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MODULATION OF CORTICAL SYNAPTIC PLASTICITY BY THE CHOLINERGIC SYSTEM AND NERVE-GROWTH FACTOR

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ABSTRACT

Long lasting modifications in the efficacy of synaptic transmission among neurons are thought to be the basic changes that account for complex processes such as learning and memory. Thus, the comprehension of the mechanisms and factors controlling synaptic plasticity is fundamental to understand higher cognitive functions. The aim of the present work was to investigate possible factors modulating synaptic plasticity in the cortex. One likely candidate is the cholinergic system, arising in the basal forebrain (BFB) and projecting to the cortex, whose deficits are known to impair cognitive functions. It has been found that either disruption of cholinergic neurons or blockade of cholinergic transmission impair Long-Term Potentiation (LTP) in rat visual cortex slices. Conversely, activation of the cholinergic receptors has a facilitatory effect in synaptic strengthening. In addition, by using a transgenic mouse expressing an anti-NGF monoclonal antibody, it has been investigated the long-term effects of Nerve-Growth Factor (NGF) deprivation in cholinergic system functionality, for which NGF is known to exert a trophic action. In these mice, it has been observed an impairment of LTP that can be rescued by application of cholinomimetic drugs. Beside its action as trophic factor, NGF is known to exert a crucial role in the activity-dependent development and plasticity of visual cortex. This observation prompted me to investigate its possible effect as modulator of synaptic plasticity. It has been found that blocking the NGF-TrkA interaction rescues the developmental loss of LTP in the rat visual cortex. In contrast, an increase in the levels of NGF reduces the capability of synapses to be potentiated. Long-Term Depression and bidirectional plasticity are unaffected. These results indicate that both the cholinergic system and NGF are effective regulators of synaptic strength in the visual cortex. Moreover, the evidence that cholinergic antagonist avoids LTP rescue mediated by blockade of NGF-TrkA interaction and that BFB lesion masks the action of NGF on LTP expression suggest that NGF modulates LTP by means of the BFB cholinergic system.

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ABBREVIATIONS

AC = adenilate cyclase

ACh = acetylcholine

αTrkA = anti-TrkA monoclonal antibody

BDNF = brain-derived neurotrophic factor

BFB = basal forebrain

CaMKII = Ca2+-calmodulin-dependent protein kinase II

cAMP = cyclic AMP

ChAT = choline acetyltransferase

CNS = central nervous system

CRE = cAMP-response element

CREB protein = CRE-binding protein

Den.NGF = heat inactivated NGF

E21= embryonic day 21

GABA = γ-aminobutyric acid

GABAergic = neuron releasing GABA

GAD = glutamic acid decarboxylase

HFS = high-frequency stimulation

IP_3 = inositol 1,4,5-trisphosphate

LFS = low-frequency stimulation

LGN = lateral geniculate nucleus

LTD = Long-Term Depression

LTP = Long-Term Potentiation

mAChR = muscarinic acetylcholine receptor(s)

MD = monocular deprivation

nAChR = nicotinic acetylcholine receptor(s)

NGF = nerve growth factor

NMDA = N-methyl-D-aspartate

NT = neurotrophin(s)

P1 = postnatal day 1

PKA = protein kinase A

PLC = phospholipase C

PNS = peripheral nervous system

VAChT = vesicular acetylcholine transporter

WM = white matter

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1.0 INTRODUCTION

1.1 Plasticity

Long lasting modifications in the efficacy of synaptic transmission among neurons are usually referred as "plasticity". The modifications the synapses undergo during these processes span from metabolic modifications both at pre- and postsynaptic sites to structural modifications of the synapses themselves, comprising creation and elimination of synaptic contacts (Bliss and Collingridge 1993, Wang et al 1997, Luscher et al 2000). These plastic changes are supposed to be the basis for the storage of information in the central nervous system. However, the question whether the modifications in synaptic transmission are the basic units for complex processes such as learning and memory in the brain, is still far from being answered (Stevens 1998).

Neural network modeling suggests that memories can be stored by coherent modifications of synapses that may be distributed among many neurons. Based on this view, the representation of any given stimulus is encoded by a unique combination of responses across the neurons in the network, and this representation can overcome the loss of individual neurons. In general, the pattern of connections encoding for memories is composed of both increased and decreased effectiveness in synaptic transmission among neurons (Bear 1996). Modeling of stimulus selectivity led to formulation of a learning rule known as the BCM algorithm (Bienenstock et al 1982). This algorithm states that active synapses can be potentiated or depressed when the total postsynaptic response exceeds or non-exceeds a critical value called "modification threshold" (θm), respectively. Moreover, if the value of θm is allowed to vary as a nonlinear function of the average postsynaptic activity, the cell response will evolve to a stable state regardless of the initial condition. This and other theoretical studies pointed out that depression, as well as potentiation, of synaptic transmission should take place in order for adaptive or plastic neuronal circuits to operate effectively (Sejnowski 1977, Bienenstock et al 1982). The experimental support to the hypothesis that synapses are bidirectionally modifiable was provided by both *in vivo* and *in vitro* studies (Bear 1996). Thus, the term plasticity accounts

for either strengthening or weakening of synaptic transmission, but in the present study I has mainly focused on the potentiation of synaptic transmission.

