Wallis test (SigmaStat, Microsoft Inc., USA). Differences between distinct experimental groups were considered significant for $P<0.05$.

To exclude possible bias due to penetration of the antibody, at each postnatal ages (two random slices/animal) BCI was calculated in different focal planes of the same slice looking for potential differences.

For immunohistochemistry, *in situ* hybridization and ELISA, experiments were conducted blind and sections taken at different postnatal ages were processed at the same time. Dark reared animals were processed with age-matched controls reared in normal (12-hr dark/ light cycle) conditions of illumination.

Riboprobes.

The rat BDNF cDNA clone pBCDPst (nucleotide 74-525, Maisonpierre et al., 1991) was kindly provided by Dr. A. Negro (Fidia Res. Lab., Padova).

5 μg of plasmid were linearised with the appropriate restriction enzyme in a 30 μl volume, and after increasing the volume to 200 μl were extracted with an equivalent volume of phenol, followed by chloroform/phenol (1:1) and chloroform alone. Plasmids were ethanol precipitated at -20 °C for 2 hours, centrifuged, speed-vac dried for 15 min, dissolved in 10 μl of diethilpirocarbolate-treated (DEPC) H2O, and stored at -20 °C. The digoxigenin labelled riboprobes were synthesised with a SP6/T7 DIG-RNA labelling kit (Boehringer) according to the manufacturer's instructions. Oligonucleotides were designed from regions not overlapping with the riboprobe sequences. The BDNF oligonucleotide probe was complementary to the nucleotides 649-694 of the coding region of the rat BDNF mRNA sequence (Maisonpierre et al., 1991). This oligonucleotide sequence has been used in previous study for *in situ* hybridisation (Ernfors et al., 1990a; 1992; Merlio et al., 1993). In order to avoid any risk of non-specific hybridization caused by the labelled tail, only a single

digoxigenin-labelled ddUTP was added with a terminal transferase at the 3' end of the oligoucleotides according to manufacturer's instructions (DIG- oligonucleotide 3' end labelling kit, Boehringer, Mannheim, Germany).

For preparation of byotinilated probes the same procedure was used with the exception that a byotin-lebelled, instead of digoxigenin-labelled, ddUTP was present in the mix.

***In situ* hybridization.**

In situ hybridization was performed after immunohistochemistry according to Tongiorgi et al., (1998). Free floating slices were washed twice in PBS/0.1% Tween 20 (PBST), quickly washed in H2O and permeabilised for 5 in at R.T. in 2.3% Na-metaperiodate (Sigma) in water, followed by a quick wash in H2O. Sections were treated then for 10 min in 1% Na-borohydride (Sigma)- 0.1M Tris/HCl (pH 7.5) and, after 3 washes in PBST, were digested with 1mg/ml Proteinase K (Boehringer-Mannheim) on ice. The duration of the digestion was dependent on the age of te animals: 10 minutes for P13 animals, 15 minutes for P23 animals and 20 miutes for adult animals. After permeabilization treatments, slices were washed for 5 min in PBST, then fixed for 5 min in 4% PFA and finally washed three times in PBST at R.T. for 10 min.

Pre-hybridisation was carried out at 55°C for 1 to 6 hours in the hybridisation solution containing: 20 mM Tris/HCl (pH 7.5), 1mM ethilenediaminetetraacetic acid (EDTA, Sigma), 1x Denhardt's solution, 300 mM NaCl, 100 mM dithiothreitol (Sigma), 0.5 mg/ml Salmon Sperm DNA (Sigma). Slices were then transferred into

the hibridisation solution, composed by the pre-hybridisation solution with 10% dextran sulphate, and 50-100 ng/ml digoxigenin labelled ribiprobes. In situ hybridisation was carried out overnight (at least 16 hours) in multiwell plates at 55°C without shaking.

After hybridisation the sections were washed twice in 2x saline sodium citrate, 0.1% tween 20 (SSCT)/50% deionised formamide at 55°C for 30 min, 20 min in 2x SSCT and twice in 0.05% SSCT at 60°C for 30 min.

Slices hybridised with digoxigenin labelled ribopobes were processed for immunodetection with an anti-DIG antibody F(ab)2-fragment conjugated with alkaline phosphatase (Boeringher), diluted 1:500 in PBST/10% fetal calf serum (FCS) at 4°C overnight. After this incubation sections were washed four times in PBST at r.t. for 10 min, then incbated in developing buffer (0.1 M Tris-HCl buffer, 0.1 M NaCl, 0.05 M MgCl2, 1mM levamisol) atR.T. for ten minutes, and finally incubated in a cromogen solution composed by the developing buffer containing 4-nitro blue-tetrazolium (NBT, Boehringer), and 5-bromo-4-chloro-3-indolyl-phopsphate (BCIP, Boehringer). The reaction was generally carried out for 2-6 hours at R.T. and was stopped by rinsing the sections in a stop-solution (10mM Tris-HCl pH 8, 1mM EDTA). Sections were then washed with PBS and mounted in water on glass slides coated with 5% gelatin (Merk). Mouted sections were then dried for 30 min in oven at 55°C, incubated for 30 seconds in methanol, for 30 seconds in a mixture of metanol/xylene (1:1) and finally for 3 min in xylene and mounted in DPX-mounting medium (BDH).

Slices hybridized with biotilinated probes, after washings with SSCT, were incubated with an avidin-biotin-peroxidase complex (1:100 dilution, ABC elite, Vector Labs,

Burlingame CA) for 1 hr at R.T. The labeling was revealed in 3-3' diaminobenzidine HCl (10 mg in 25 ml Tris /HCl pH 7.5, Sigma, St Louis, MO) for 20 min at R.T. Sections were then washed and mounted as previously mentioned.

RESULTS

BDNF REGULATES A NEW FORM OF SYNAPTIC PLASTICITY DURING POSTNATAL DEVELOPMENT.

Searching a parameter for synaptic plasticity in rat visual cortex.

The degree of plasticity of thalamo-cortical synapses changes during development. Long Term Potentiation (LTP) and Long Term Depression (LTD) are two classical forms of long term synaptic plasticity. Several works have shown that after appropriate stimulation, neuronal responses can be potentiated or depressed according to the received stimuli, and that these properties depend on the circuitry investigated, and on the age of the animal. A synapse can be considered even more plastic if it can be depressed and soon after potentiated, and we have chosen this parameter to establish the degree of plasticity of rat visual cortex during postnatal development. The stimulating micropipette was placed at the border between white matter and layer six, whereas the recording electrode was placed in cortical layers II-III. Extracellular field potentials were evoked as explained in materials and methods. Rats of different postnatal ages were examined.

The first group of animals was P17 rats, i.e. two days after eye opening. . In our experimental procedure, we first induced LTD by LFS (900 pulses at 1 Hz) stimulating the white matter and recording in layers II-III (PCCB= 69 ± 4%, n=5); we followed the depression for 40 min, and then elicited stable LTP by a subsequent HFS (100 Hz), 1s). In all slices (Fig 3A, n=5/5) we were able to observe a stable potentiation after depression. Potentiated responses were equal or higher to the

Fig.3

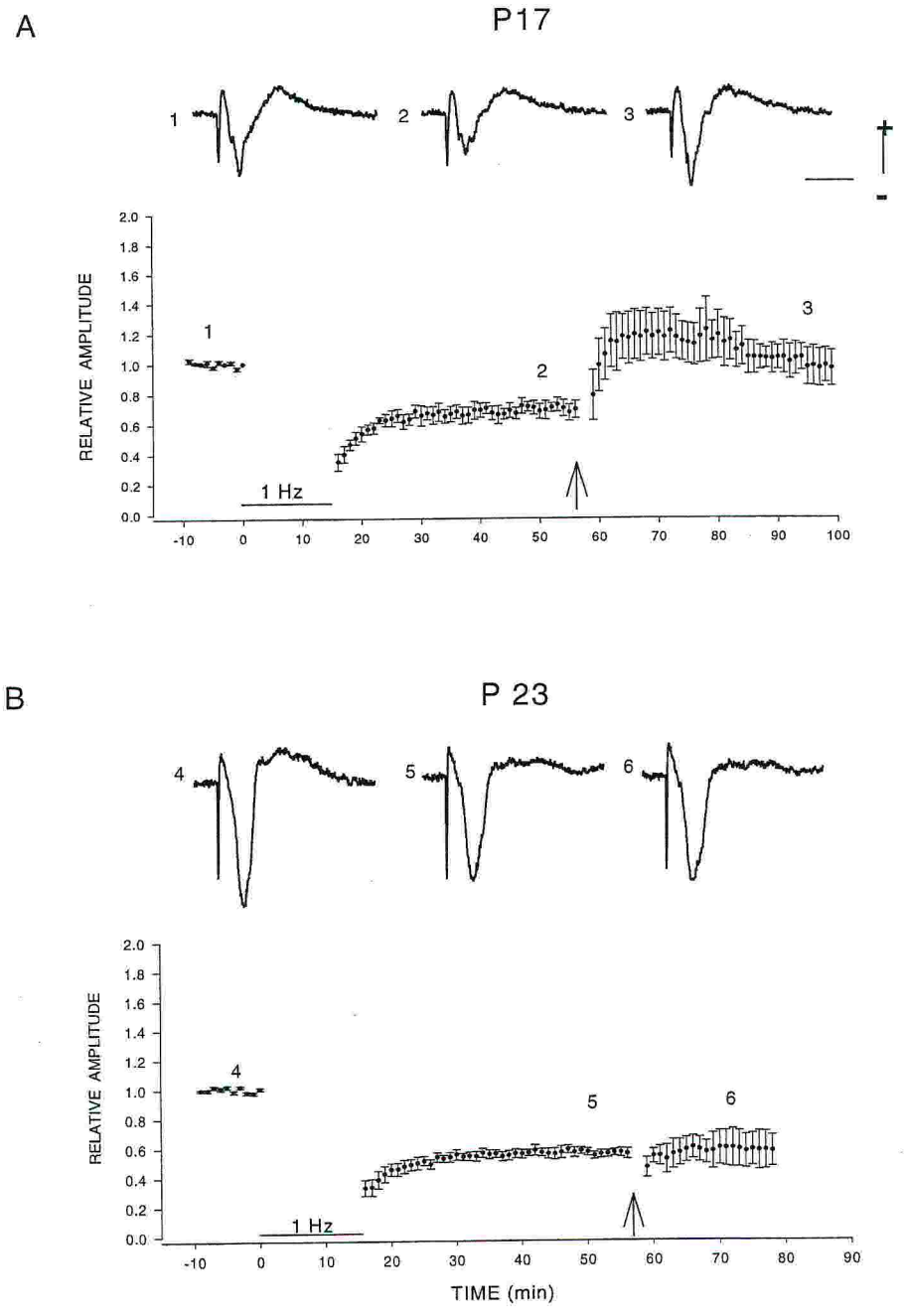

A
P17
1
2
3
+
-
RELATIVE AMPLITUDE
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 Hz
-10
0
10
20
30
40
50
60
70
80
90
100
B
P 23
4
5
6
TIME (min)

Fig. 3: Reversal of LTD at P17 and P23.

Averaged (± s.e.m.) responses representing the amplitude of the maximum negative field potential recorded in layer 2/3 following stimulation of white matter. Values were normalized to average baseline. Bar and arrow indicate the conditioning protcol for LTD (1 Hz, 900 pulses) and LTP (three bursts of 1 s at 100 Hz), respectively. (A) Effects of an LTD protocol (bar) followed by an LTP protocol (arrow) in slices of P17 rats. Top traces are representative field potentials taken at the time indicated by the numbers. Horizontal bar= 10 ms; verical bar= 0.4 mV. Initial downward deflections are stimulation artifacts. (B) Effects of an LTD protocol (bar) followed by an LTP protocol (arrow) in slices of P23 rats. Top traces are representative field potentials taken at the time indicated by the numbers. Scale bars as in A. Note that at this age it was no longer possible to evoke potentiation after LTD (dedepression).

control values recorded prior the LTD protocol (PCCB= 105 ± 10%, n=5). The opposite phenomenon, i.e. the depression of cortical synapses after induction of LTP, was equally observed in slices from P17 rats (LTP= 179 ± 17%, Depotentiation = 107 ± 4%, n=3). However de-potentiation was not further investigated at later ages, as it is known that LTP expression declines during development.

We repeated this experimental procedure in rats 23 days old. At this age (Fig. 3B) the effects of LFS were similar to those evoked at P17 (PCCB= 62 ± 4%, n=8). In fact, comparison of LTD amplitude at these two ages revealed no statistically significant difference. However, in sharp contrast to that observed at P17, at P23 it was never possible (n=8/8) to potentiate the evoked responses after the induction of LTD (PCCB= 56 ± 7%, n=8). In order to verify a possible delay in the onset of potentiation after LTD at P23, in two cases the recording was continued beyond 30 min after the tetanic stimulation. However, in accordance to that observed in the other cases, no potentiation was noted.

Similar experiments performed in P35 animals (n=2, LTD PCCB= 70%, dedepression PCCB= 66%), confirmed that this form of plasticity is present at P17 but declines rapidly thereafter.

These results show that there is a form of synaptic plasticity which is present soon after eye opening and disappears after few days.

Control experiments.

In control experiments at P23 we checked whether the inhability of synapses to be potentiated after depression was a consequence of a general inability of the visual cortical slices to undergo potentiation. To test this hypothesis, we first repeated the

same experimental protocol of evoking LTP having followed a LTD for 40 min (Fig 4A, position 1). Having verified the lack of potentiation with this electrode position, the recording electrode was moved to a new location in the same slice, within layers II-III of the primary visual cortex. In these conditions, after at least 30 min of control stimulation in order to exclude a run down of the ability to undergo LTP, we were able to induce LTP (Fig. 4A, position 2, 5/6 slices). Thus at P23 it was possible in the same slice to induce an LTP but not to potentiate a previously depressed evoked response.

In a further set of control experiments at P23, after 40 min of LTD we increased the current used for the HFS of the white matter. In order to avoid any massive stimulation of previously unrecruited fibers, the current was only increased up to evoke responses of the same amplitude as the control pre LTD values. HFS with increased current induced in three cases a temporary increment in the responses which lasted less than 25 min but in nine of these experiments it was possible to induce long-term potentiation after the depression (Fig 4B, n=4/4). Recordings were continued for at least 30 min after the tetanic stimulation. These results demonstrate that the synaptic inability to potentiate previously depressed responses observed at P23, is not due to the small amplitude of the test response *per se*.

Effects of dark-rearing on reversal of long-term depression.

The inability to reverse a depression observed at P23 could be part of a developmental process under the control of visual experience. If this hypothesis were true then it could be possible to maintain the P17 thalamo-cortical synaptic behavior

Fig.4.

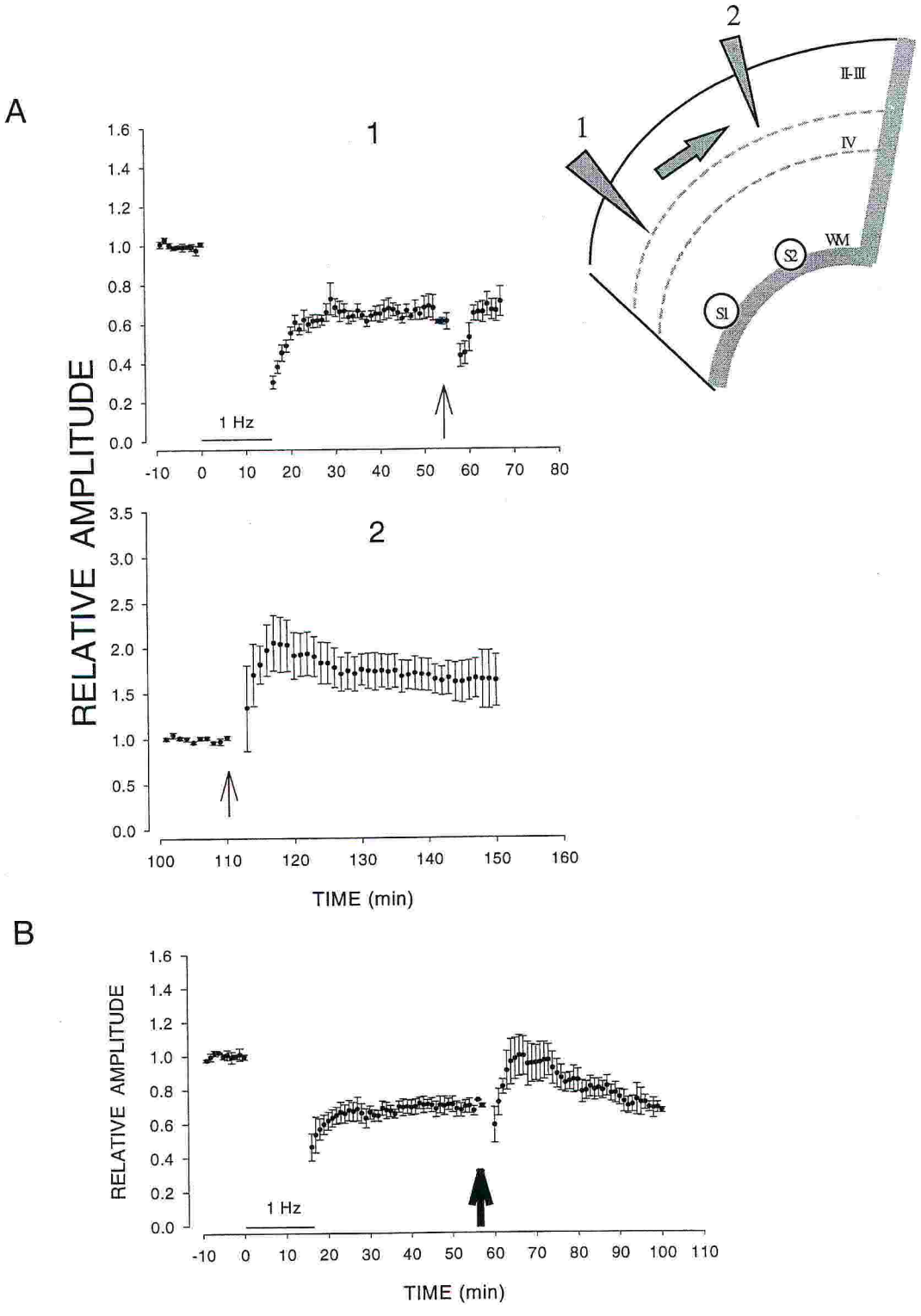

A
1
2
II-III
IV
WM
S2
S1
RELATIVE AMPLITUDE
1 Hz
TIME (min)
B
RELATIVE AMPLITUDE
1 Hz
TIME (min)

Fig. 4: Control experiments.