The first sensory system studied for developmental plasticity was the visual cortex of kitten, where manipulation of visual input during a restricted time window of early postnatal development (called the critical period), causes a dramatic change in cortical connectivity (Hubel and Wiesel 1970, 1998). Similarly, although in the rat visual cortex is not found an anatomical segregation of neurons such as the ocular dominance columns seen in cats and primates, neurons in rat visual cortex do have specific functional properties and exhibit a certain degree of eye preference, as well as monocular deprivation (MD) during the critical period shifts the response of neurons in the binocular region toward the open eye (Fagiolini et al 1994, Gordon and Stryker 1996b). Moreover, it was shown that dark rearing interferes with normal development of cat and rat visual cortex, leading to the alteration of several functional properties of visual cortical neurons (Fregnac and Imbert 1984, Fagiolini et al 1994). Thus, in the visual system neural activity generated by both eyes during the critical period, drives the final pattern of neuronal connections (Katz and Shatz 1996). Nevertheless, recent findings suggest that intrinsic signals by themselves are responsible for the formation of ocular dominance columns in the visual cortex before the onset of visual activity (Crowley and Katz 2000).

Despite the great amount of knowledge acquired on visual cortical plasticity, the preferred *in vitro* preparation for studying plasticity is, for several reasons, the hippocampus (Bliss and Lomo 1973), and in particular the CA1 area (Bliss and Collingridge 1993). In the hippocampus, the Schaffer collateral commissural fibers form a well-defined pattern of axons, projecting to the apical dendrites of the CA1 pyramidal neurons. This pattern of connections allows evoking orthodromic monosynaptic potentials devoid of contamination by antidromic action potentials. Moreover, commissural fibers stimulation allows a reliable induction of an activity-dependent plasticity referred as Long-Term Potentiation (LTP) (Bliss and Collingridge 1993).

The neocortex, which should be the privileged site for learning and memory, reveals a much complex and undefined organization. In it, the thalamic input relies mainly on layer IV, and in turn, neurons of layer IV send projections to layer II/III as well as several extracortical modulatory inputs ascend parallel and intermingled to the axis of pyramidal neuron apical dendrites, making hard the selective stimulation of different afferent pathways. In addition, different layers are populated by different classes of neurons increasing the complexity of the cortical circuitry. Therefore, it is probable that in the neocortex heterosynaptic and associative

forms of plasticity take place (Brown et al 1990). It was shown that in the visual cortex an NMDA-dependent LTP can be obtained by stimulation of either the underling white matter (WM) (Artola and Singer 1987) or the layer IV (Kirkwood et al 1993), with the current sink peaking in the layer II/III, where it can be recorded extracellularly as a negative deflection of potential, which is polysynaptic in nature (Bode-Greuel et al 1987, Aizenman et al 1996). However, at least in rat, the expression of LTP is developmentally regulated disappearing at the age of 30-35 days, which almost coincide with the end of the so-called "critical period" (Perkins and Teyler 1988, Kato et al 1991, Kirkwood et al 1995). Blocking the GABAergic intracortical inhibitory system allows the rescue of LTP. In fact, LTP can still be induced in adulthood by perfusing slices with low doses of GABA receptor antagonist bicuculline (Artola and Singer 1987), or by local delivery of such antagonist (Kirkwood and Bear 1994). Conversely, an accelerated maturation of intracortical GABAergic system produced a precocious down regulation of LTP during development of mice visual cortex (Huang et al 1999).

LTP induction requires Ca^{2+} influx, mainly through the NMDA receptors (Madison et al 1991, Bliss and Collingridge 1993). The activation of metabotropic glutamate receptors (mGluRs) also seems to play a key role in synaptic strengthening (Bashir et al 1993, Bortolotto et al 1994, Anwyl 1999). A rise in intracellular Ca^{2+} triggers the activation of several second-messenger systems, such as protein kinase A (PKA), Ca^{2+}/calmodulin dependent protein kinase II (CaMKII), and the cyclic adenosine monophosphate (cAMP) pathways (Bliss and Collingridge 1993, Lismann et al 1997, Abel and Kandel 1998). These second-messengers induce short- and long-term intracellular modifications responsible for the enhancements of synaptic transmission. One of the long-term effects is activation of immediate early genes. For instance, activation of the cAMP-responsive element binding protein (CREB) by cAMP can induce the transcription of genes that contain a CRE site in their promoter region (Bourtchuladze et al 1994, Pham et al 1999), allowing the expression of genes responsible for the long lasting modifications that are hallmarks of plasticity.

Recent development of gene transfer technologies in mice opened the possibility to investigate the role of gene products possibly involved in plasticity. For example, the α-CaMKII knockout mice exhibit normal visual cortical responses, but the shift of neuronal selectivity for the open eye in monocular deprivation experiments is reduced in about half of the animals, and a similar fraction shows impaired spatial learning tasks (Gordon et al 1996a). Visual cortex LTP is reduced both in probability of expression and in magnitude (Kirkwood et al 1997). Mice

deficient for two isoforms of PKA appear normal in spatial and contextual learning tasks (Huang et al 1995), as well as in visual cortical responses; a normal shift is also seen in MD experiments. In contrast, *in vitro* synaptic plasticity assessed in visual cortical slices manifests total absence of LTP, Long-Term Depression (LTD) or paired pulse facilitation (Hensch et al 1998b). On the contrary, in mice lacking the 65 Kd isoform of glutamic acid decarboxylase (GAD), LTP and LTD are normal, despite the animals showed severe deficits in developmental ocular dominance plasticity (Hensch et al 1998a). Very recently, it was shown that in mice, in which the postnatal rise of BDNF level in the forebrain is genetically accelerated, both age-dependent decline of cortical LTP induced by WM stimulation and termination of the critical period for ocular dominance plasticity are accelerated at the same pace (Huang et al 1999). Taken together, these results support the idea that these molecules are involved in plastic phenomena to one side, but provide also evidence of at least partial mismatching between *in vivo* synaptic mechanisms of plasticity and the predictive role of *in vitro* models of synaptic plasticity, such as LTP and LTD. Nevertheless, it has to be considered that in complex systems, like organisms are, the knockout of a gene could be compensated by up or down regulation of other gene products, masking the role of the disrupted gene.

1.2 Neurotrophins

The neurotrophins (NT) comprise a family of related proteins, whose prototype is Nerve-Growth Factor (NGF), discovered more than 50 years ago (Levi-Montalcini 1951, Cohen 1960, Levi-Montalcini 1987). Since that time, other molecules of the same family were described. These are Brain-Derived Neurotrophic Factor (BDNF; Leibrock et al 1989), NT-3 (Hohn et al 1990, Maisonpierre et al 1990), NT-4/5 (Hallböök et al 1991, Berkemeier et al 1991) and possibly NT-6 (Götz et al 1994). NT are secretory proteins that share about 50% of homology in their aminoacidic sequence (Hallböök et al 1991); in particular, three disulfide bridges are conserved at the same positions (Acklin et al 1993). The NT form homodimers through noncovalent bonds, which are stable at low concentration (Bothwell and Shooter 1977, Arakawa et al 1994). The NGF monomer consists of three anti-parallel β strands forming a flat surface where conserved hydrophobic residues can interact with the corresponding aminoacids of the

adjacent monomer (McDonald et al 1991, McDonald and Hendrickson 1993). It was shown that also heterodimers can be formed, having mixed biological activities *in vitro*, but lower potency (Arakawa et al 1994, Jungbluth et al 1994). Whether such heterodimers are functional *in vivo* is not known. Sequence comparisons suggest that NT-3 could be the most conserved neurotrophin with respect to the ancestor gene (Barde 1994, Hallböök 1999). In particular, the replacement of some aminoacids in a peculiar location of NT-3 gives a molecule displaying all the biological activities of NGF, BDNF and NT-3 (Urfer et al 1994).

The receptors for neurotrophins can be divided into two groups according to their binding affinity properties (Chao and Hempstead 1995, Bothwell 1995). The first receptor to be cloned binds to all neurotrophins with low affinity, and it was called the p75 receptor (Johnson et al 1986, Radeke et al 1987). The second group comprises the Trk-family of NT receptors. In mammals, three different Trk receptors were identified: TrkA (Kaplan et al 1991, Klein et al 1991a), TrkB (Klein et al 1991b, Middlemas et al 1991), and TrkC (Cordon-Cardo et al 1991, Lamballe et al 1991), which bind NGF, BDNF and NT-4/5, and NT-3, respectively (Barbacid 1995, Bothwell 1995).

Studies in transfected cells suggested that p75 is able to accelerate the on-rate binding of NGF to the tyrosine kinase receptor TrkA, thus leading to the formation of high-affinity receptors (Mahadeo et al 1994). In the last few years several molecules related to the p75 receptor were identified. They are transmembrane proteins, which share common features such as cysteine-rich motifs in the extracellular domain, a single transmembrane domain and a short cytoplasmic domain referred to as the death domain, because its activation induces programmed cell death. Indeed, the intracellular domain of p75 is only distantly related to the death domain and there is no evidence, at this time, for a death signal transduction mediated by the p75 receptor (Feinstein et al 1995, Nagata 1997, Wallach 1997). However, there is an active role of p75 in programmed neuronal death, following interaction with NGF, in neurons lacking the co-expression of TrkA receptor (Van der Zee 1996, Frade and Barde 1999), that is, TrkA-NGF interaction would block the proapoptotic effect of NGF-p75 interaction.