Averaged (± s.e.m.) responses representing the amplitude of the maximum negative field potential recorded in layer 2/3 following stimulation of the white matter. Values were normalized to average baseline. Bar and arrow indicate the conditioning protocol for LTD (1 Hz, 900 pulses) and LTP (three trains of 1s at 100 Hz) respectively. (A) Control experiments in slices of P23 rats. (1) Effects of an LTD protocol (bar) followed by a tetanic stimulation (arrow) with no LTP as shown in Fig. 3B. The recording electrode was then placed in a new position (2) not previously stimulated (S2). (2) Effects of tetanic stimulation (arrow) given at S2 eliciting an LTP. Thus at P23 it was possible in the same slice to induce an LTP, but not to potentiate a previously depressed evoked response. S1 and S2 represent the two stimulation sites relative to the recording positions 1 and 2. (B) Control experiments in slices of P23 rats. Effect of an LTD protocol (bar) followed by a tetanic stimulation (arrow). The current used for the tetanic stimulation (LTP protocol) was increased to a level able to evoke responses of the same amplitude as the control values. In these conditions only a temporary increment in the responses was observed.

by blocking sensory input. To verify this we reared normal P17 rats in the dark for 12 days. Experiments performed using visual cortex slices from these animals (Fig. 5) showed that it was again possible to see potentiation of the evoked responses after LTD (PCCB= 111 ± 6%, n=5/5); this is even more important considering that these animals were P29: infact at this age it is not possible to induce this phenomenon. LTD amplitude, although slightly reduced, was still considerable (PCCB= 82 ± 2%, n=5). Clearly we were able to block or delay the developmental loss of this form of synaptic plasticity suggesting that it relies on visually evoked activity. From these data appears that this form of synaptic plasticity is modulated by visual experience.

Effects of brain-derived neurotrophic factor on reversal of long-term depression.

To examine the possible effect of exogenous BDNF on cortical synaptic plasticity, we filled the recording electrode with 1M NaCl solution containing BDNF (100 ng/ml). This method was used in order to localize BDNF release and study its action in a restricted area of the visual cortex. To ensure leaking of BDNF from the recording electrode we slightly broke the tip of the pipette immediately before the beginning of the recording session. The resistance of BDNF filled electrodes was verified not to be grossly different with respect to that of the electrodes used in control experiments. BDNF released from the pipette induced a gradual increase in the size of the responses as already shown when BDNF was applied to the slice by perfusion. This effect, not observed in control slices, reached a plateau in around 15min when control responses started to be recorded. In these experimental conditions we found that at P23 it was again possible to induce LTP after LTD (Fig. 6). LTD size (PCCB =

Fig.5.

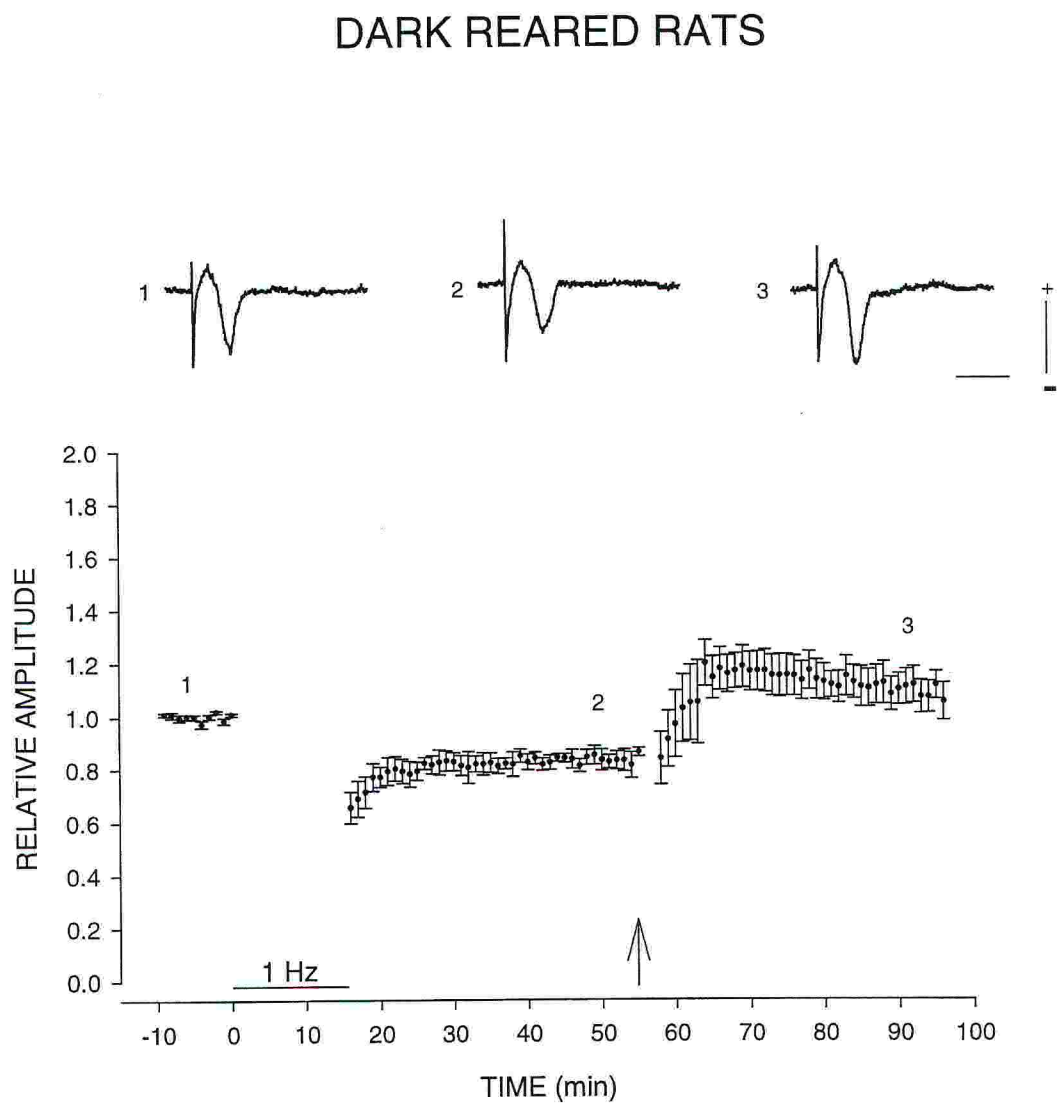

DARK REARED RATS
1
2
3
+
-
RELATIVE AMPLITUDE
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
1 Hz
-10
0
10
20
30
40
50
60
70
80
90
100
TIME (min)

Fig. 5: Effects of an LTD protocol (bar) followed by an LTP protocol (arrow) in slices of dark-reared from P17 to P29.

In these slices it was again possible to observe potentiation after LTD. Top traces are representative field potentials taken at the times indicated by numbers. Horizontal bars= 10ms, vertical bars= 0.5mV. Initial downward deflections are stimulation artifacts.

Fig.6.

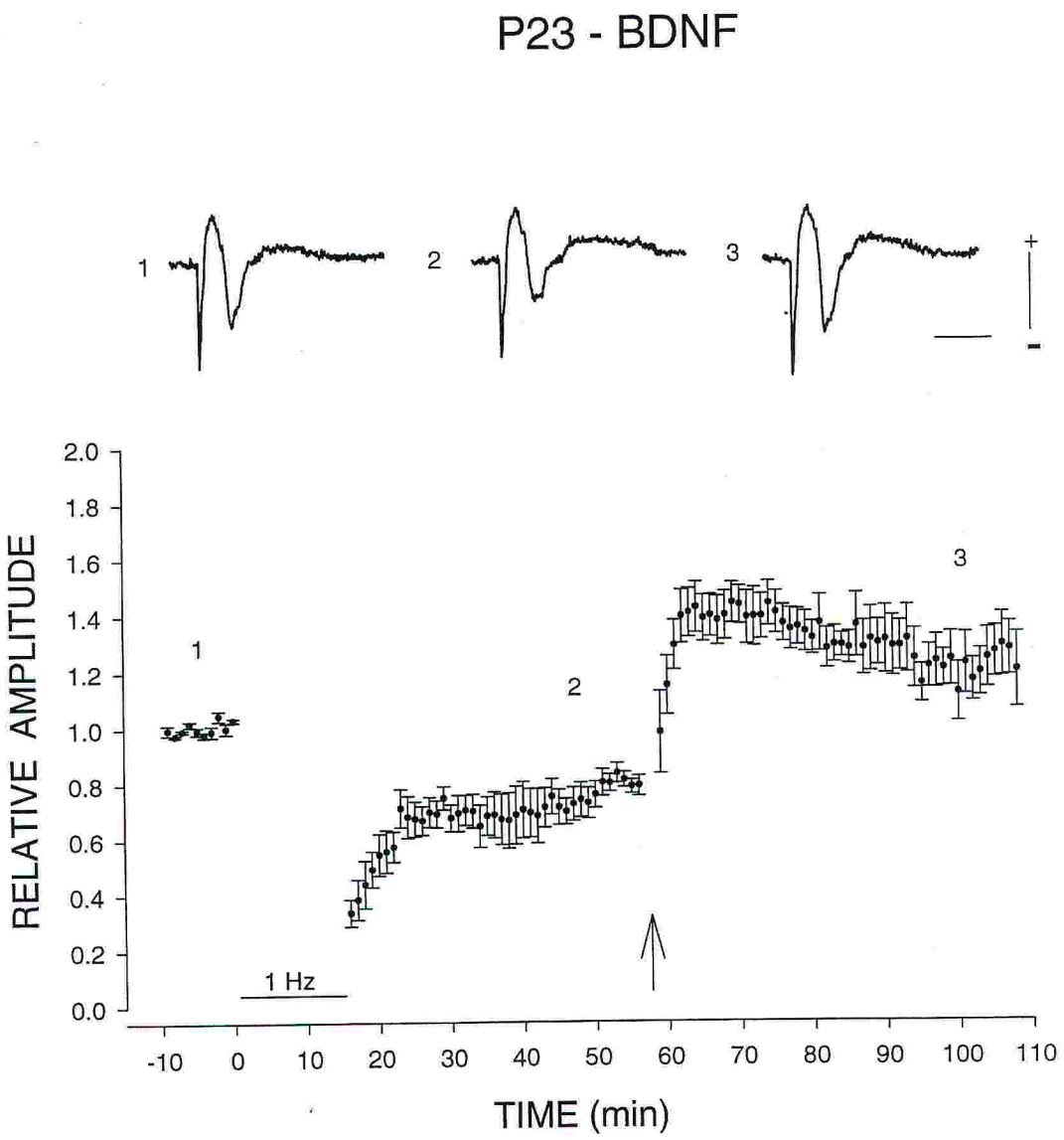

P23 - BDNF
1
2
3
+
-
RELATIVE AMPLITUDE
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
1 Hz
-10
0
10
20
30
40
50
60
70
80
90
100
110
TIME (min)

Fig. 6: Effects of an LTD protocol (bar) followed by an LTP protocol (arrow) in slices of P23 rats.

In these experiments the recording electrode was filled with BDNF solution (100 ng/ml in 1M NaCl). In contrast to that normally observed at this age it was again possible to reverse a depression. Top traces are representative field potentials taken at the time indicated. Horizontal bar= 10 ms, vertical bar= 0.4 mV.

Initial downward deflections are stimulation artifacts.

73 ± 6%, n=6) was not significantly reduced in comparison to P23 control slices. In marked contrast, HFS to the white matter was again capable of inducing a stable dedepression (n=6/6) whose magnitude (PCCB= 125 ± 8%) was not significantly different from that found at P17. We thought it was important to check whether applied BDNF was directly responsible for restoring at P23 the potentiation after LTD observed at P17. In order to do this we used K252a, an inhibitor of neurotrophin receptor tyrosine kinase activity, at a concentration (200 nM) known to block BDNF action on hippocampus and visual cortex synaptic plasticity. From the results reported in Fig. 7 it is clear that perfusion of P23 slices with K252a (200nM) blocked the action of the BDNF contained in the recording electrode (PCCB during LTD=70 ± 1%, PCCB after High- frequency protocol= 67 ± 5%, n=4). However, the effect of K252a on BDNF action was specific to the dedepression phase as the amplitude of LTD did not change.

These results prompted us to test the possible role of endogenous tyrosine kinase activated by neurotrophins in reversing LTD at P17. To do this we perfused P17 slices with K252a (200 nM) and applied the reversing LTD protocol. Contrary to that previously observed in control P17 preparations, the presence of K252a blocked the potentiating effects of the tetanic stimulation (Fig. 8) while LTD amplitude did not differ significantly (PCCB during LTD= 63 ± 3%, PCCB after high frequency protocol=70±3%,n=5). It has to be noted that K252a blocks all tyrosine kinases, not only those of neurotrophins intracellular pathway.

These data show that the lack of plasticity can be prevented with local application of BDNF. The action of neurotrophins in this form of synaptic plasticity is shown with the use of K252a which prevents plasticity to occur even at P17.

Fig.7.

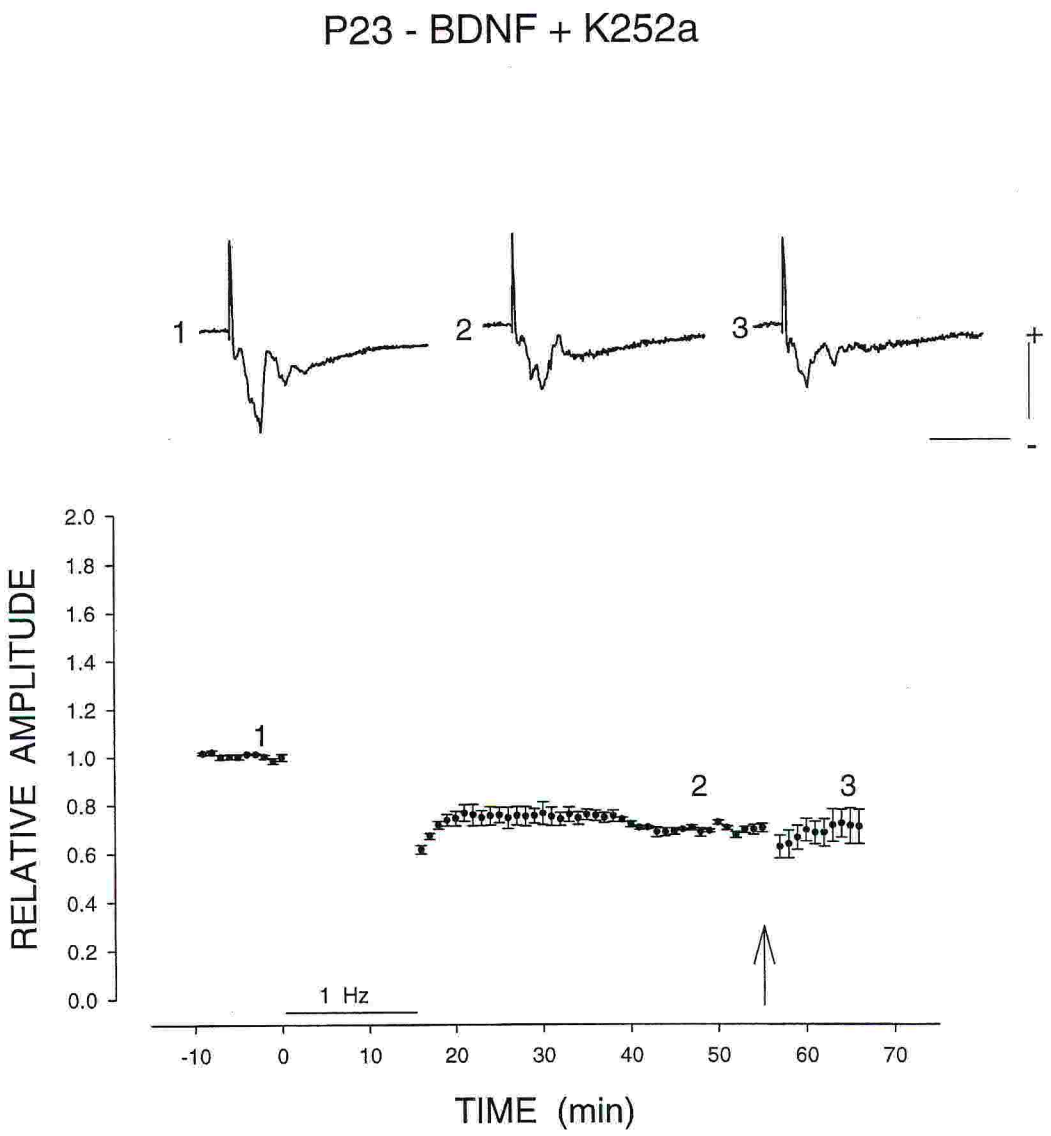

P23 - BDNF + K252a
1
2
3
+
-
RELATIVE AMPLITUDE
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
1 Hz
-10
0
10
20
30
40
50
60
70
TIME (min)

Fig. 7: Effects of an LTD protocol (bar) followed by an LTP protocol (arrow) in slices of P23 rats.

In these experiments the recording electrode was filled with BDNF solution (100 ng/ml in 1M NaCl) while slices were perfused with K252a (200 nM). K252a completely blocked any BDNF-induced potentiation after LTD. Top traces are representative field potentials taken at the time indicated by numbers. Horizontal bar= 10 ms, vertical bar= 0.6 mV. Initial upward deflections are stimulation artifacts.

Fig.8.