The Trk receptor family comprises a group of transmembrane proteins that in the extracellular domain possess two cysteine-rich domains, one leucine-rich domain and two IgG-related domains, while the tyrosine kinase domain characterized the cytoplasmic region (Barbacid 1994). The interaction of these receptors with the proper neurotrophin leads to the autophosphorylation of the receptors and triggers a cascade of intracellular events responsible

for regulating cell morphology, growth and survival (Kaplan and Miller 2000). The genes coding for each Trk receptor can be alternatively spliced and they produce distinct isoforms with variable kinase domains as well as forms of the receptors TrkB and TrkC with truncated intracellular domain (Middlemas et al 1991, Valenzuela et al 1993). These truncated forms might have a role in modulating the local availability of neurotrophins, and it seems likely that they have other signaling functions as well.

In the mammal peripheral nervous system (PNS), different neurotrophins are required for the survival and differentiation of distinct subset of sensory neurons, and the functional identity of these neurons can be biased to a different phenotype depending on which neurotrophin is available. In mammalian central nervous system (CNS), many neurons have receptors for, and respond to, the neurotrophins. However, the effects of NT in regulating cell number that can be readily demonstrated in the PNS, are not evident in the CNS. Nevertheless, the action of neurotrophins on neuronal survival in the brain was widely reported. Moreover, data obtained on the rapid actions of NT on synaptic efficacy and their ability to modulate cortical plasticity in the developing visual system suggest that neurotrophins might play an important role in regulating neuronal connectivity and synaptic strength in the brain (Lewin and Barde 1996). In fact, besides its actions as trophic factor, BDNF has been widely studied as potential regulator of plasticity in the cortex, due to its high expression in brain regions, which are known to have a role in learning and memory such as hippocampus and cerebral cortex (Lu and Figurov 1997, Lu and Chow 1999, Schuman 1999).

NGF was the first trophic factor to be identified. It is required for the development and survival of sympathetic neurons of the paravertebral chain in the peripheral nervous system (Levi-Montalcini and Booker 1960). More recently, it was shown that a neuronal subpopulation of the PNS concerned with nociceptive information is either redirected to a proprioceptive phenotype during development or is lost in adulthood when endogenous NGF is depleted by anti-NGF antibody injection (Lewin and Mendell 1993, 1994). In addition, it was observed that blockade of endogenous NGF prevents both heat and mechanical hyperalgesia (Lewin et al 1994b, Woolf et al 1994) and conversely exogenous supply of NGF can cause increase sensitivity to painful stimuli (Lewin et al 1994b).

In the CNS of rat, both mRNA in situ hybridization (Lauterborn et al 1994) and immunohistochemistry (Nishio et al 1994) showed that NGF is mainly expressed in the cerebral cortex, in the hippocampus, and the basal forebrain. The expression of NGF, in the neocortex,

appears to be developmentally regulated: in the neocortex NGF mRNA is not detected till postnatal day 6 (P6), when deeper layers V and VI show a light labeling, while at P12 the labeling reaches the superficial layers, mainly layer II. By P20-25, the pattern of distribution resembles that of the adult with labeling restricted mainly to layers II-III and layers V-VI (Lauterborn et al 1994). Consistently, NGF immunoreactivity in adult rat is restricted mainly to layers II-III and layers V-VI (Nishio et al 1994). By in situ hybridization, it was observed that the NGF mRNA is transiently expressed in several nuclei of the thalamus in the early postnatal period (from P1 to P20-25). However, the expression of NGF is retained only in the lateral geniculate nucleus (LGN) in the adulthood (Lauterborn et al 1994). The main target of NGF produced in the cortex is the cholinergic neuronal population that resides in the basal forebrain region (see paragraph 1.5).

1.3 Cholinergic system

In this work, the term "cholinergic system" indicates the cholinergic neuronal population that resides in the basal forebrain region (BFB). In the rat, these neurons spread from the anterior medial septal nucleus rostrally to the rostral portion of the lateral hypothalamus caudally. Ectopically located cholinergic neurons are also considered part of the BFB. The cholinergic neurons are from medium to large sized, long axoned, and multipolar. Most of the cell bodies have an oval or fusiform shaped (Sefton and Dreher 1995). They display intense immunoreactivity for acetylcholinesterase (AChE), choline acetyltransferase (ChAT), and vesicular acetylcholine transporter (VAChT), as well as staining for ChAT and VAChT mRNA (Sefton and Dreher 1995, Ichikawa et al 1997). Based on their projection area, the BFB population gives rise to at least four different pathways: 1) the basalo-olfactory bundle, projecting to the olfactory bulb and associated nuclei; 2) the basalohippocampal bundle, innervating the hippocampal formation and nearby limbic cortex; 3) the medial cortical pathway, originating mainly in the vertical and horizontal limbs of diagonal band, nucleus basalis, and partially in the magnocellular preoptic area and substantia innominata. These nuclei project to medial cortical regions, including the medial frontal, cingulate, retrosplenial and medial occipital cortices; 4) the lateral basalocortical and basaloamygdalar tracts, supplying afferents to the