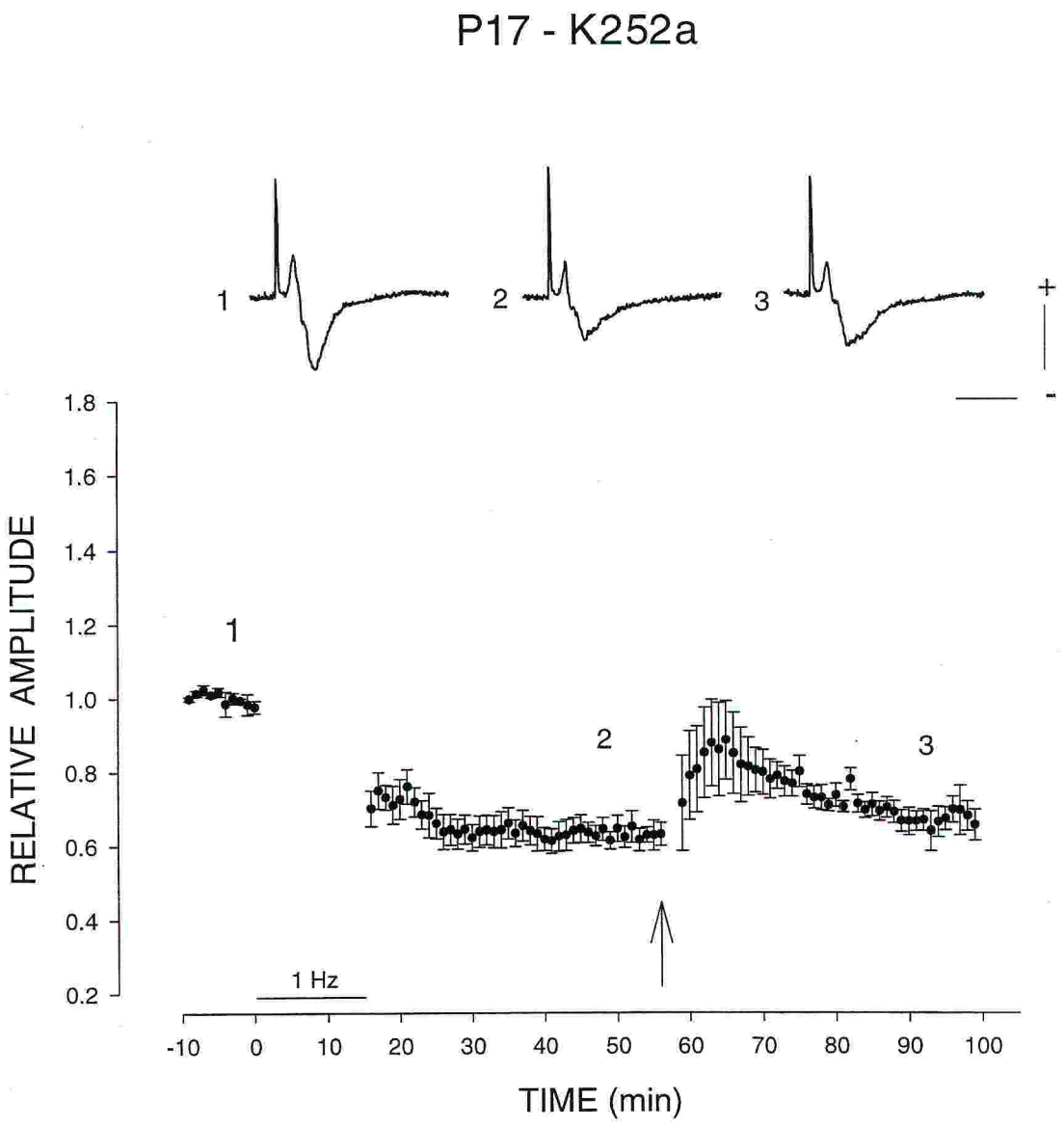

P17 - K252a
1
2
3
+
-
RELATIVE AMPLITUDE
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1 Hz
-10
0
10
20
30
40
50
60
70
80
90
100
TIME (min)

Fig. 8: Effect of an LTD protocol (bar) followed by an LTP protocol (arrow) in slices of P17 rats.

In these experiments slices were perfused with K252a (200 nM). K252a (200nM) completely blocked potentiation after LTD. Top traces are representative field potentials taken at time indicated. Horizontal bar=10 ms, vertical bar= 0.4mV. Initial upward deflections are stimulation artifacts.

ENDOGENOUS BDNF EXPRESSION DURING THE POSTNATAL DEVELOPMENT OF RAT VISUAL CORTEX.

There are many experimental evidences (Timmusk et al., 1995; McAllister et al., 1999) suggesting that BDNF is involved in the modulation of synaptic plasticity. The data previously reported support this idea. For this reason many authors have investigated the expression of BDNF during development of rat visual cortex. These studies have shown that BDNF mRNA expression changes during postnatal development. Moreover, it has been demonstrated that BDNF mRNA expression is influenced by light. However, no work has been done with the aim to clarify wether BDNF mRNA corresponds to BDNF protein.

To check the endogenous expression of BDNF protein both qualitatively and quantitatively we used specific immunoessays.

Control experiments

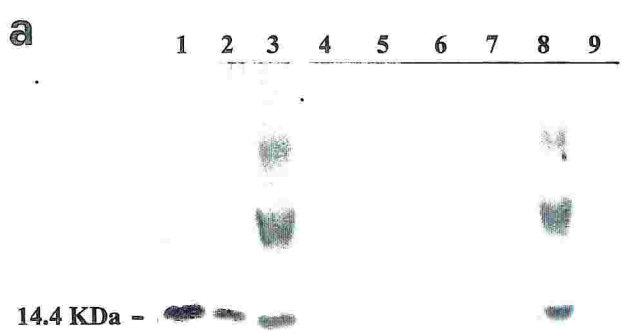
The specificity of the anti-BDNF antibody used in this study (Chemicon, C-RAb) was verified by Western blot. Fig. 9 (a) shows that this antibody recognizes BDNF but not the other neurotrophins. The patterns of immunoreactivity obtained by using two different antibodies were compared. Similar patterns of immunoreactivity were obtained by using the BDNF antibody C-RAb and an affinity purified antibody (Yan et al., 1997) (Amgen, A-Rab, Fig. 9b and 9c). In a second group of experiments cortical sections through the primary visual cortex were incubated with three different concentrations of BDNF antibodies (C-RAb), 1 μg/ml, 2.5 μg /ml and 10 μg /ml. Concentrations of 1 μg /ml allows to reveal only moderately labeled cells (data not

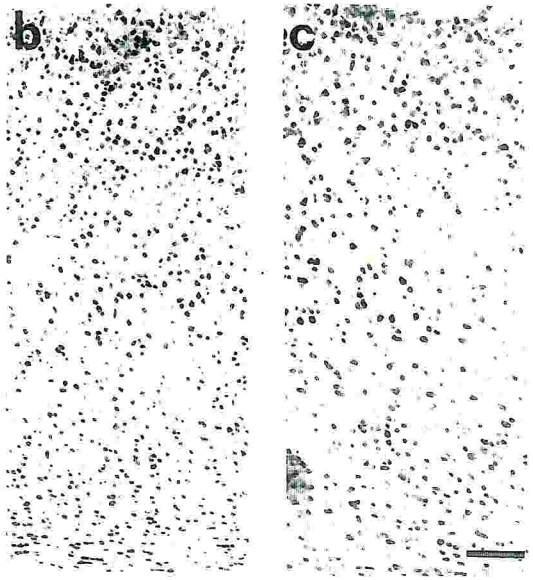
shown), while the concentration of 2.5 μg /ml showed weakly stained cells in addition (Fig. 9 d). Further increase of antibody concentration (10 μg /ml) gave a pattern similar to that obtained using a concentration of 2.5 μg /ml. The specificity of the antibody was further assessed by pre-adsorbing the antibody with BDNF and by omitting the secondary antibody (Fig. 9 e and f).

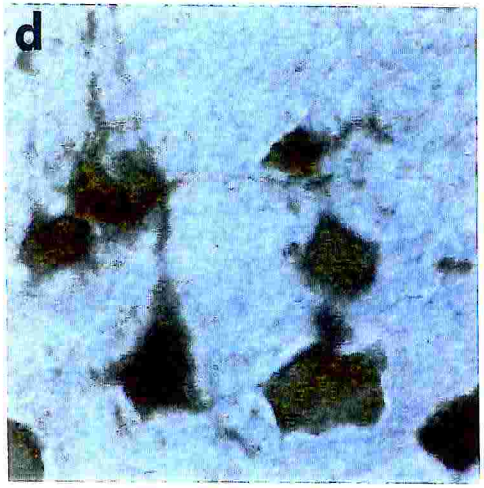
BDNF protein changes during postnatal development

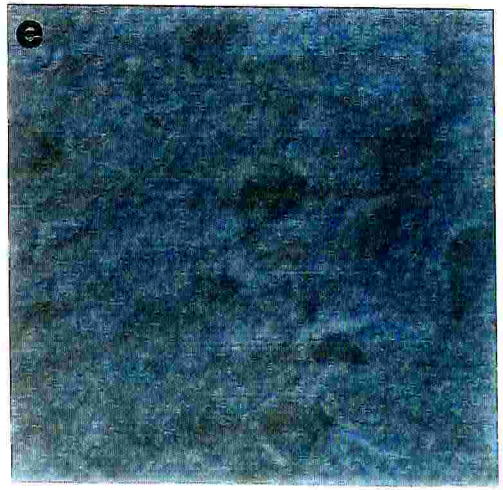
Experiments were carried out at a concentration of 2.5 μg/ml by using C-Rab antibody. At the cellular level, BDNF labeling appeared to be mostly confined to neurons. The labeling was diffuse and homogenous in the neuronal perikarya (Fig. 9d). Proximal dendrites of neurons were intensely labeled (Fig. 9 d), expecially in a few cells of layer V, where it was possible to follow the apical dendrites for several tens of μms (Fig. 10, P13). Two populations of cells were distinguished on the basis of the high or low levels of staining (Fig. 10a).

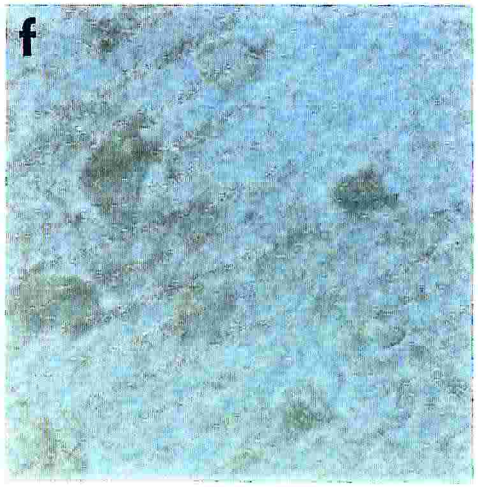
Cells immunostained with anti-BDNF antibody were present in all cortical layers and at all postnatal ages considered (Fig. 10). The intensity of staining in neurons of layers IV and VI of P13 animals was weaker with respect to the labeling of neurons in the same layers at later ages. In the neuropil, the intensity of staining at P13 was weaker than at later postnatal ages. At all postnatal ages, immunopositive neurons were more numerous in supragranular and infragranular layers than in layer IV (Fig. 10). Proximal dendrites were stained at all post-natal ages.


a
1 2 3 4 5 6 7 8 9
14.4 KDa –


b
c


d


e


f

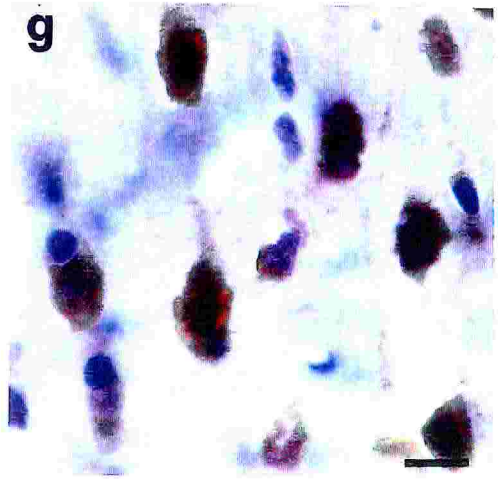

g

Fig. 9: Specificity of BDNF antibody used for immunohistochemistry.

a. Western Blot of neurotrophins. Lanes 3 and 8 contain Molecular Weight Marker (MWM). Lanes 1, 2 contain BDNF, 100 and 30 ng, respectively. Lanes 4 and 5 contain NT-3, 100 and 30 ng, respectively. Lanes 6 and 7 contain NT 4, 100 and 30 ng, respectively. Lane 9 contains NGF 150 ng.

b,c. Comparison between the pattern of expression of two different antibodies (Chemicon: 2.5 μg/ml (b), Amgen: 1.6 μg/ml (c)) in adult rat primary visual cortex. The two distribution patters are similar. Scale bar = 330 μm.

d. Enlargement of layer V of adult rat primary visual cortex showing BDNF immunostaining. Neurons present different levels of staining. In many neurons, both soma and dendritic processes are labeled.

e. Enlargement of layer V of adult rat primary visual cortex showing immunostaining obtained by using the primary antibody preadsorbed on an Elisa plate coated with BDNF at saturating concentrations. The preadsorbed antibody does not recognize neurons.

f. BDNF immunostaining obtained when the primary antibody was omitted. No specific labeling can be observed.

g. BDNF immunostaining in cresyl violet counterstained slices. Brown staining corresponds to BDNF protein, while blue staining characterizes cells stained with cresyl violet.

d,e,f,g: scale bar = 24 μm.

Fig.10.

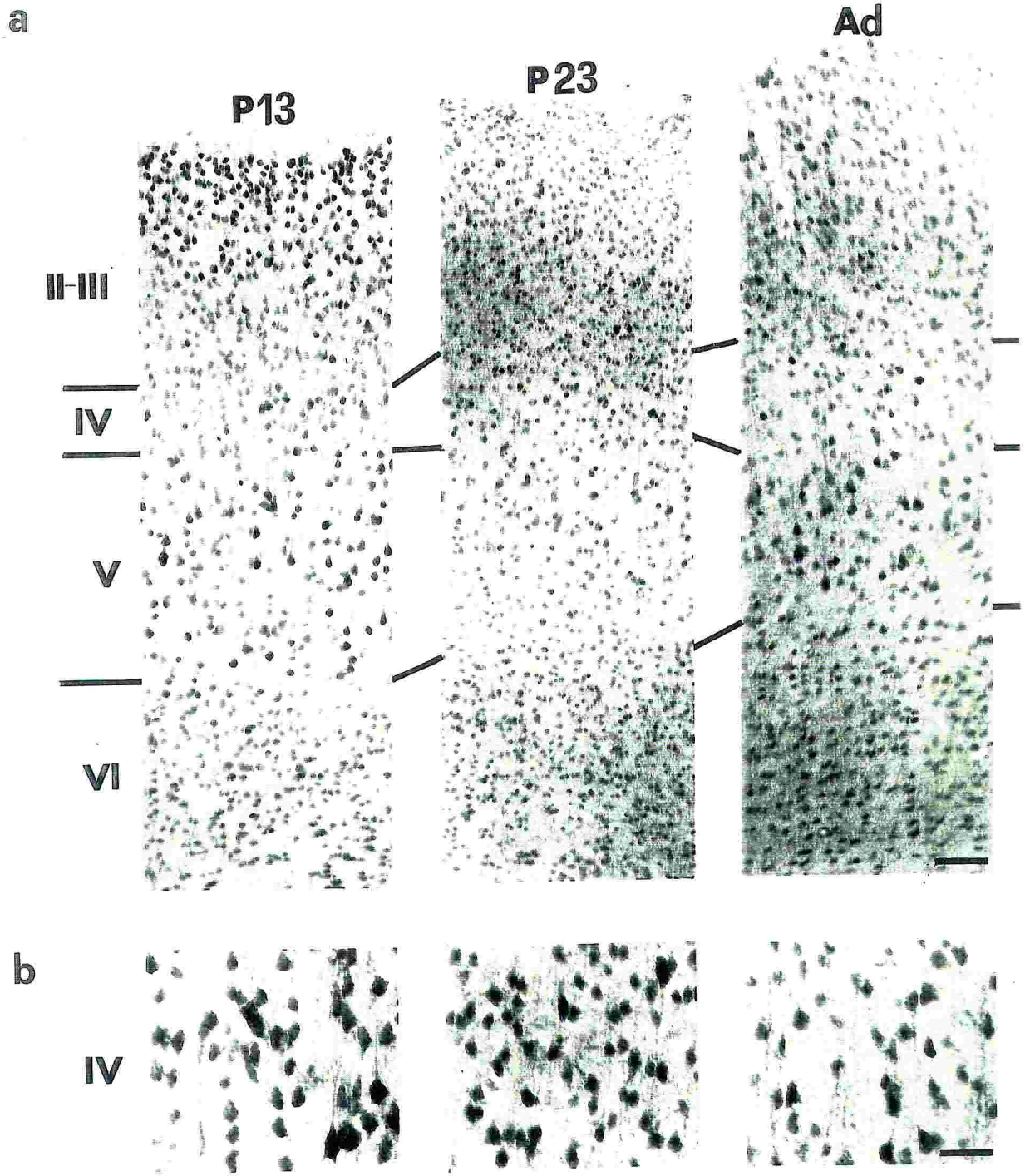


Fig. 10: BDNF-like immunoreactivity in rat primary visual cortex during postnatal development.

a. Immunostaining for BDNF in the primary visual cortex of P13, P23 and adult rats. At P13, before eye opening, there are immunostained cells scattered throughout the different cortical layers. Neurons in layer II-III and V appear more intensively stained. At P23, neurons expressing BDNF protein are more numerous with respect to P13 in all cortical layers. In adulthood (P90), neurons are distributed throughout all cortical layers. In layer IV of P90 animals, there are less immunopositive neurons with respect to the same layer at P23 and to supragranular and infragranular layers of the corresponding age. Scale bar = 230 μm

b. Enlargement of layer IV. Scale bar = 97 μm.

Changes in the number of BDNF labeled neurons occurring during postnatal development were quantified using an unbiased random uniform method (Howard & Reed, 1998) and expressed as a ratio with respect to cresyl violet stained cells (BDNF Cell Index = BCI). Penetration of the antibody was assessed at each postnatal age. We found that BCI was not different between superficial focal planes and the central core of each slice at all postnatal ages analyzed.

In Fig. 9g there is an example of double stained cells. BCI was reported in Fig. 11, for each cortical layer, at the three postnatal ages. In all layers there was a significant increase in the percentage of BDNF positive cells passing from P13 to P23 animals. This difference was more pronounced in layers IV and VI. The BCI did not change significantly after P23.

The total amount of BDNF protein detectable in different layers at different postnatal ages was evaluated by a sensitive ELISA immunoassay. We dissected the visual cortex in order to separate layer IV from supragranular and infragranular layers (Bolz et al, 1996). In table 1 we show that the amount of BDNF is higher at P23 than at P13 in all cortical layers analyzed. (These data were consistent with the BCI calculated at the cellular lever). After P23, in layer IV and infragranular layers the amount of BDNF decreased to levels lower than those found at P13.