remaining allocortex, to the lateral isocortex, and to the amygdala (Mesulam et al 1983, Butcher and Semba 1989, Sefton and Dreher 1995). It is proposed that cholinergic neurons in BFB provide the majority of the cholinergic innervation to the telencephalon (Rye et al 1984). Interestingly, despite the fact that ChAT-immunoreactive neurons were identified by using a variety of antibodies in the neocortex, both in situ hybridization and Northern-blot analysis failed to detect either ChAT or VAChT mRNAs (Sefton and Dreher 1995, Ichikawa et al 1997). A prominent morphologic feature of the majority of cholinergic neurons is the existence of long isotropic dendrites emanating from the cell body. These processes intersect the soma, dendrites, and axons of surrounding neurons; moreover some neuronal projections appear to be closely associated with blood vessels. This anatomical arrangement suggests that cholinergic neurons can interact among themselves both synaptically and in an endocrine fashion. In the cortex the main synaptic targets of cholinergic innervation are pyramidal neurons, but also other morphologies are represented.

The basal forebrain is typically defined as diffusely projecting system. This feature, however, seems to rely on the diffuse dendritic arbor of cholinergic neurons while their axons innervate relatively small areas of the cortex (Sefton and Dreher 1995). The neocortex is divided into cytoarchitectonic regions (modules) that are constrained by the sensory modality of their thalamic inputs, and in turn each sensorial modality is organized in a point to point representation. The cholinergic projections of the BFB obey to this principle of modular organization. Thus, these modules of at most 1-2 mm^2 (in the rat brain) are innervated by discrete groups of cholinergic cells. It was reported that the number of cholinergic neurons clustered to form a cortical projecting module is greater in primates (average size > 15) than in rat or cats (3-5). It was speculated that this could represent a structural feature accounting for the greater cognitive abilities of primates (Woolf and Butcher 1991).

The precise neuroanatomical profile of the cholinergic projections as well as the developmental maturation of these fibers are important in understanding the functional role of the cholinergic system in modulating the activity of the neocortex. Several techniques were used to describe these aspects: retrograde and anterograde tracer studies, laminar distribution of cholinergic markers such as ChAT, VAChT, and AChE by both immunodetection and mRNA in situ hybridization. Neurogenesis of the rat cholinergic neuronal population begins at caudal level at embryonic day 12 (E12) and is completed at the most rostral level by E17 (Semba and Fibiger 1988). By using an anterograde tracer method, BFB axons projecting to the cortex are

detected in the subcortical WM under the occipital cortex at P0; at this age the occipital cortex comprises the subplate, layer VI, an emerging layer V, an undifferentiated cell dense cortical plate, and the marginal zone. At P3, when layer IV starts to differentiate from the cortical plate, BFB axons are seen in layers VI and V, while by P4 they reach layer IV. At P6, all layers of the occipital cortex are differentiated and the BFB axons are seen throughout all layers, although the vast majority of labeled axons are still confined in the infragranular layers. During the second postnatal week the axons continue to develop to reach a mature pattern of distribution by P11 (Calarco and Robertson 1995). Retrograde labeling of neurons from the cortex to the basal forebrain shows that already at P0, the number and pattern of distribution is similar to the adult. However, at this time the neurons are significantly smaller and faintly stained. Marked changes occur by P14 when the morphological features of the BFB neurons approach the adult ones (Dinopoulos et al 1989, Calarco and Robertson 1995). The main limitation in using tracer methods is due to the unspecific labeling of fibers projecting to the cortex; however, the observation that more than 80% of projecting neurons in the BFB express a cholinergic phenotype (Rye et al 1984) strongly suggests that the results described above are realistic.

A better method to identify cholinergic BFB-projecting neurons is the detection of markers involved in the metabolism of the neurotransmitter characterizing cholinergic neurons, i.e. acetylcholine. In the BFB, the cholinergic marker ChAT is already detected at this embryonic stage of development (Dinopoulos et al 1989, Gould et al 1991) and the immunoreactivity reaches intensity comparable to the adult by the end of the second postnatal week (Dinopoulos et al 1989). In adult rat somatosensory cortex, ChAT immunoreactive fibers with periodic varicosities appear to form a loosely organized network throughout all cortical layers. ChAT terminals are found in association with dendrites of pyramidal neurons and somata of non-pyramidal neurons (Houser et al 1985). ChAT activity in the rat visual cortex cannot be detected until P8 when a low level of activity can be observed in all layers. Thereafter, the level of activity increases until P24. The pattern of ChAT activity in adult rat visual cortex shows no statistical difference among layers, however the peaks of activity are observed in layers I and V and the lowest in layer IV (Mc Donald et al 1987). Another possible marker of cholinergic fiber distribution is represented by AChE activity. At the end of the second postnatal week, AChE activity was shown to peak in layers I, and IV and the deeper part of layer III. In the third week, a peak of activity also appears in layer V. In the adult, the pattern of AChE activity is high in layer I, in deep layer IV and in layer V.