Fig.11.

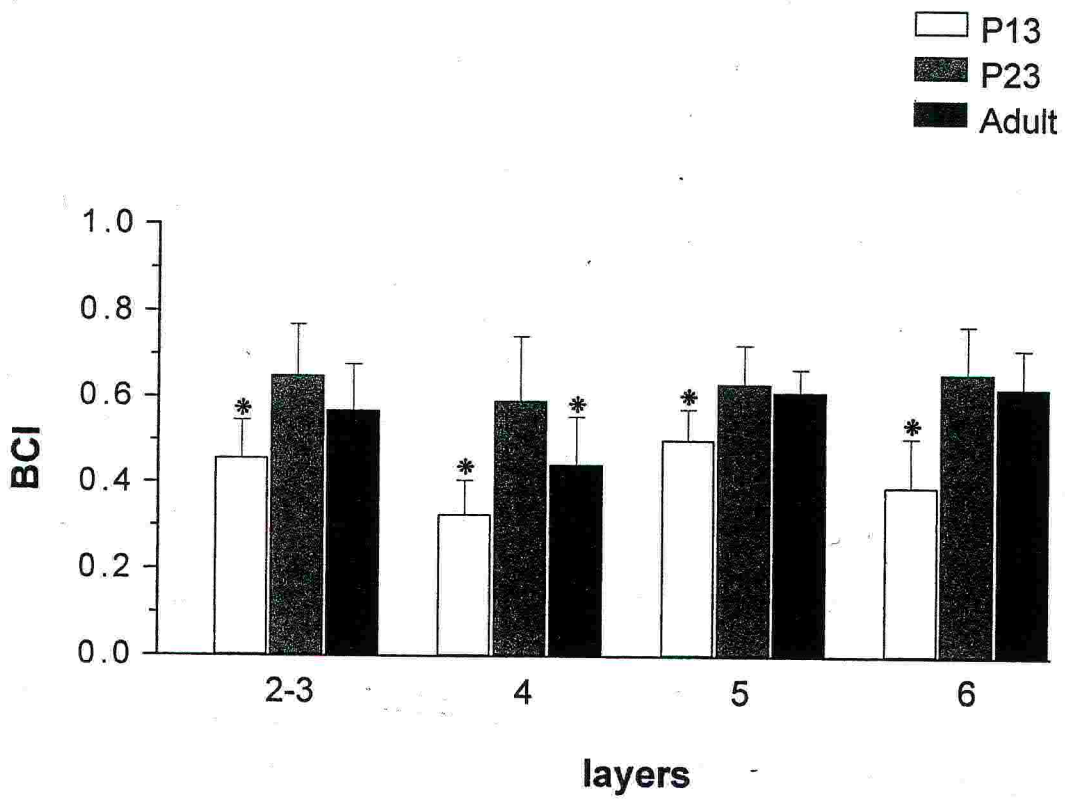


Fig. 11: Quantitative analysis of BDNF immunopositive neurons.

BCI at P13 (n=6) is significantly lower ($P < 0.05$) than BCI at P23 (n=6) and at P90 (n=6) in all cortical layers. BCI between P23 and P90 is not significantly different with the exception of layer IV. In this layer BCI is decreased with respect to P23 ($P < 0.05$). These results show that the number of neurons expressing BDNF protein changes during postnatal development.

Table1.

Changes in BDNF protein amount in different cortical layers during postnatal development

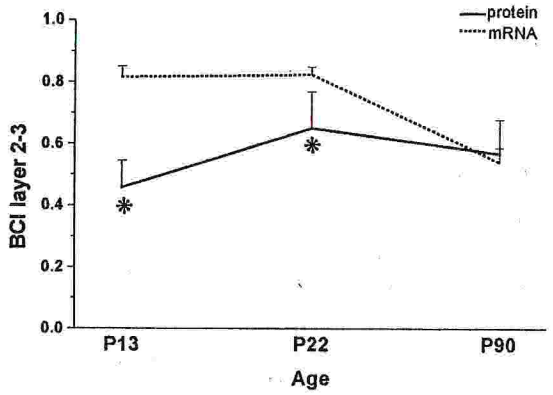
	P12	P23	P90
II-III	40.3	48	48.2
IV	31.2	48.6	19.6
V-VI	42.7	46.8	23.5
tot	114.2	143.4	91.3

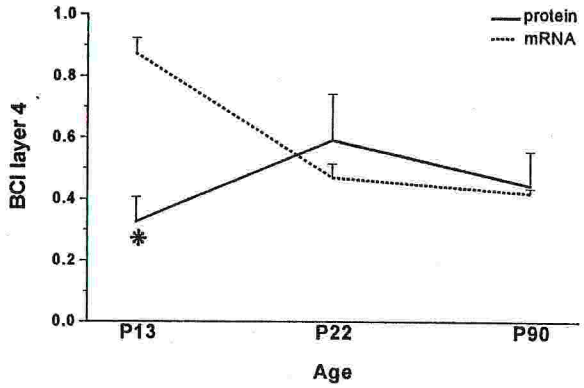
Table 1: Numbers represent pg of protein.

Mismatch of BDNF protein and mRNA in visual cortical neurons.

The percentage of neurons expressing BDNF mRNA at different postnatal ages and in adulthood has been previously studied and reported by our group (Capsoni et al., 1999 a, b). Now we compared the two sets of data for BDNF mRNA and for BDNF protein. This was possible because the analysis methods followed in the present and in previous papers (Capsoni et al., 1999 a, b), were strictly comparable. The data (Fig. 12) show that at P13, before eye opening, in cortical layers II-III, IV, V and VI, there is a significant difference between the percentage of neurons stained for BDNF mRNA with respect to those stained for BDNF protein. This difference is larger in layer IV. In this layer, neurons stained for BDNF mRNA are more numerous by a factor of two than BDNF immunopositive cells. During postnatal development this difference disappeared: at P23 and P90 the BCIs for mRNA and protein were similar in all cortical layers with the exception of layer V. In this layer, immunopositive neurons were more numerous with respect to those stained for BDNF mRNA at P90. This discrepancy could be due to neurons expressing TrkB receptors and able to take up BDNF from external environment, but not to synthesize it (Cellerino et al., 1996 b). An example of these cells is represented by parvalbuminergic neurons as shown in Figure 13.

Mismatch between BDNF mRNA and protein distribution at an early stage of postnatal development was proven within the same neuron by using a double *in situ*/immunohistochemistry to detect BDNF mRNA and BDNF protein. In Fig. 14 we


protein
mRNA
BCI layer 2-3
1.0
0.8
0.6
0.4
0.2
0.0
P13
P22
P90
Age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mRNA
BCI layer 4
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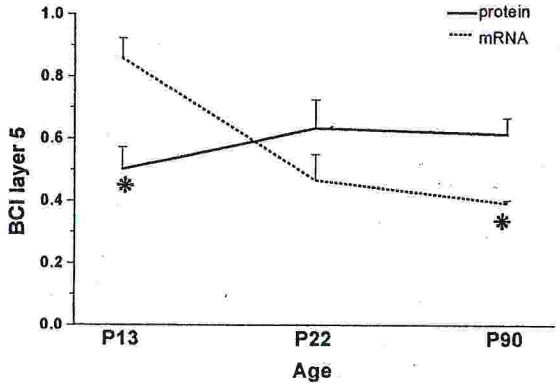
0.4
0.2
0.0
P13
P22
P90
Age


protein
mRNA
BCI layer 5
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0.8
0.6
0.4
0.2
0.0
P13
P22
P90
Age

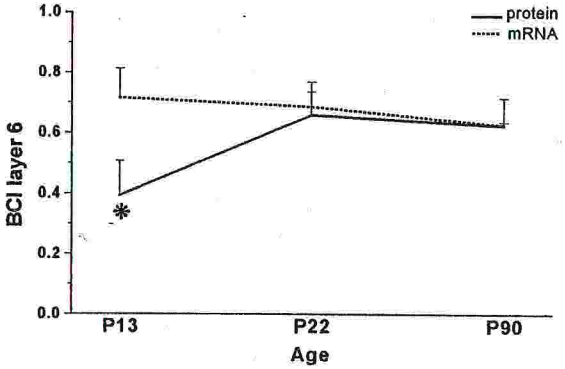
protein
mRNA
BCI layer 6
1.0
0.8
0.6
0.4
0.2
0.0
P13
P22
P90
Age

Fig. 12: Comparison between BCI for BDNF mRNA and BDNF protein in rat primary visual cortex during postnatal development.

Data for BDNF mRNA were taken from Capsoni et al (1999a, b). and plotted as a scatter histogram. At P13 (n=3 for mRNA, n=6 for protein) BCI for BDNF mRNA is significantly higher compared to BCI for BDNF protein. This difference is stronger in layer IV. At later ages BCIs for BDNF mRNA and protein are not significantly different with two exceptions: in layer II-III of P23 animals (n=3 for mRNA, n=6 for protein) there are less cells expressing BDNF protein compared to cells expressing BDNF mRNA; in layer V of adult rats (n=3 for mRNA, n=6 for protein), BCI for BDNF protein is higher than the corresponding BCI for BDNF mRNA. Both differences are statistically significant.

Fig.13.

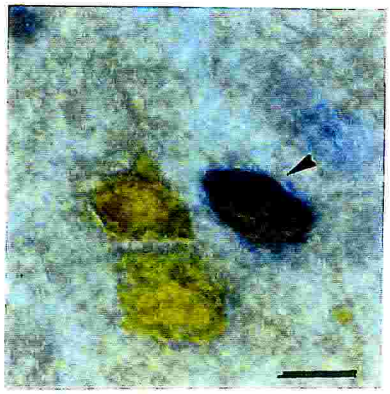

Fig. 13: Parvalbumine positive neurons coexpress BDNF protein in adult rat visual cortex.

Cells were double-labeled with a polyclonal antibody for BDNF and a polyclonal antibody for parvalbumin. Cells stained for BDNF appear brown, while cell double-stained appear blue-brown (arrowhead). Scale bar = 24μm

show that some neurons are labeled for protein (light brown staining, arrowheads), other neurons are labeled for BDNF mRNA only (Fig. 14, blue staining, arrows) and a third group of neurons contained both BDNF mRNA and protein (BDNF protein: in the figure light brown staining is more visible in the center of cell body; BDNF mRNA: blue labeling more evident at the periphery; overlapping colours appear as bluisch). At P13, a conspicuous number of neurons was stained only for BDNF mRNA, while only few cells were double labeled. At later ages (P23, P90) BDNF protein and its mRNA were co-expressed in almost all neurons.

Thus, before eye opening there is a mismatch between BDNF mRNA expression and protein distribution within visual cortical neurons.

In particular, there are more neurons containing BDNF mRNA but not BDNF protein. After eye opening, the number of cells expressing BDNF mRNA and protein, reach similar levels.

Visual experience controls BDNF expression in developing visual cortex.

Since visual experience influences the general level of BDNF mRNA and its cellular expression (Capsoni et al., 1999a, b) we predicted that the absence of light would affect the cellular content of BDNF protein. Animals were kept in a dark room from birth until P39. Fig. 15a shows that in P39 dark reared rats (P39 DR) the percentage of BDNF immunopositive neurons was reduced throughout the cortex with respect to P39 normally reared rats (P39 NR). Moreover, in all cortical layers the BCI of P39 DR rats was identical to that of P13 normally reared animals (Fig. 15b). Thus, in dark reared rats the number of neurons expressing BDNF protein is low, similarly to what found before eye opening.

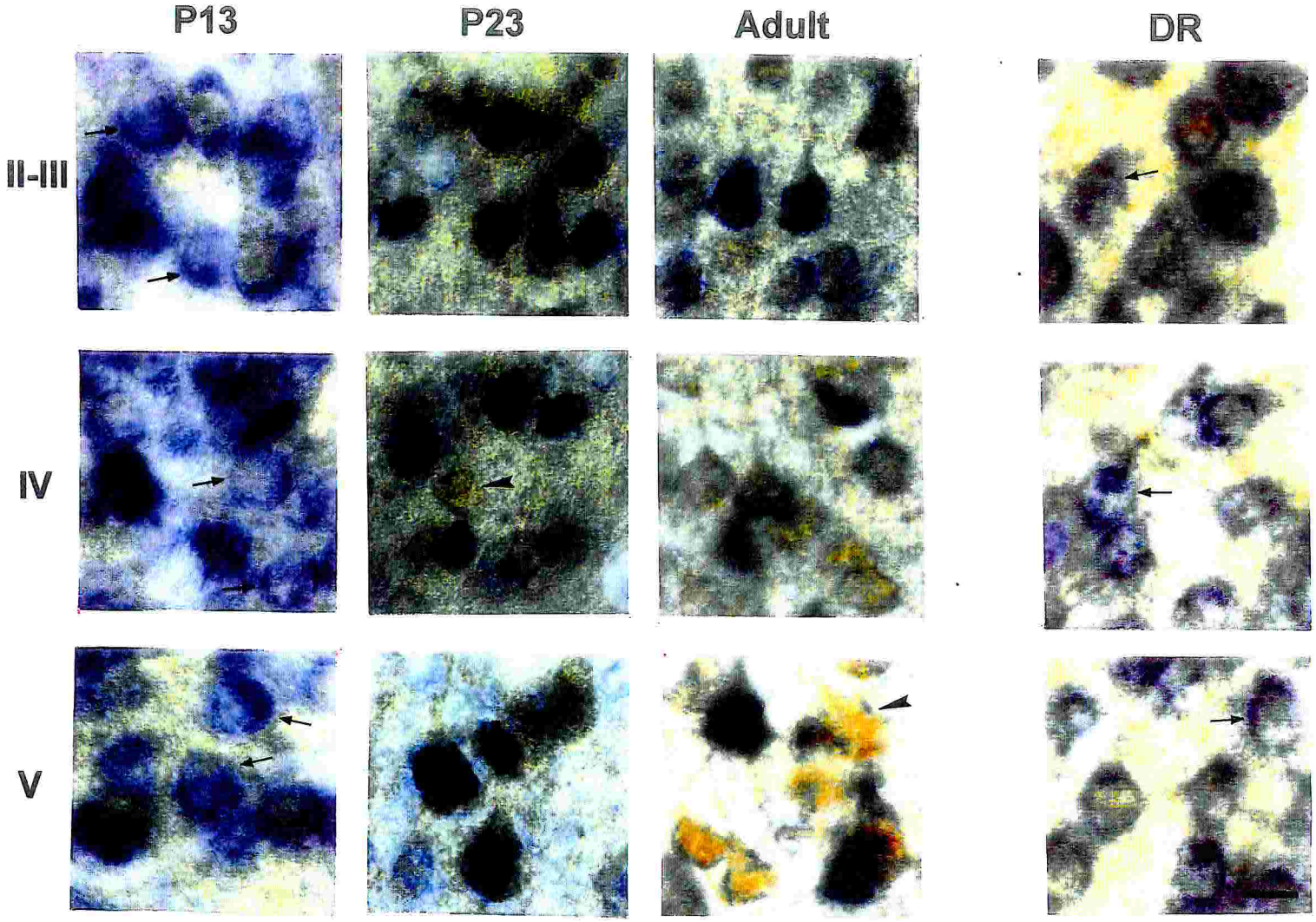
P13
P23
Adult
DR
II-III
IV
V

Fig. 14: Double labeling for BDNF mRNA and protein in rat primary visual cortex during postnatal development and in dark reared animals.

Enlargement of layers II-III, IV and V at P13, P23, P90 of normally reared rats and at P39 of dark reared rats. Cells were double labeled with a biotinilated riboprobe for BDNF mRNA (blue) and a polyclonal antibody (Chemicon) for BDNF (light brown). Arrows indicate cells stained for BDNF mRNA. Arrowheads indicate cells stained for BDNF protein. Double stained cells appear brown-blue. At P13 of normally reared rats and at P39 of dark reared rats there are more cells stained for BDNF mRNA than cells co-expressing protein and mRNA and cells stained for protein alone. At P23 and P90 of normally reared rats most neurons co-express BDNF mRNA and protein. In layer V of P90 rats there are several cells stained only for protein. Scale bar= 24 μm.

Fig.15.

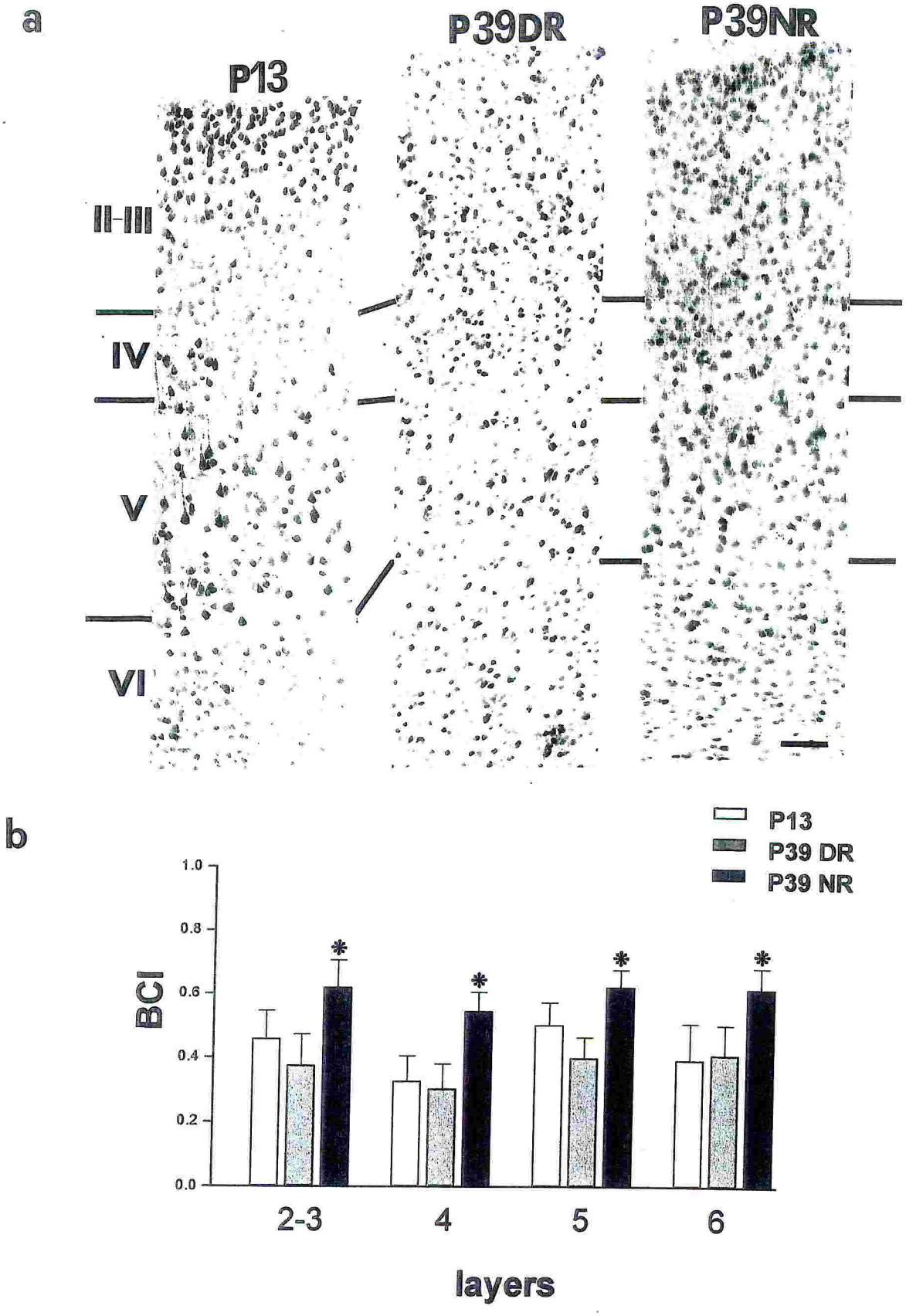
a
P13
P39DR
P39NR
II-III
IV
V
VI
b
P13
P39 DR
P39 NR
BCI
1.0
0.8
0.6
0.4
0.2
0.0
2-3
4
5
6
layers

Fig. 15: Visual experience controls the expression of BDNF protein in rat primary visual cortex.

a: BDNF-like immunoreactivity in the rat primary visual cortex of P13, P39 dark reared rats (P39DR) and P39 normal reared animals (P39NR). Scale Bar = 260 μm.

b: Quantitative analysis of neurons expressing BDNF protein in rat primary visual cortex. BCI at P39 is significantly lower in dark reared rats (n=6) with respect to normally reared rats of corresponding age (n=5).

No statistically significant difference was found between BCI of P39 dark reared rats and BCI of P13 normally reared rats.

Double *in situ* hybridization/immunohistochemistry showed that in all layers of P39 dark reared animals a conspicuous number of neurons were labeled for BDNF mRNA but not for BDNF protein (Fig. 14, DR).

A distinct group of animals were reared in dark for ten days from P13 to P23. We compared the BCI values of P39 and P23 dark reared animals: in all cortical layers the relative number of BDNF immunopositive neurons was not significantly different between the two groups of animals, thus indicating that even a short period of light deprivation was sufficient to reduce the percentage of BDNF positive neurons.

To further prove that light may modulate BDNF protein expression we reared rats in darkness from P13 to P23 and then we exposed them to normal light environment for a brief period. We have found that two hours of light were sufficient to restore the normal BCI values in all cortical layers (Fig. 16).

It has been previously reported (Capsoni et al., 1999a) that dark rearing does not homogeneously affect BDNF mRNA cellular expression in all the cortical layers. Indeed, BCI for mRNA was significantly higher only in cortical layers IV and V of P23 dark reared rats with respect to age matched controls. In the present paper we confirmed these data (table 2) and we extended our investigation to the effects produced by two hours of illumination in layer IV and V. In these layers BCI for mRNA of dark reared animals exposed to light was significantly different from that of normally reared animals and almost identical to that measured in dark reared rats. These results suggest that two hours of light were not sufficient to restore the normal number of BDNF mRNA expressing cells in dark reared rats.

Comparing BCI for BDNF mRNA and protein (Fig. 16, Table 2), it appears

Fig.16.

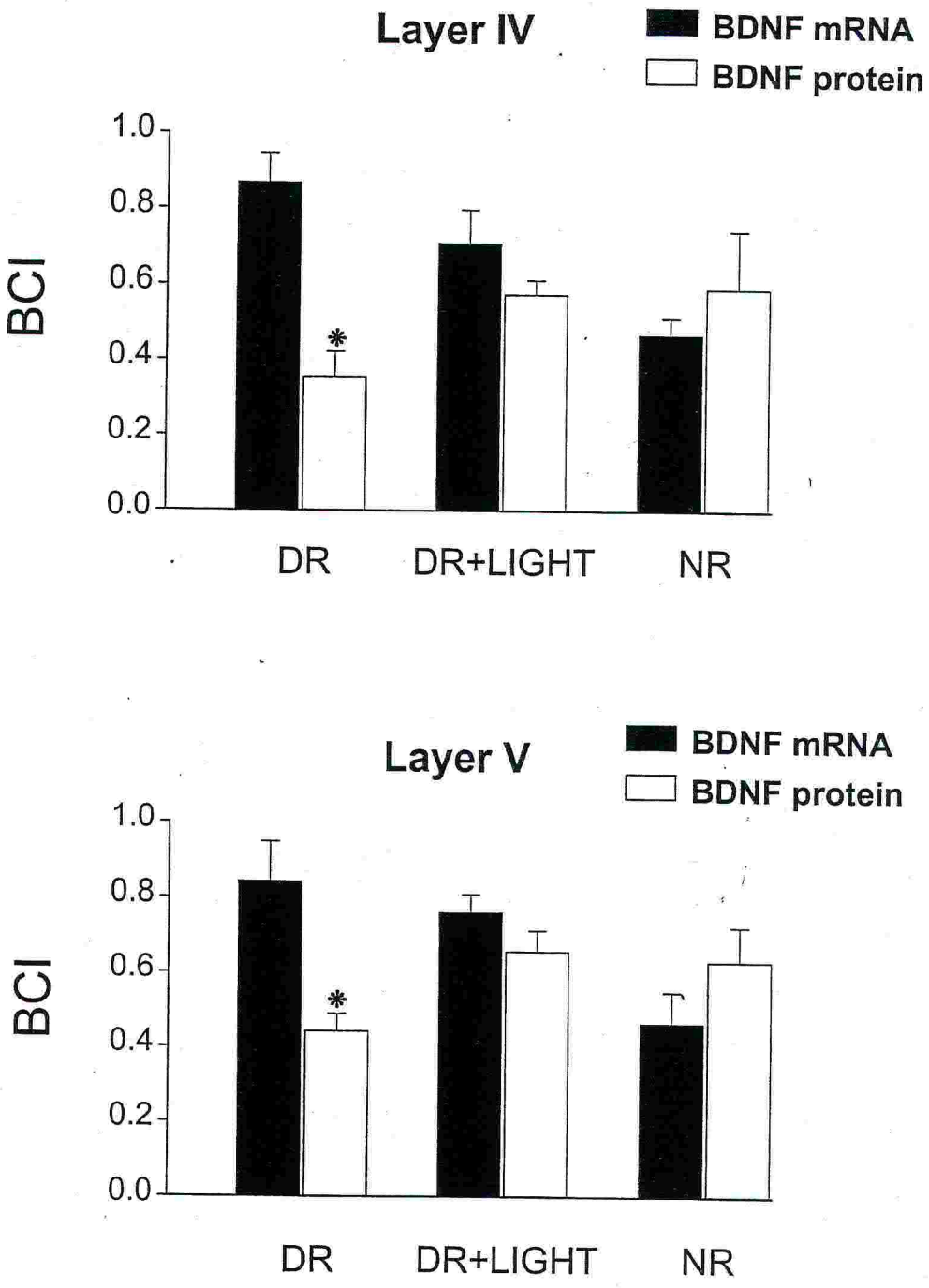
Layer IV
BDNF mRNA
BDNF protein
BCI
1.0
0.8
0.6
0.4
0.2
0.0
*
DR
DR+LIGHT
NR
Layer V
BDNF mRNA
BDNF protein
BCI
1.0
0.8
0.6
0.4
0.2
0.0
*
DR
DR+LIGHT
NR

Fig. 16: Visual experience differentially regulates BDNF mRNA and protein.

BCI values are expressed as the means ± SD. In layer IV (top panel) and V (bottom panel) BCI for mRNA is significantly different ($P<0.05$) between P23 dark reared (DR, n=4) and P23 normally reared (NR, n=3) rats; the difference between P23 DR and P23 Dr exposed to 2 hours of light (DR+light, n=4) is not significant. In layer IV and V BCI for protein is significantly different ($P<0.05$) between DR and DR +light rats and between DR (n=6) and NR (n=6) rats. In both layers of DR rats the difference between BCI for mRNA and BCI for protein is significant ($P<0.05$).These results indicate that 2 hours light are sufficient to restore both a normal number of neurons expressing BDNF protein and a good match between neurons expressing BDNF mRNA and protein.

Table2.

BCI for mRNA and protein in dark reared (DR) and normal reared (NR) P23 rats.

LAYERS	BDNF	P23 DR	P23NR
II-III	**mRNA**	**0.89 ± 0.01**	**0.82 ± 0.02**
	PROTEIN	0.39 ± 0.04	0.65 ± 0.12
IV	**mRNA**	**0.87 ± 0.08**	**0.47 ± 0.04**
	PROTEIN	0.36 ± 0.07	0.59 ± 0.15
V	**mRNA**	**0.84 ± 0.1**	**0.47 ± 0.09**
	PROTEIN	0.44 ± 0.04	0.63 ± 0.09
VI	**mRNA**	**0.73 ± 0.07**	**0.69 ± 0.05**
	PROTEIN	0.39 ± 0.05	0.66 ± 0.11

Table 2. BCI for mRNA and protein in dark reared (DR) and normally reared (NR) P23 rats.

BCI values are expressed as the mean ± SD. For mRNA, differences between DR and NR rats are significant ($P<0.05$) only in layers IV and V. For protein, differences between DR and NR rats are significant in all cortical layers. BCIs for protein and mRNA are significantly different in all cortical layers of DR rats.

that i) in dark reared animals there is an higher percentage of neurons expressing BDNF mRNA with respect to those with a detectable level of BDNF protein in all cortical layers; ii) two hours of light were sufficient to restore a normal percentage of neurons containing BDNF protein in layers IV and V while recovery of BDNF mRNA positive neurons was incomplete.

Our findings indicate that visual experience controls BDNF mRNA and protein cellular content in developing visual cortex.

TRKB RECEPTORS EXPRESSION DURING POSTNATAL DEVELOPMENT OF VISUAL SYSTEM. DARK REARING EFFECTS.

Since BDNF exerts its action by binding TrkB receptors, we studied receptors expression in rat visual cortex, considering both the cellular expression and protein levels. To address this issue we used two different antibodies: TrkB+ and TrkB- that recognize, respectively, the full length and the truncated form of TrkB receptor.

We considered TrkB expression at different postnatal ages: P13, P23, and P40.

To detect the protein level, a western blot was performed in homogenates of rat primary visual cortex, taken at the ages considered (Fig. 17)

The western blot shows that the expression of both forms of TrkB receptors increases during development going from P13 to P40.

Thus, the endogenous level of TrkB receptors is regulated during postnatal development.

Next question is whether visual experience may influence the endogenous level of TrkB receptors. From the Fig. 17 it is evident that deprivation of visual experience from P13 does not affect the expression of TrkB. This is true both for animals kept in the dark until P23 and until P40 (data not shown). Thus, contrary to what found for BDNF mRNA and BDNF protein the development of TrkB, as measured by western blot, does not depend on visual experience.

In a second group of experiments the same antibodies were used to investigate the cellular pattern of TrkB expression during postnatal development.

For TrkB full length (Fig. 18) the cellular distribution changes going from P13 to later ages. Before eye opening most of the stained cells are localized in layers II-III and V.

After eye opening cells positively labeled for TrkB+ are distributed throughout all cortical layers and this distribution is maintained also in P40.

For the truncated form of TrkB (Fig. 19) the cellular distribution overlaps that of the full length form: before eye opening labeled cells are mainly localized in cortical layers II-III and V. At P23 the distribution of TrkB- cells is more homogeneous, and in P40 animals many more cells are labeled and are localized in all cortical layers.

We went on into characterize the effects of dark rearing on TrkB+ and TrkB- expression. Also at cellular level there are no differences between dark reared animals and aged matched controls (P23) (Fig. 18; Fig. 19).

Similar results were obtained by comparing P40 dark reared animals with their age-matched controls (data not shown): we can conclude that dark rearing does not influence the normal developmental cellular distribution of TrkB receptors.

To conclude the cellular expression and the endogenous level for TrkB receptors changes during development: both forms of the receptor are initially concentrated in layers II-III and V and then spreads through all cortical layers, although many more cells are positively stained for the full length form of the receptor.

For both forms of the receptors the protein levels increase during postnatal development while it is not altered by the deprivation of visual experience. Also at a cellular level, the distribution of TrkB receptors is not different between dark reared rats and age matched controls.

Fig.17.

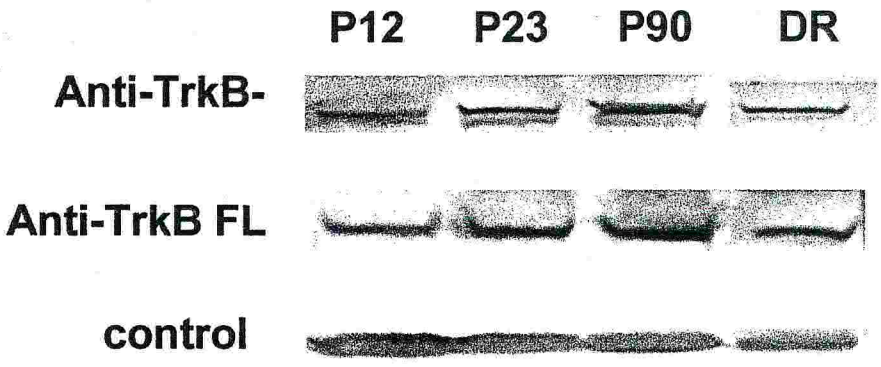

Fig. 17: Western blot analysis of the full length (FL) and truncated (TkB-) forms of TrkB receptors during development of rat visual cortex.

The first two lanes show the total amount of truncated and full length of TrkB receptors, respectively measured at P12, P23, P40 and in dark reared animals.

Western blot analysis clearly shows that both forms of TrkB receptor increase in rat visual cortex going P13 to P40. On the contrary, there is no difference between TrkB levels at P23 normally reared compared to P23 dark reared animals.

The last line shows the amount of control protein tubulin at the same age.

The amount of control protein does not change at the different ages considered, contrary to what was observed for TrkB proteins.

Fig.18.

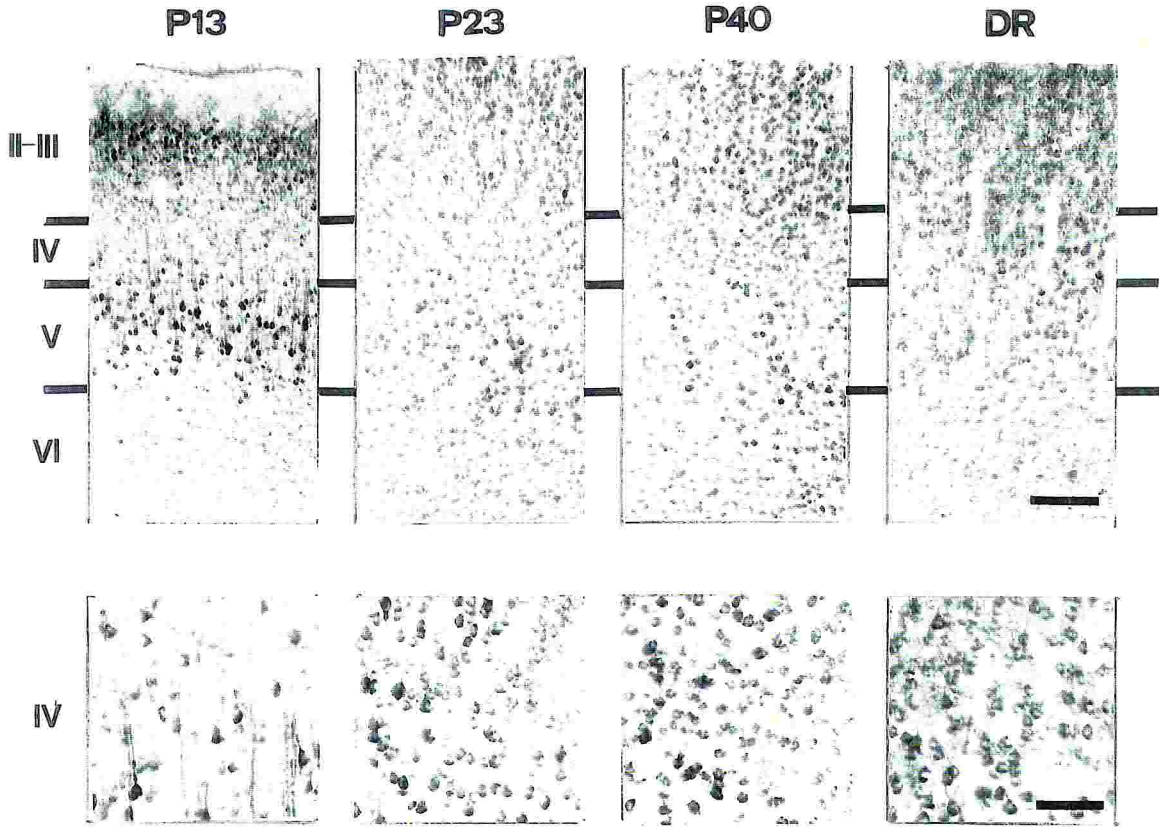

Fig. 18: Cellular expression of full length (FL) TrkB receptor during development of rat visual cortex and in dark reared animals.

Top: Cellular expression of FL TrkB receptor in all layers of rat visual cortex at different postnatal ages and in dark reared animals. At P13 positively labeled cells are mainly concentrated in layers II-III and V, while later in development the staining spreads throughout the cortical layers. There is no variation in the cellular expression between dark reared animals and age-matched controls. Scale bar= 250 μm.

Bottom: High magnification of layer IV at the corresponding ages. Scale bar=78μm.

Fig.19.