Lesions of either lateral geniculate nucleus or the BFB reveal that in the first three weeks of postnatal development, the peaks of AChE activity are due to the LGN projecting neurons, with a gradual shift toward a BFB origin of the peak of AChE activity between the fourth postnatal week and the adulthood (Hanes et al 1992). The latter findings suggest that, since geniculocortical pathway develops before the cholinergic projections, the peaks of AChE activity could reflect an instructive role of the thalamic input in the formation of cholinergic synapses. More recently, the marker VAChT was used to identify unambiguously the cholinergic terminals. VAChT is responsible for concentrating ACh in synaptic vesicles. Unfortunately, in the rat somatosensory cortex VAChT-immunoreactive fibers and varicosities were found alternatively more concentrated in layers I, IV and V (Gilmor et al 1996) or in layers II and V (Ichikawa et al 1997).

In summary, no univocal laminar distribution of cholinergic fibers can be assigned by using the markers ChAT, AChE and VAChT, with all layers revealing a significant level of each one of the markers examined. However, the results suggest that: i) the BFB cholinergic neurons complete their development in the third postnatal week, and this timecourse is paralleled by ChAT immunoreactivity and activity maturation; ii) the BFB cholinergic terminals make synaptic contact mainly in layer I and V, and possibly in deep layer IV of adult rat visual cortex.

The cholinergic markers described so far are characteristic of the presynaptic terminals (with the exception of AChE which is synthesized both at pre- and postsynaptic sites). Another way to define the pattern of innervation of cholinergic fibers consists in determining the localization of ACh receptors. The cholinergic receptors can be divided into two classes: the metabotropic or muscarinic receptor (mAChR), and the ionotropic or nicotinic receptor (nAChR) classes. The metabotropic/muscarinic receptor family of ACh receptors comprises five different subtypes (m1-m5 or pharmacologically defined M_1-M_4, but see Caulfield and Birdsall 1998). They are coupled to G-proteins, which upon binding to ACh, activate phospholipase C (PLC) (M_1, M_3, M_5) and hence inositol 1,4,5-trisphosphate (IP_3) turnover, or inhibit the adenylate cyclase (AC) (M_2, and M_4) and in turn the formation of cAMP (Ashkenazi et al 1989, Caulfield and Birdsall 1998). The M_3 and M_5 subtypes are expressed at very low levels in the brain, while the relative abundance in the cortex of the other three subclasses is $M_1 >> M_2 > M_4$ (Levey et al 1991, Levey 1996, Caulfield and Birdsall 1998). In the neocortex of different species of mammals (rat, mouse, and monkey) a good overlapping was found among the immunoreactive patterns of the different receptor subclasses. Highest levels for M_1 are detected in layers II/III, and VI, where

virtually all pyramidal neurons are stained in their cell bodies and proximal dendrites, non-pyramidal neurons are also stained. The M_2 labeling is denser in layers IVA, IVC and at the border of layers V/VI with immunoreactivity concentrated in spines and small dendrites of mostly interneurons (Levey et al 1991, Mrzljak et al 1993, Hohmann et al 1995, Tigges et al 1997). The M_4 immunoreactivity, is lower as compared to the M_1, M_2 receptor subtypes, with highest staining in neuropil of supragranular layers, layer V, and patches in layer IV (Levey et al 1991, Hohmann et al 1995, Tigges et al 1997). Equivalent results were obtained by using quantitative receptor autoradiography in the rat visual cortex: the number of M_1 binding sites is higher in upper layer III and deeper layer VI. In contrast, highest binding for M_2 is present in upper layer IV and upper layer VI (Schliebs et al 1994). The laminar distribution of the different receptor subtypes in rat brain correlates well with the cellular localization of their respective mRNAs (Weiner and Brann 1989). Interesting insights came from the autoradiographic study of the ontogenetic profile of muscarinic receptors. The binding sites for the total population of muscarinic receptors increase steeply between E20 and P21, while a slower increment is detected thereafter. At E20, the putative M_1 binding sites in the neocortex are on average 11% of the adult values. The supragranular layers of the occipital cortex increase the number of binding sites from E20 to P35, and partially decrease thereafter. The other layers stop the increment of their binding site at P21 and decrease slightly thereafter. In occipital cortex, the putative M_2 binding sites are still very low at P7; from P7 to P60 the density of the binding sites increases steadily in all layers (Aubert et al 1996). A different temporal development of muscarinic receptor subtypes was described in mouse forebrain by using subtype specific antibodies. In this case, while M_2, as well as M_1, immunoreactivity reach a staining pattern characteristic of the adult by P14, the intensity of the cortical immunoreactivity for M_2 increase further until P30 (Hohmann et al 1995). Despite these discrepancies, if we consider that, M_2 receptor is assumed to be mainly an autoreceptor at cholinergic presynaptic terminals, or that in any case it is thought to mediate inhibitory signals (Levey 1996), the difference in the pace of temporal maturation between M_1 and M_2 receptors subtype could be particularly meaningful.

The neuronal ionotropic/nicotinic receptor class comprises 11 subunit genes, including $\alpha 2$ through $\alpha 9$, which encode the ligand binding subunits, and $\beta 2$ through $\beta 4$, which may represent the structural subunits. The receptor appears to be a pentameric ligand-gated ion channel with low selectivity for cations, and with high permeability to calcium. Data support the idea that the vast majority (> 90%) of neuronal nicotinic receptors in the rat brain are composed by $\alpha 4$, $\beta 2$

subunits (possibly with the combination $(\alpha4)_2(\beta2)_3$ and are α-bungarotoxin insensitive. The second most represented receptors in the cortex contain α7 subunits (possibly homomeric) and are α-bungarotoxin sensitive. The α7 receptor exhibits an extraordinary permeability to calcium ions and is particularly enriched in rat hippocampus (Sargent 1993, Seguela et al 1993, Mc Gehee and Role 1995, Role and Berg 1996, Jones et al 1999). Immunohistochemical observations revealed that α4-immunoreactivity is distributed through layers II to VI of the rat cerebral cortex. The immunolabeling is present in somata and apical dendrites, and absent in basal dendrites and axons of the pyramidal neurons (Nakayama et al 1995). The distribution of β2 subunit was mapped in rat and mouse brain by using iodinated monoclonal antibody. β2 immunolabeling is centered over layers I, IV and V (Swanson et al 1987). Autoradiographic analysis of tritiated ACh and nicotine known to bind preferentially the α4β2 complex (Sargent 1993) reveals that cortical layers I, III and IV are preferentially labeled (Clarke et al 1985). In a study employing monoclonal antibodies against α4 and β2 subunits in the rat brain, immunoreactivity is found in all cortical regions. The most prominent immunoreactive neurons are the pyramidal ones of layers II-III and V (Bravo and Karten 1992). By using quantitative in vitro autoradiography, the densities of α4β2 nicotinic receptor binding sites in the cerebral cortex at E20 are found to represent on average 36% of the levels observed in the adult. In the occipital cortex all layers show an increase in the binding site densities from E20 to P21. Between P21 and P35 the levels decrease in supra and infragranular layers, while they remain relatively constant in the granular layer. Thereafter the binding densities remain roughly stable up to P60 (Aubert et al 1996).

The isocortex of the rat brain is characterized by a remarkable homogeneous distribution of α7 immunolabeling with the highest concentration in layer V pyramidal neurons, α7 immunoreactivity is much weaker in other layers. The immunostaining is present both on somata and dendrites of pyramidal neurons, but also other cell classes are lightly stained (Dominguez del Toro et al 1994). In rat visual cortex, the appearance of α7 mRNA starts as early as E13 in the ventricular zone. Between P5 and P10 both mRNA and binding sites densities reach their maximal values. The adult pattern of α7 mRNA expression is reached after the second postnatal week and it shows two bands of highest expression in layers II-IV and layer VI (Broide et al 1995, 1996).

In summary, the reported distribution of nicotinic neuronal receptors and their mRNAs is puzzling, since different works have often reported complementary pattern of distribution. Taken together, these studies indicate a distribution of nAChR spanning all cortical layers. These studies also suggest that neuronal nicotinic receptors start to be expressed already during embryonic development in contrast to the muscarinic receptors, and could play an important role in early cortical development.

1.4 Cholinergic system and plasticity

Cholinergic transmission in the cortex has a long and well-established role in memory and selective attentional processes (Deutsch 1971, Everitt and Robbins 1997, Sarter and Bruno 1997, Wenk 1997). Basal forebrain dysfunction is associated with cognitive deficits in both normal aging and in age-related, as well as genetic, pathological conditions. Atrophic processes in the BFB cholinergic system, and decline in spatial learning ability, follow a parallel course during aging (Decker 1987, Gallagher and Colombo 1995). Similarly, cholinergic deficits are a prominent hallmark of age-related pathologies such as senile dementia (Perry et al 1978, Flicker et al 1985) and Alzheimer's disease (Bierer et al 1995, Mesulam 1996, Francis et al 1999). In particular, a decrease in both TrkA mRNA expression and choline acetyltransferase activity were found in postmortem BFB tissue from Alzheimer's disease subjects (Boissiere et al 1997). Moreover, defects in cholinergic innervation during ontogeny can play a key role in abnormal cortical neuronal morphogenesis of developmental brain disorders, such as Down Syndrome (Yates et al 1980, Fiedler et al 1994) and Rett Syndrome (Wenk and Hauss-Wegrzyniak 1999), thus resulting in persistent cognitive disabilities.