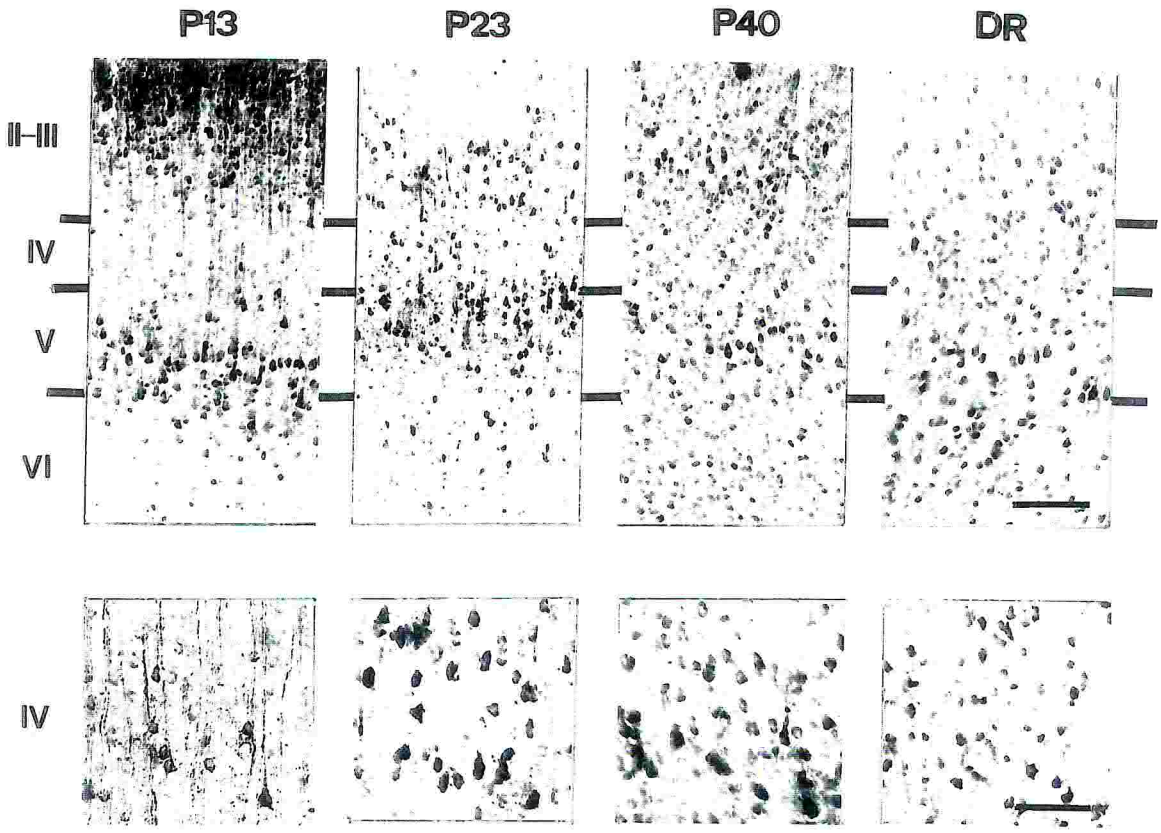

Fig. 19: Cellular expression of truncated TrkB receptor during development of rat visual cortex and in dark reared animals.

Top: Cellular expression of truncated TrkB receptors in all layers of rat visual cortex at different postnatal ages and in dark reared animals. At P13 positively labeled cells are mainly concentrated in layers II-III and V. At later ages the number of positively stained cells progressively increases and the staining is present in all cortical layers. There is no variation in cellular expression between dark reared animals and age-matched controls. Scale bar= 250 μm.

Bottom: High magnification of layer IV at the corresponding ages. Scale bar=78μm.

DISCUSSION

ROLE OF BDNF IN SYNAPTIC PLASTICITY

A new form of synaptic plasticity (de-depression) is expressed in the developing rat visual cortex.

The initial aim of this study was to investigate the mechanisms involved in activity-dependent development and plasticity of visual cortex, aiming to propose a cellular model of synaptic plasticity. Two well-studied forms of long term synaptic plasticity are LTP and LTD. Several studies have proposed LTP and LTD as models of synaptic plasticity and suggested that they are involved in plastic processes such as learning and memory.

In the visual cortex LTP and LTD can be elicited by stimulation of white matter. For LTP it has been shown that its amplitude is high soon after eye opening, progressively decreasing thereafter. After the end of the critical period for monocular deprivation it is not possible to evoke LTP (Kirkwood et al., 1995). For LTD, the situation is more complex. Recording field potentials, LTD expression does not change greatly during the critical period; at the end of the critical period LTD amplitude is only slightly diminished.

In the present work we have described a new form of synaptic plasticity expressed during an early phase of postnatal development. To date, in the visual cortex all studies have dealt with either LTP or LTD but not with the two forms together. During the first phase of postnatal development synapses are highly modifiable by visual experience. Therefore visual cortical neurons must be able to integrate different synaptic inputs before choosing the set of inputs to be strengthen, and it is necessary to revert the sign of long term synaptic change. We have chosen to

study the ability of visual cortical synapses to be first depressed and then potentiated as a specific indicator of the degree of cortical synaptic plasticity (de-depression). This form of synaptic plasticity has been investigated in adult hippocampus where long-term changes in synaptic effectiveness can be modified in a bi-directional fashion: this phenomenon is known as meta-plasticity.

It is important to relate the plastic behavior of a synapse to its physiological role during development. In this respect, the maintenance of highly plastic characteristics in the adult hippocampus suits its physiological role in learning and memory.

Our study has been performed in cortical slices containing the primary visual cortex. We analyzed the amplitude and shape of field potentials elicited in cortical layers II-III by stimulation of white matter. One of the technical constrain of this recording technique is that white matter stimulation should stimulate fibers widely. To minimize this possibility the stimulating electrode was placed at the border between white matter and layer VI in a way that according to Kenan-Vaknin and Teyler (1994) and Woodward et al. (1990) facilitates the stimulation of vertical pathways projecting to layers IV and II-III. To avoid polysynaptic potentials and back-propagation of the stimulus we ensured that potentials i) were monophasic, ii) their waveshape was not changed by stimulation frequencies higher than 1Hz. To control for the possibility that spike responses might influence the recorded response, at the beginning of the recording session we moved back and forth the recording electrode into the tissue in order to ensure that the electrical stimulation was not evoking spike population.

Our results demonstrate the presence of a short time window, between P17 and P23, during which visual cortical synapses rapidly lose their ability to reverse a given LTD. Although potentiation of depressed responses is no longer inducible

at P23 we and others have shown that LTP *per se* is still present at this age. Indeed in control experiments LTD does not prevent the possibility of evoking LTP in a different location within the primary visual cortex. This observation suggests that, in spite of their similarity, LTP and potentiation after LTD might rely on partially different mechanisms. Alternatively, LTD might trigger an inhibitory mechanism, not yet present or active at P17, preventing potentiation. It is interesting to notice that in the visual cortex the inhibitory circuitry using GABA as neurotransmitter is not yet mature at this age (Luhmann and Prince, 1991). In any case the fact that LTD amplitude does not change significantly between P17 and P23 indicates that potentiation does not primarily depend on the magnitude of LTD.

The way that this plasticity property changes during development points to a fundamental role in the shaping of visual cortical circuitry. Malleability of cortical synapses is present soon after eye opening when selectivity for orientation and movement direction to visual stimuli is almost absent: most cells receive binocular input and their receptive fields are highly immature. At this developmental stage neurons in the visual cortex receive many different inputs which they integrate. The ability to reverse the sign of information storage coming from a given synaptic input suits the needs of visual neurons at this age. The dramatic decline in synaptic flexibility observed over a few days coincides with the functional maturation of cortical circuitry. For example the functional properties of visual cortical neurons such as ocular dominance and orientation selectivity show a high rate of maturation between P17 and P23, reaching adult-like properties between P26 and P30 (Fagiolini et al., 1994). It has already been suggested that the decline of visual cortical LTP runs in parallel with the maturation of functional properties of rat visual cortex. We propose that the

ability of a synapse to reverse a long-term change in synaptic efficacy represents an intrinsic and crucial neuronal characteristic of an early stage of visual cortex functional development. As maturation proceeds under the control of visual input, a gradual decline in the degree of synaptic plasticity occurs, first with the loss of the capacity to reverse an LTD. After P35- P49, when all the visual cortex characteristics are adult-like, LTP also disappears while LTD is still present, albeit at lower amplitude. In Fig.20 the different forms of synaptic plasticity are temporally related to the time course of the critical period for monocular deprivation and to the periods of maturation of several functional properties such as orientation selectivity, receptive fields, ocular dominance distribution and visual acuity. Thus, although there is no direct experimental evidence establishing a casual relationship between the main features of cortical development and the different forms of cortical synaptic plasticity, the temporal correlation described here might be a good model.

Role of visual experience in the developmental expression of de-depression.

Most evidence indicates that visual experience plays an important role in the functional and structural maturation of the visual cortex. In the rat visual cortex, soon after eye opening visual cortical functions are immature. For example, selectivity for orientation is almost absent, most cells receive a binocular input and the receptive fields are larger than adult size. These functional properties mature progressively with different time periods. This functional maturation is dependent on visual experience. Indeed, rats deprived of visual input from birth and studied well after the end of maturation periods are characterized by cortical functions with the properties of an immature stage of postnatal development. Thus, in the rat visual experience also drives the functional maturation of primary

Fig. 20.

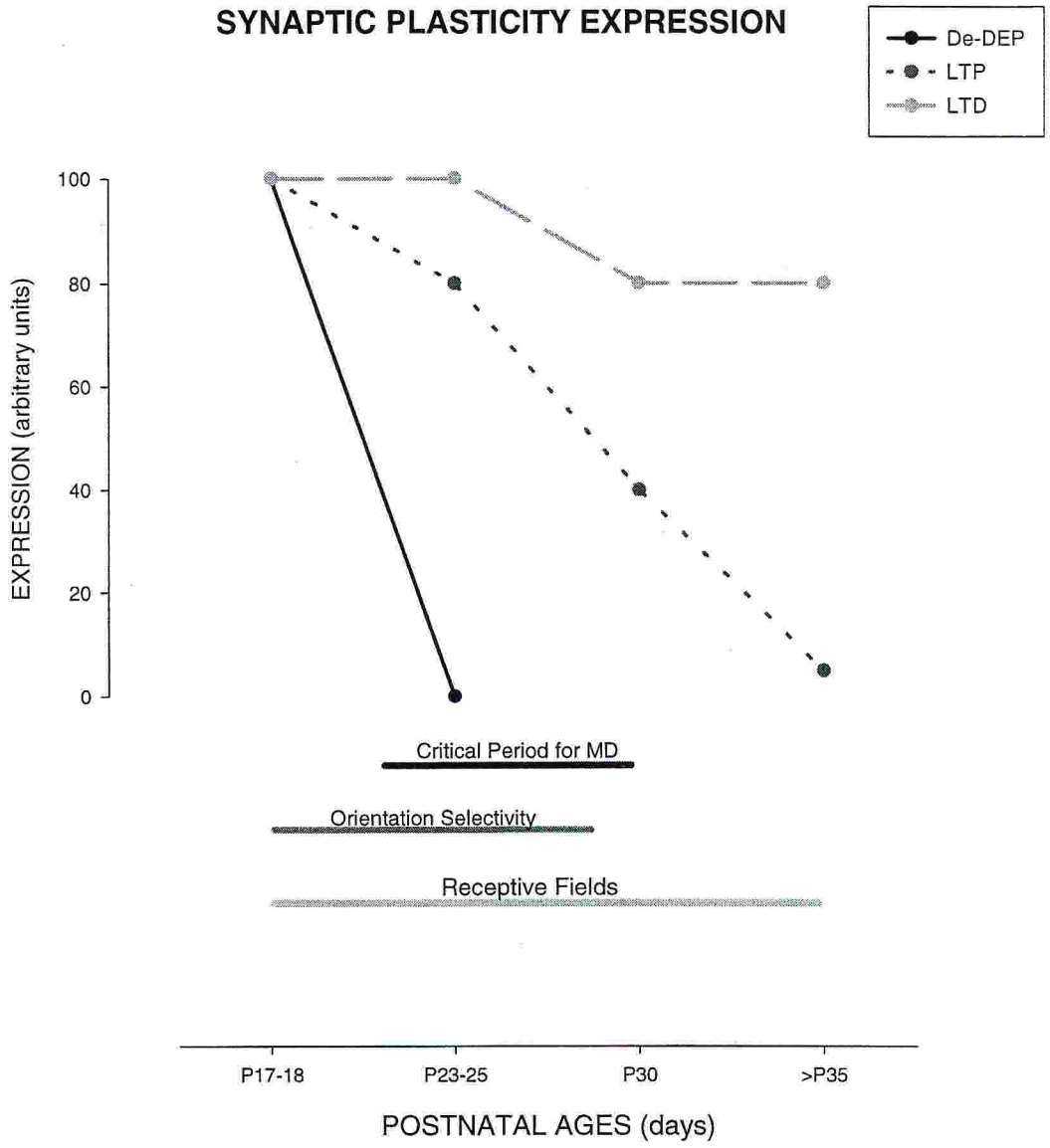

Fig. 20: Synaptic plasticity expression in rat visual cortex. Time course expression of three different forms of synaptic plasticity: LTP, LTD and de-depression during the development of rat visual system.

visual cortex.

In order to further relate de-depression to cellular mechanisms involved in activity-dependent development of visual cortex, it was important to know whether this form of synaptic plasticity, in addition to being developmentally regulated, might be influenced by visual experience.

Our study indicates that in rats reared in darkness from P17 to P29, visual cortical synapses maintain the capacity to be potentiated after LTD well beyond the age when this property is normally lost. This loss can therefore be considered part of a developmental process and, in particular, of an activity-dependent development which is known to control most of the ontogenic changes in rat visual cortex. Dark rearing is able to prevent both the loss of LTP in the adult as well as LTD reversal at P23, indicating that the absence of sensory triggered synaptic activity maintains the plastic properties characterizing the early stage of development.

Contrary to what is observed in animals light-deprived from birth, where LTD amplitude is extremely reduced, in rats deprived of sensory input at P17 it was still possible to induce a robust LTD.

Having established that this form of synaptic plasticity is developmentally regulated in an activity-dependent manner we tried to identify possible factors modulating it. Several factors have been proposed to be involved in activity dependent plasticity and postnatal development of the visual cortex

BDNF is a likely candidate to translate neuronal activity into synaptic plasticity. It has been reported that BDNF mRNA is expressed in an activity-dependent manner in the rat visual cortex. In addition, BDNF modulates both synaptic currents and LTP elicited by stimulation of cortical layer IV in the rat visual cortex (Akaneya et al., 1997).

Recently, it has been reported that TrkB ligands, NT-4 and BDNF, are capable of modulating the amplitude of late phase LTP elicited by stimulation of white matter during postnatal development of the rat visual system (Sermasi et al., 2000).

In order to study the effects of BDNF on potentiation after depression, we released it through the recording pipette. The aim of using this technique was to apply BDNF to the portion of the slice we were recording from, allowing us to record from a limited number of activated cells (Pesavento et al, 2000) and avoiding any undesired general activation of TrkB receptors expressed throughout all layers of the primary visual cortex.

We have shown that an exogenous supply of BDNF is able to restore the capability of cortical synapses to be potentiated after LTD at a postnatal age when this property is normally lost. Our data indicate an involvement of BDNF in modulation of this form of synaptic plasticity and suggest a role for endogenous BDNF and its receptor TrkB. In a second set of experiments we went on to clarify whether BDNF effects are mediated by TrkB receprors expressed in slices of visual cortex. The possible involvement of endogenous TrkB receptors in potentiating depressed responses was tested by blocking the tyrosine kinases activated by binding of BDNF to its receptor. The experiments were performed at P17 when de-depression is highly expressed. We used K252a to block the tyrosine kynases associated with neurotrophins receptors. In these conditions it was no longer possible to potentiate previously depressed synapses.

Further studies in our laboratory have shown that blockade of endogenous TrkB-ligands by applying TrkB immunoadhesins at P17 prevents the potentiation of previously depressed synapses and also potentiation after LTD.

These results clearly indicate that endogenous TrkB ligands are necessary

for the expression of de-depression during early postnatal development.

Recent results obtained on LTP have shown that blockade of TrkB ligands by the same immunoadhesin used in the present study does not abolish the capacity of synapses to be potentiated, but only diminishes the amplitude of late phase LTP. This suggested to the hypothesis that TrkB ligands, together with other factors are involved in mechanisms underlying the maintainance of LTP once elicited. However, it also suggests that endogenous TrkB ligands act as a permissive factor on this form of synaptic plasticity. But this is not true for de-depression in which is completely blocked by blockade of TrkB ligands. We suggest that, during an early phase of postnatal development, BDNF instructs visual cortical synapses with the capacity to reverse a long-term change of synaptic efficacy in response to specific electrical stimuli.

BDNF EXPRESSION IN DEVELOPING VISUAL CORTEX IS REGULATED BY VISUAL EXPERIENCE

Since electrophysiological data have shown that BDNF is able to control different forms of synaptic plasticity (Akaneya et al., 1997, Korte et al., 1995, 1996), segregation of LGN inputs in ocular dominance columns (Cabelli et al., 1995) and ocular dominance distribution (Lodovichi et al., 2000) in primary visual cortex, we decided to investigate whether BDNF and its receptors are expressed in the proper sites of the visual cortex and at the right time.

BDNF expression is regulated during postnatal development

We looked at the distribution of BDNF in the rat visual cortex by using an immunohistochemical approach.

Much it is known about the distribution of BDNF mRNA in the visual cortex during both postnatal development and adulthood. Before eye opening, the total level of BDNF mRNA is low (Bozzi et al., 1995) and neurons expressing BDNF mRNA are evenly distributed throughout the cortical layers (Capsoni et al., 1999a). After eye opening, BDNF mRNA levels increase (Bozzi et al., 1995) while the number of neurons expressing BDNF mRNA in layer IV and V decreases (Capsoni et al., 1999a). Alteration of normal visual experience influences the endogenous level of BDNF mRNA in the visual cortex (Castren et al., 1992; Bozzi et al., 1995; Schoups et al., 1995; Capsoni et al., 1999a, 1999b). In particular, deprivation of visual experience from birth (dark rearing) induces a reduction: in neurons of layers IV and V, BDNF mRNA is reduced but distributed in the great majority of neurons (Capsoni et al., 1999a). These studies indicate that cortical expression of BDNF mRNA undergoes developmental changes and is regulated by visual experience. In general, in the rat visual cortex, it appears that the endogenous level of BDNF mRNA increases during the first three postnatal weeks, reaching a plateau around P23-30. The cellular expression also changes during the same postnatal period: before eye opening BDNF mRNA is distributed in almost all neurons but at very low level of staining, while after eye opening there two opposite processes: i) in supragranular layers the intensity of staining is higher with respect to earlier ages, ii) in a subgroup of neurons in layers IV and V mRNA becomes undetectable determining the reduction of the number of cells having the machinery to produce BDNF. However, whether developmental changes of BDNF mRNA result in actual changes of the protein is an unanswered question. This is crucial because it is possible that mRNAs and proteins are differently regulated by neuronal activity. Indeed, BDNF mRNA and protein levels have been shown to be regulated differently after kainate-induced seizures

in the hippocampus (Wetmore et al., 1994).

Several studies have analyzed the distribution of BDNF protein in the visual cortex of adult animals (Kawamoto et al., 1996; Conner et al., 1997; Yan et al., 1997; Furukawa et al., 1998). In rat visual cortex BDNF is prevalent in layers II-III and V. At the cellular level, most pyramidal neurons in different layers are stained. Inhibitory interneurons are also immunopositive for BDNF, but there is evidence that at least some of them are not able to produce BDNF themselves ,but instead express BDNF receptors on cell membrane and take up BDNF from the environment (Cellerino et al.,1996).

Studies on BDNF protein in developing visual cortex are less prevalent (Rossi et al., 1999). In our study we showed that the total amount of BDNF protein and its cellular distribution undergo developmental changes. In particular, we provide evidence that in all cortical layers of primary visual cortex the amount of protein increases between P13, i.e. before eye opening, and P23 as measured by ELISA and the percentage of immunopositive neurons. This process is particularly pronounced in layer IV, the principal target of thalamic afferents.

We are confident that the differences in BCI are not due to changes in the penetration of the antibody. The issue is that penetration of antibodies decreases with increasing postnatal age. However, if this were the case, more labeled cells should be observed in younger animals than in older ones; on the contrary we observed a higher number of labeled cells at P23 compared to P13 rats.

After P23, the total amount of protein in layer IV and infragranular layers decreased to levels lower than at P13 while the number of BDNF immunopositive neurons is higher in adult animals than P13 animals. It has to be noted that the ELISA assay measures not only the intracellular content of BDNF in neurons but also the total amount of the protein in the tissue, thus taking into account BDNF

trafficking and BDNF contained in glial cells. It is possible that during postnatal development release and/or uptake of BDNF could be differently regulated with respect to protein synthesis, thus accounting for the discrepancy between cellular expression and total amount of BDNF.

BDNF expression is regulated by visual experience

Since BDNF seems to be involved in activity-dependent development and plasticity it is important to know whether BDNF cellular expression is influenced by light. To this aim we deprived animals of light following two different protocols. One group of rats was reared in the dark from birth to P23 and the second group from P13 (immediately before eye opening) to P23. It has been reported that dark rearing from birth may result in altered development of cortical circuitry and to avoid this undesired effect, we used a shorter period of dark rearing starting immediately before eye opening. The results obtained by two protocols were almost identical. In both cases visual deprivation reduced the percentage of neurons expressing BDNF in all cortical layers. Thus, in the visual cortex the cellular expression of BDNF during postnatal development is influenced by the absence of light. The second conclusion is that dark rearing effects are distributed in all cortical layers without major differences. This might seem surprising since the effects of dark rearing are expected to be more prominent in layer IV which is where most of LGN fibers terminate. A possible explanation for this result is that, using our approach, we are looking at chronic effects of visual deprivation which possibly are distributed throughout all cortical layers. It would be nice to prove that a brief period of visual deprivation induces stronger effects in cortical layer IV.

To further test whether light is controlling the development of BDNF

cellular expression we exposed dark reared rats to a brief period illumination (two hours). The aim was two fold: i) to clarify if a period of illumination was able to accumulate BDNF into cells and therefore to restore the normal percentage of cells expressing BDNF; ii) to avoid possible interference with accumulation of BDNF depending from anterograde or retrograde transport. Indeed, it is extremely unlikely that in such short period intracellular transport of BDNF can alter its cytoplasmatic level. The results were clear and indicate that two hours of light is a period sufficient to restore the normal cellular expression of BDNF by increasing its synthesis.

The emerging evidence is that BDNF protein, in addition to BDNF mRNA, is regulated by light during postnatal development in the rat visual cortex.

Mismatch between BDNF mRNA and protein expression in the developing visual cortex.

A consistent observation is that before eye opening many neurons contain BDNF mRNA but not the protein. After eye opening almost all neurons containing BDNF mRNA co-express BDNF protein. Comparing the percentage of neurons containing BDNF protein in each cortical layer with the percentage of neurons expressing BDNF mRNA (obtained by our group by using a BDNF riboprobe, Capsoni et al., 1999 a,b), we showed that before eye opening neurons expressing BDNF mRNA are more numerous than those containing BDNF protein in all cortical layers. Together these results suggest that at an early stage of postnatal development there is a mismatch between BDNF mRNA and protein distribution within visual cortical neurons. After eye opening the percentage of BDNF immunopositive neurons increases and matches the percentage of neurons expressing BDNF mRNA in all cortical layers except layer V. Indeed, in adult

animals layer V BDNF immunopositive neurons are more than those expressing BDNF mRNA. This could be due to an increase in the number of cells able to take up and retrogradely transport BDNF but not to synthesize it. For example, it has been shown that parvalbuminergic neurons of the adult visual cortex are immunopositive for BDNF and express its receptor TrkB, but do not contain BDNF mRNA (Cellerino et al., 1996). In the present study we confirm this idea, showing that in our experimental conditions parvalbuminergic neurons also contain BDNF protein.

One possibility to explain the observed mismatch is that at an early stage of postnatal development, before eye opening, BDNF protein might disappear from cell perikaria to be rapidly targeted to dendrites or axons for release. This hypothesis is unlikely, since i) targeting of BDNF to neuronal processes, in particular dendrites, requires high neuronal activity, a situation opposite to that present before eye opening and in dark reared rats, ii) targeting of BDNF to neuronal processes does not lead to BDNF disappearance from neuronal somata.

A model consistent with the results reported here is that at an early stage of postnatal development, in many visual cortical neurons, BDNF mRNA is expressed at a low level, leading to BDNF accumulation below the detection threshold for immunohistochemistry. It has been previously reported that before eye opening almost all neurons throughout the cortical layers express BDNF mRNA but at a low level (Capsoni et al., 1999 a). After eye opening, neuronal activity triggered by visual input would shape the cortical circuitry expressing the BDNF mRNA. Indeed, the proportion of neurons expressing BDNF mRNA decreases in layers IV and V (Capsoni et al., 1999 a). In addition the intracellular level of BDNF mRNA increases. Thus, neurons which continue to express BDNF mRNA after eye opening express it at a high intracellular level, and therefore the

protein within these neurons can be detected. However, we can not eliminate the additional possibility that at least part of the increase of the cellular content of BDNF could be due to take up and internalization of the protein by neurons expressing BDNF receptors (Cellerino et al., 1996).

Several previous papers (Bozzi et al., 1995, Castren et al. 1992, Schoups et al., 1995) as well as studies performed in our laboratory have shown that the total amount of BDNF mRNA increases during development, while the number of cells that are labeled for this molecule decreases in the same period. This apparent discrepancy can simply be explained by assuming that before eye opening cortical cells have not yet imprinted by visual input. We suggest that at this immature stage, a low level of BDNF is necessary to maintain malleable cortical connections capable of responding to dynamic changes occurring in the environment. When visual input comes, this modulates cortical circuitry expressing BDNF. On this view visual cortical neurons receiving strong visual input synthesize more BDNF mRNA while those receiving faint input stop producing BDNF mRNA. This hypothesis predicts that electrical stimulation of LGN fibers or of optic nerve fibers changes the expression of BDNF mRNA when the rat's eyes are still closed.

For BDNF protein expression a similar behavior can be hypothesized. Before eye opening many cells produce BDNF mRNA, but not all of them translate it into protein. When visual input comes, BDNA mRNA increases and as a consequence more protein is synthesized (Fig.21).

We previously mentioned that in the visual cortex there is at least a subgroup of GABAergic cells, namely the parvalbuminergic cells, that are able to take up BDNF but not to synthesize it: in these neurons BDNF is due to internalization from the environment. Future experiments should clarify whether

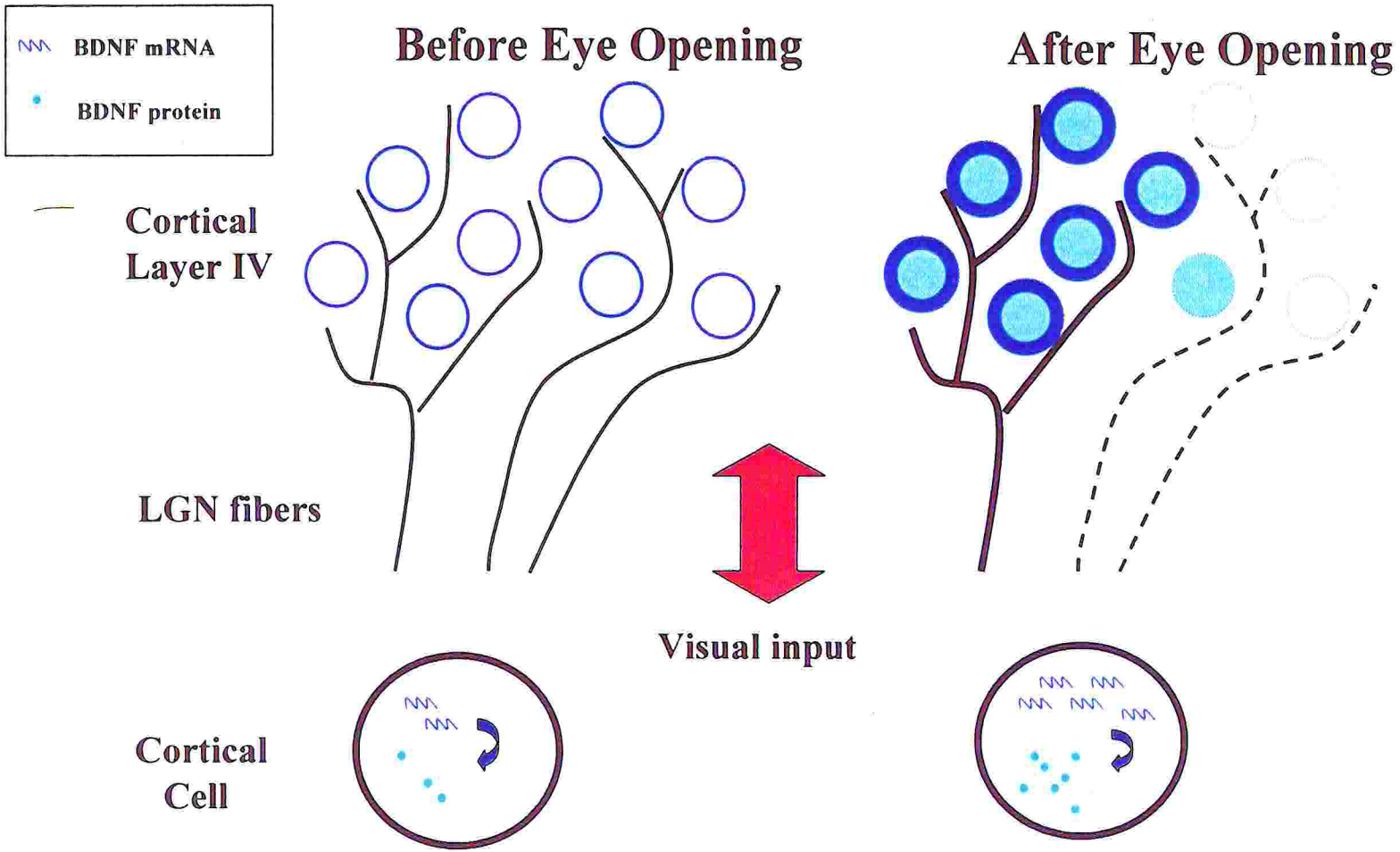
BDNF mRNA
BDNF protein
Before Eye Opening
After Eye Opening
Cortical Layer IV
LGN fibers
Visual input
Cortical Cell

Fig. 21: Model for BDNF action.

In this scheme LGN fibers and cortical cells are shown before and after eye opening. Top: LGN fibers contact cortical cells in layer IV. Circles represent the amount of BDNF mRNA produced in the cell, while the amount of protein contained in the cells corresponds to the filling. Before eye opening most of the cells contain low levels of BDNF mRNA while fewer cells contain BDNF protein. When visual input occurs fibers that carry the electrical signal induce the stimulated cells to produce more BDNF mRNA and to express BDNF protein; on the other hand inactive fibers lead cortical cells to stop BDNF mRNA expression, so the protein contained in these cells comes only from the external environment. Bottom: intracellular content of BDNF mRNA and protein.

After eye opening there are fewer cells stained for BDNF mRNA, but the total amount of the molecule is increased. Visual input also induces an increase in the production of BDNF protein, so cells that contain mRNA start to translate it into protein

the internalization and/or transport of BDNF are also regulated by light in addition to BDNF synthesis.

In agreement with the hypothesis that visual experience controls the cellular content of BDNF mRNA and protein we reported that in rats deprived of light from birth there is an high percentage of neurons expressing BDNF mRNA but not the protein; a situation which is similar to that present before eye opening. In addition, in many visual cortical neurons of dark reared rats BDNF mRNA but not the protein was detected, thus reproducing the mismatch observed before eye opening. Re-exposition of dark reared rats to a brief period of light (two hours) was sufficient to restore a normal percentage of neurons with a detectable level of BDNF protein and to re-establish a good match between neurons expressing BDNF mRNA and neurons immunolabelled for BDNF.

It is particularly interesting that two hours of restoration are sufficient to restore the cellular expression of BDNF protein, but not that of BDNF mRNA. This suggests that, although BDNF behaves as an immediate early gene, visual input is also able to rapidly regulate protein expression. It is unlikely that anterograde or retrograde transport of BDNF taken up from the external environment has a part in these rapid changes of BDNF protein. Indeed cellular transport is an inefficient system for accumulating protein in brief time periods (Foletti et al., 1999).

An intriguing possibility is that visual experience, in addition to controlling the cellular level of BDNF mRNA, might regulate the efficiency of BDNF mRNA translation and therefore the cellular accumulation of protein. Consistent with this idea, both before eye opening and in dark reared animals BDNF mRNA is expressed at a low level and is poorly translated into the protein. After eye opening visual input regulates the expression of BDNF mRNA and

increases its translation into the protein. It is also possible that BDNF mRNA and protein synthesis are differently regulated by visual experience. Indeed, we showed that in dark reared rats two hours of light are sufficient to restore a normal percentage of neurons containing BDNF protein while the process of restoring BDNF mRNA takes longer. It is noteworthy that regulation of translational efficiency by visual experience has been shown to occur in the visual cortex for Ca^{++} calmodulin-dipendent kinase (Wu et al., 1998).

A third possibility is that the mismatch between BDNF mRNA and protein could be due to differences in the turnover of BDNF mRNA, in the degradation rate of BDNF protein, or some combination of the two. For example, in our experimental conditions, before eye opening and in dark reared rats, the BDNF protein might be rapidly degraded, thus leading to a decreased cellular content of BDNF. For this reason the number of immunopositive neurons could be lower at P13 than at P23 and P90.

Moreover it has to be considered that the mRNA probe used in the present study recognizes all the splicing forms expressed in the visual cortex. In the hippocampus not all mRNA splicing forms lead to BDNF synthesis (Metsis et al., 1993). Thus, it is possible that a differential regulation of the various splicing forms involved in BDNF synthesis could account for the mismatch between BDNF mRNA and its protein. A corollary of this hypothesis is that different splicing forms might have distinct turnover properties, targeting to subcellular compartments, or translational competence. However, the role of different splicing forms in the differential regulation of BDNF translation or targeting must be investigated by using different experimental assays. Some of the experiments required are in progress in our laboratory.

TRKB RECEPTORS ARE REGULATED DURING POSTNATAL DEVELOPMENT. VISUAL DEPRIVATION DOES NOT AFFECT THE DEVELOPMENTAL PATTERN OF TRKB.

Since neurotrophins act on neurons by binding to specific receptors expressed on cell membranes, it was important to study the distribution of TrkB, the receptor of BDNF, in the visual cortex, considering both the total amount of the protein and its cellular expression. TrkB receptors are produced primarily as full length receptor tyrosine kinases. They, as well as other neurotrophin receptors, also exist in a truncated form. Two truncated isoforms for TrkB are expressed in the CNS, TrkB-T1 and –T2 isoforms, which bind ligands but lack most of the intracellular portion. In the present work we used one antibody recognizing the full length form of TrkB and a second antibody recognizing both the truncated forms of TrkB.

A few studies have been performed on TrkB receptors. Cabelli et al. (1996) reported that in the ferret visual cortex, the number of TrkB positively stained cells increases during postnatal development. A study on the total content of both the full length form of the receptor, and the truncated form were carried out by Allendoerfer et al., (1994). In developing ferret visual cortex starting from E30 to adulthood. For detection of TrkB receptors, they used the cross-link with iodinated neurotrophins: this technique measures directly the amount of neurotrophin linked to the receptors and indirectly receptor concentrations. They reported that the amount of the truncated form of the receptor increases during postnatal development, while the ratio of the full length to the truncated form decreases.

Concerning cellular localization, the two forms of TrkB receptors have

been found in both pyramidal cells and interneurons, thus allowing its ligand to act on both excitatory and inhibitory circuitry (Cabelli etal., 1996; Cellerino et al., 1996). In particular immunoreactivity for the full-length form was present in some interneuron axon initial segments and in axon terminals that formed inhibitory - type synapses characteristic of GABAergic, cholinergic, and monoaminergic terminals. (Drake et al., 1999). The presence of TrkB on parvalbuminergic neurons (Cellerino et al., 1996) suggests that TrkB ligands are able to modulate the acivity of inhibitory, as well excitatory neurons.

In the rat visual cortex we found that the levels of both the full length and the truncated form of the TrkB receptor increase during postnatal development. We observed a parallel increase of both forms of the receptors, suggesting they are regulated in the same way. However, we cannot say if the ratio between the two forms changes and therefore we are unable to confirm the results by Allaendoerfer et al.(1994) in the rat visual cortex.

The pattern of cellular distribution of TrkB receptors also changes during postnatal development. Before eye opening, immunostained cells are localized in layers II-III and V while later in development they are distributed throughout all cortical layers.

The cellular distribution of truncated and full length form of TrkB throughout the cortical layers is almost identical at all postnatal ages, suggesting that the two forms are expressed within the same neurons. This is supported by results obtained in double staining experiments showing that some cells co-express both forms of TrkB. These results are in accordance with those reported in previous papers (Rudge et al., 1994; Armanini et al., 1995; Wetmore and Olson, 1995). This study showed that full length and truncated TrkB are co-expressed within the same neurons in different areas of the CNS.

Several hypotheses have been proposed to explain the functional relevance of the truncated forms of TrkB. Here we discuss the most relevant.

The first hypothesis postulates that TrkB truncated receptors act as a nonfunctional sink for ligands. According to this hypothesis, truncated TrkB serves to limit the action of TrkB ligands during development. In this regard, Knusel et al. (1994) have demonstrated a progressive reduction of auto-phosphorylation of the full-length TrkB protein in response to BDNF as development proceeds, suggesting that an increase in truncated TrkB, as shown by Allaendoerfer et al, (1994) would account for such result.

In an experiment performed on the Xenopus oocyte (Eide et al., 1996) it has been shown that the truncated form of TrkB inhibits the intracellular cascade of events induced by BDNF binding. In fact, while the expression of full length TrkB was able to elicit Ca^{++} efflux responses after stimulation by BDNF, the induced co-expression of truncated TrkB in these cells completely prevented this effect.

Thus there is increasing evidence that truncated TrkB oppose the normal intracellular cascade of events occuring when neurotrophin binds to the full length form. This is consistent with evidence that neurotrophins induce the intracellular cascade of events only when TrkB homodimers are expressed on cell membrane. The presence of a truncated form of the receptor opens the possibility that functionless TrkB heterodimers can be inserted into the cell membrane. Thus, functional homodimers and functionless heterodimers can be present within the same neurons in this way modulating the response to BDNF and/or NT-4.

A variant of the previous hypothesis takes into account a possible role of truncated TrkB in spatially restricting the access of BDNF to the full length receptors.

Studies performed in adult rat hippocampus have shown that the TrkB truncated receptors are mainly distributed in the somata and the dendritic shafts. In contrast, thr full length TrkB receptors appear predominantly to be concentrated in axons, terminals, and dendritic spines (Drake et al., 1999). Some hypotheses concerning TrkB actions have been proposed in relation to its ultrastructural localization: truncated receptors may limit the diffusion of TrkB ligands (Bothwell et al., 1995), preventing access to distant terminals and/or spines. Although data on the subcellular localization of TrkB during development are not available, the developmental increase in the ratio of truncated to full length TrkB (Allendoerfer et al., 1994) is consistent with the idea that truncated TrkB restricts access to TrkB ligands during maturation. The subcellular localization of TrkB changes as a function of the level and possibly of pattern of neuronal activity. In cultured retinal neurons, excitatory stimuli (depolarization or cAMP elevation) trigger the translocation of full length TrkB from cytoplasm to plasma membrane (Meyer-Franke et al., 1998). This could represent a mechanism for relatively rapid enhancement of postsynaptic sensitivity to incoming signals.

A recent paper by Biffo et al. (1995) proposed a different role for truncated TrkB. They showed that truncated TrkB takes BDNF into the cell after external binding, possibly releasing BDNF at different intracellular sites, thus activating other intracellular pathways that modulate, but do not necessarily oppose, normal BDNF action.

An intriguing finding in our work is that BDNF expression is modulated by visual experience, while neither full length TrkB, nor its truncated form is affected by visual deprivation. This is true both for the endogenous level of TrkB receptors and for their cellular expression. This result can be explained in different ways: first, TrkB is the high affinity receptor not only for BDNF, but

also for NT-4. Thus it is possible that NT-4 expression, or that the combination of NT-4 and BDNF expression, require the permanent expression of their receptor. Although this hypothesis cannot be ruled out, a recent study by Minichiello et al. (1998) has shown that the intracellular pathways activated by the two neurotrophins are different.

Second, it can be less expensive, for the system, to modulate only one factor, in this case, the ligand, than both the ligand and its receptors.

Differential regulation of ligand and its receptor has been observed at the mRNA level in the rat hippocampus (Mudo et al., 1996). The authors observed that induction of status epilepticus induced an increase in the mRNA levels of NGF, but no change was observed in TrkA mRNA levels. In visual cortex Bozzi et al. (1995) observed that monocular deprivation induced a decrease in BDNF mRNA levels with no change in TrkB expression.

MODELS FOR BDNF ACTION AND CRITICAL ISSUES

Neurotrophins are a family of secreted proteins known for their ability to regulate the survival and differentiation of nerve cells. Recent evidence indicates that neurotrophins are also involved in neuronal plasticity.

In 1991 it was proposed that in the CNS neurotrophins act to regulate neuronal plasticity (Domenici et al., 1991). The authors tested this hypothesis in the visual system using the classical paradigm of monocular deprivation during the critical period, proposed by Hubel and Wiesel during early 60s. They showed that NGF supplied into the lateral ventricle is able to prevent the functional and morphological effects of monocular deprivation such as the shift of ocular dominance towards the undeprived eye, the lost of acuity of the deprived eye and the shrinkage of LGN neurons receiving input from the deprived eye. The idea was that LGN fibers compete for NGF released by cortical neurons the synthesis and/or release of which is under the control of neuronal activity. To show that NGF regulates activity-dependent plasticity of the geniculo-cortical system it is necessary to demonstrate that NGF is controlled by neuronal activity and that TrkA, the NGF receptor is present in LGN neurons. In the visual cortex NGF is partially controlled by light but TrkA is not expressed or expressed at an undetectable level in LGN neurons.

More recently, it has been reported that an exogenous supply of BDNF can prevent the formation of ocular dominance columns. In addition, NT-4, provided by local cortical injections, attenuates the shrinkage of LGN neuron cell bodies receiving an input from the deprived eye. Moreover, in organotypic slices of visual cortex, McAllister et al. (1995; 1996) showed that BDNF controls the dendritic growth of neurons in cortical layer IV. These results lead to the

hypothesis that BDNF and possibly NT-4 play a fundamental role in activity-dependent segregation of LGN fibers and, more generally in the postnatal development of visual cortex. However, as in the case of NGF, BDNF must satisfy specific requisites which are essential to demonstrate its role in activity-dependent plasticity of the geniculo-cortical system. In the present thesis we have reported that BDNF and its receptors satisfy the following:

•BDNF regulates forms of long term synaptic plasticity in the developing visual cortex, including a new form described in the present work;

• BDNF is present in the visual cortex and its expression changes during postnatal development;

• expression of BDNF mRNA and protein is regulated by visual input;

• BDNF receptors are present in the visual cortex and their expression change during postnatal development.

These results suggest that BDNF is a likely candidate to link neuronal activity with developmental plasticity, although some crucial issues are still unclear. In the following paragraphs some of these issues including possible model of BDNF action, will be briefly discussed.

The evidence that BDNF is involved in geniculo-cortical plasticity has been gained by using prolonged application of ligands. This implies functional and structural rearrangement of geniculo-cortical or cortical circuitry over a period of days, on a similar time scale to effects induced by neurotrophins on neuronal survival and differentiation. It appears difficult to reconcile the slow in vivo effects of BDNF with the rapid changes observed in cell cultures and in vitro slices. For example neurotrophins can acutely modulate synaptic processes by acting on the secretion of neurotransmitters (Carmignoto et al., 1997) and can cause membrane depolarization (Kafitz et al., 1999). These processes are

regulated on a timescale of milliseconds to minutes. Also, forms of synaptic plasticity such as LTP and the new form of synaptic plasticity described in the present work are regulated by BDNF within minutes to hours. Difference in time/scale of BDNF action could also imply different mechanisms and this makes it difficult to translate in vivo effects into cellular models of BDNF action on synaptic plasticity and neuronal function.

Concerning synaptic plasticity, more effort must be devoted to clarify the relation between long term synaptic plasticity, neuronal plasticity induced by monocular deprivation and developmental rearrangements of visual cortical circuitry. For this aim it is important to clarify whether the different forms of long term synaptic plasticity are timely related with the whole critical period or with a specific phase of the critical period to understand the link between synaptic and rearrangement of visual cortical circuitry. De-depression is selectively expressed during an early phase of postnatal development under the control of visual experience. Also LTP is expressed only during the critical period. Beyond the evidence that some forms of synaptic plasticity studied *in vitro* are timely related with specific phases of postnatal development the evidence is still lacking that the same forms are expressed in the developing visual cortex of freely moving animals.

At a cellular level it is not yet clear if BDNF regulates synaptic plasticity, in particular the de-depression form, by acting at pre-synaptic and/or post-synaptic sites. It will be important to further investigate whether the release of neurotransmitters such as glutamate and/or modifications of glutamatergic and GABAergic receptors are involved in the mechanisms underlying synaptic strengthening driven by BDNF.

The presence of BDNF mRNA and protein in the rat visual cortex during

its postnatal development, and the finding that visual experience does regulate the global level and the cellular expression of both BDNF mRNA and protein, clearly suggest that BDNF is involved in activity-dependent refinement of cortical circuitry. An important question is whether activity regulation of BDNF mRNA occurs rapidly and at the right subcellular domain. In the present work we have shown that BDNF synthesis is regulated by light on a temporal scale of 1-2 hours. This opens the possibility that intracellular BDNF content may rapidly change upon arrival of the appropriate input. Interestingly, recent work by Tongiorgi et al. (1997) and Righi et al., (2000) has shown that BDNF mRNA is targeted towards dendrites and that this dendritic targeting is controlled by neuronal activity. Thus, synthesis and targeting of BDNF can be regulated by neuronal activity on a time scale compatible with the temporal changes of synaptic efficacy. It is conceivable that BDNF mRNA can be targeted to synaptic domains and rapidly translated there upon arrival of specific inputs, following the model reported in Fig.22.

The present model could also help to explain how neuronal activity is translated into long term changes of synaptic efficacy. Post-synaptic sites receiving inputs which result in coordinated activity of pre- and post-synaptic elements would activate the machinery leading to rapid synthesis and release of BDNF and this could be a critical step for synapse strengthening. A recent report showed that LTP elicited by high frequency stimulation in hippocampus is associated with an increasing BDNF expression (Castren et al.,1998). (For a general scheme of cellular BDNF action see Fig. 23.)

Concerning TrkB receptors, we have reported that both the full length and the truncated form change in the visual cortex during postnatal development but that their expression is not regulated by visual input. These results indicate that TrkB receptors are at the right sites to mediate the action of BDNF during

mRNA
protein

Fig. 22: Scheme of dendritic targeting after stimulation.

External arrows indicate points of stimulation. Stimulation is an input that produces long-lasting changes i.e., a high frequency stimulation train.

After stimulation mRNA is transported to the terminals where it can be translated into protein.

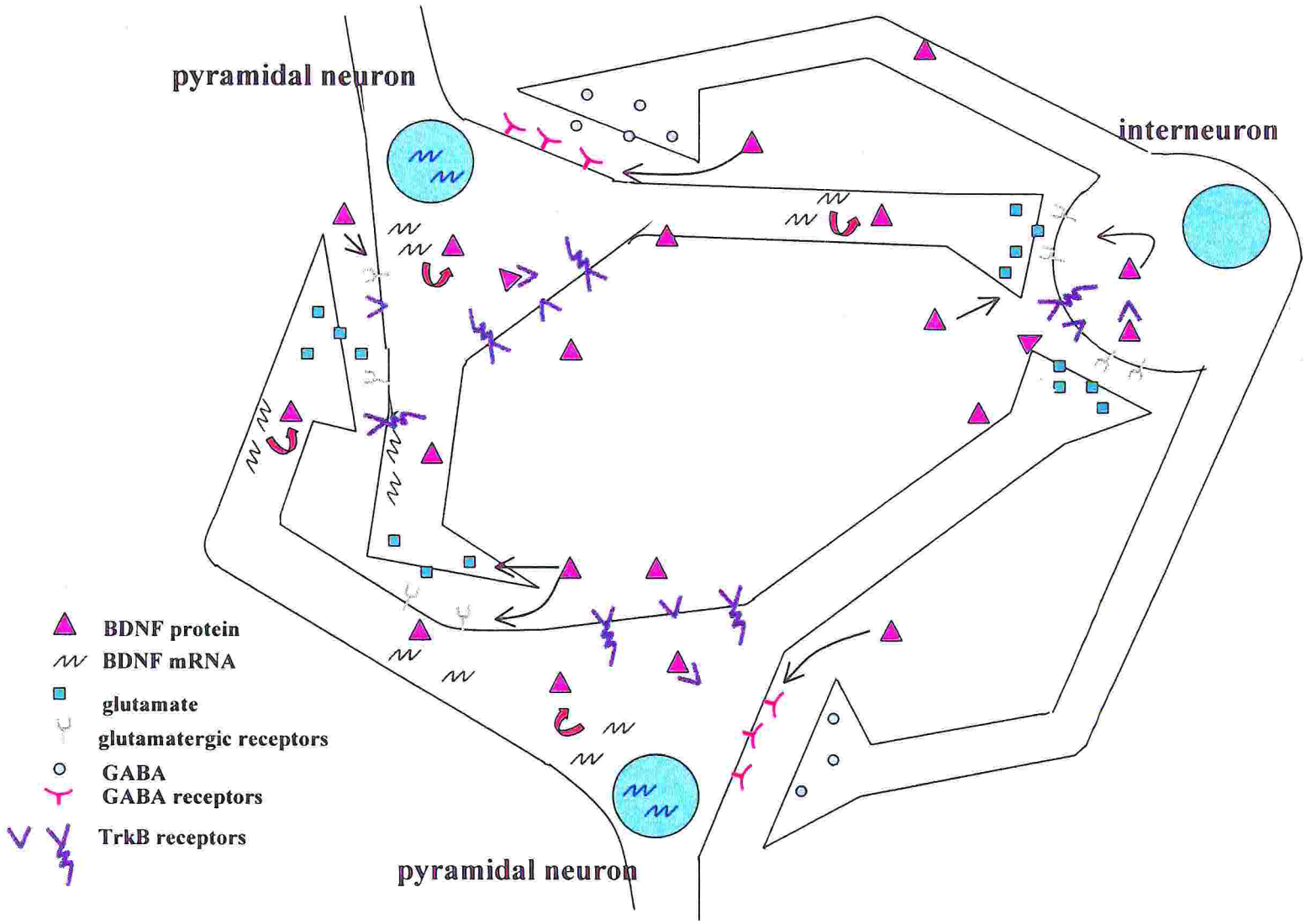
pyramidal neuron
interneuron
pyramidal neuron
BDNF protein
BDNF mRNA
glutamate
glutamatergic receptors
GABA
GABA receptors
TrkB receptors

Fig. 23: Summary scheme of cellular BDNF action.

BDNF mRNA is produced by pyramidal neurons and translated into protein both in soma and dendrites. BDNF protein is released in the extracellular environment where it binds to TrkB receptors, both in the full length and in the truncated form. BDNF can be internalized through truncated Trk receptors, so it can be present also in interneurons, which do not produce BDNF mRNA. The binding of BDNF to its receptors influences the release of neurotransmitters such as glutamate and modulates the electrical properties of glutamatergic and GABA receptors.

postnatal development. TrkB receptors are expressed in visual cortical neurons (present work) and in LGN neurons (Cabelli et al., 1996). It is quite possible that TrkB signalling may be involved in many functions in developing visual cortex. For example, TrkB ligands could influence the segregation of LGN inputs and this could explain the results obtained on both segregation of ocular dominance columns (Cabelli et al.,1996) and ocular dominance plasticity (Lodovichi et al., 2000).Unfortunately, it is not yet known if BDNF can act directly on LGN neurons.

In the visual cortex TrkB is expressed in both pyramidal neurons and interneurons (Cellerino et al., 1996) in addition to LGN fibers. Beyond obvious implication for the balance between excitation and inhibition within the cortical network, this observation also raises several questions concerning the interpretation of results obtained by using high doses and prolonged application of BDNF. Indeed, it is possible that prolonged application of BDNF, in addition to influencing the segregation of geniculate fibers, could alter the maturation of glutamatergic and GABAergic circuitry. Interestingly, it has recently been reported that over-expression of BDNF in transgenic mice accelerates the maturation of GABAergic circuitry (Huang et al., 1999). This could be the reason why monocular deprivation effects do not occur in these animals (Hanover et al., 1999). Thus, it seems important to differentiate BDNF action in the visual cortex as a function of its neuronal target.

An additional question is whether TrkB, in addition to binding ligands and activating the intracellular cascade of events, could also control the local action of BDNF and perhaps NT-4. One possibility is that TrkB in the visual cortex, and possibly also in LGN fibers, is regulated in an activity dependent way. Following this hypothesis LGN fibers characterized by high electrical activity would express

high levels of TrkB and in this way BDNF released by cortical neurons reward the most active fibers. However, the result that TrkB receptors in the visual cortex are not influenced by visual input does not support such possibility. But we can not eliminate completely the possibility that TrkB signalling could also be regulated at the pre-synaptic site. Indeed, active fibers should switch on an activity-dependent intracellular mechanism interacting with the cascade activated by BDNF and to synaptic strengthening. In this case, the amount of TrkB receptors on active and non-active fibers would be the same but the response of cells would be regulated at the pre-synaptic site. Anyway experimental evidence for this model is still lacking.

The described models of BDNF action on neuronal plasticity and activity dependent development assume that BDNF is synthesized and released by cortical neurons acting as a retrograde message to afferent fibers. Recent findings provide evidence that BDNF is anterogradely transported in several areas of the CNS (Altar et al., 1997; Yan et al., 1997) and the PNS (Zhou and Rish, 1996; Michael et al., 1997). This anterograde transport could be important for the trafficking of BDNF and may constitute an important means for regulating synaptic strength. However, it is essential to demonstrate that BDNF is anterogradely transported by LGN neurons and/or intracortical neurons of the visual cortex and to demonstrate that anterogradely transported BDNF is capable of being released to activate target cells expressing BDNF receptors.

These and others issues need to be addressed in order to understand how BDNF participates in various forms of synaptic plasticity. Nevertheless, about 40 years after the experiments of Hubel and Wiesel, after many efforts to clarify the cellular mechanisms of visual cortical plasticity, at the moment there are good models to explain these processes, but more molecular investigations are needed.

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