In vivo and *in vitro* studies concerning the role of cholinergic transmission in modulating activity-dependent plasticity in hippocampus reported apparently conflicting results. Fimbria/fornix lesions impair spatial performance but do affect neither Short-Term Potentiation (STP) nor LTP in CA1 area (Kleschevnikov et al 1994). The same results were obtained with lesions restricted to the cholinergic neurons by using the selective neurotoxin 192 IgG-saporin (Jouvenceau et al 1996). Similarly, lesions restricted to the medial septum induce deficits in working/spatial memory but do not impair LTP in CA3 area (Feasey-Truger et al 1992). By

employing pharmacological tools, it was observed that muscarine impairs LTP in CA3 (Williams and Johnston 1988) area, while stimulation of cholinergic receptors enhances LTP in CA1 (Blitzer et al 1990). Physostigmine, a cholinesterase inhibitor, induces an LTP-like phenomenon in hippocampus (Ito et al 1988). From these data it can be argued that if cholinergic transmission acts as neuromodulator of synaptic plasticity, the facilitatory or inhibitory effects depend on several parameters, such as the neural network underlying the synapses involved and the stimulus inducing plasticity. This idea is supported by a study in which the homosynaptically evoked LTP in CA1 area is not suppressed, but rather enhanced by the muscarinic antagonist atropine. In contrast, associative LTP is significantly reduced by application of atropine (Sokolov and kleschevnikov 1995). Moreover, Huerta and Lisman (1995) showed that the same brief high frequency burst can lead to either depression or potentiation of synaptic strength, depending on the synchronization between the cholinergic dependent theta oscillations of the network and the tetanic stimulus.

In the neocortex, homogeneous results were reported in assigning a relevant role to cholinergic innervation in modulating plastic phenomena. Depletion of cholinergic fibers, induced by 192 IgG-saporin immunolesion of the BFB nuclei, exhibits reduced activity-dependent plasticity in the somatosensory cortex (Baskerville et al 1997, Zhu and Waite 1998). Similarly, BFB cholinergic neurons modulate the reorganization of the sensory map in auditory cortex (Kilgard and Merzenich 1998). In the visual cortex, combined destruction of the cortical noradrenergic and cholinergic innervations reduces the shift in ocular dominance induced by monocular deprivation, although the alternate lesion of each system is ineffective (Bear and Singer 1986). Pharmacological blockade of cholinergic transmission by muscarinic antagonists infused into the visual cortex is by itself sufficient to suppress ocular dominance changes (Gu and Singer 1993). In 1992, Bröcher and colleagues reported that agonists of cholinergic and noradrenergic receptors synergistically facilitate the induction of LTP in slices of rat visual cortex. This cannot be observed when the slices are alternatively treated with either cholinergic or noradrenergic agonists. The authors suggested that the facilitated potentiation of synapses, induced by these compounds, acts at postsynaptic level and that NMDA receptor-gated conductance or other voltage dependent channels need to be activated in order to induce LTP. The possible subcellular mechanisms, accounting for the facilitatory effect of cholinergic transmission in LTP induction, probably converge to induce an increase in intracellular calcium at postsynaptic site. This aspect is known to play a key role in synaptic plasticity phenomena

(Bliss and Collingridge 1993). M_1 cholinergic receptors, which are coupled to PLC, can increase intracellular Ca^{2+} directly by mobilizing this ion from intracellular stores (Caulfield 1993, Cox et al 1994). In addition, it was shown that the IP_3 pathway mediates cholinergic potentiation of rat neuronal responses to NMDA (Markram and Segal 1992, Aramakis et al 1999). Muscarinic receptor stimulation was also reported to reduce voltage- as well as Ca^{2+}-activated K^+ conductances (Benardo and Prince 1982, Cole and Nicoll 1984, Caulfield 1993, Cox et al 1994), resulting in an increase of cellular excitability. In summary, acetylcholine acting through muscarinic receptors induces depolarization, upregulates NMDA channels and activates phosphoinositide cascade in pyramidal neurons, thus facilitating the activity–dependent synaptic plasticity (Jerusalinsky et al 1997).

Despite the evidence that nAChR are involved in a variety of brain functions, including neuronal development, learning and memory formation, a clear role for neuronal nicotinic receptor in mediating synaptic transmission is not yet well established in the cortex. Indeed, it was recently shown that cholinergic transmission can directly excite both interneurons and pyramidal cells in the cortex (Jones et al 1999). Much well defined is the function of nAChR in modulating transmitter release at presynaptic terminals (McGehee et al 1995, Wonnacott 1997, Jones et al 1999, Radcliffe et al 1999). In addition, it is known that α7 subtype nAChR has a permeability to calcium ions three times higher as compared to NMDA receptors and it is not blocked by magnesium at resting membrane potential (Seguela et al 1993, McGehee and Role 1995). Hence, it appears that neuronal nicotinic receptors potentially possess all the features necessary to play a key role in the modulation of plastic phenomena (Role and Berg 1996, Wonnacott 1997, Broide and Leslie 1999, Jones et al 1999).

1.5 Neurotrophins and cholinergic system

The possibility that basal forebrain cholinergic neurons could be a target for the trophic factor NGF was supported by several studies, ranging from neuroanatomical observations to functional effects of NGF on survival and differentiation of cholinergic neurons.

In 1984, Seiler and Schwab showed that iodinated NGF injected in the occipital cortex of adult rats is retrogradely transported to large, presumably cholinergic neurons in the nucleus

basalis region of the basal forebrain. Other authors (DiStefano et al 1992, Domenici et al 1994b) confirmed these findings in similar experiments. Immunohistochemical studies reported that virtually all neurons, within the basal forebrain region, show colocalization of ChAT and TrkA, as well as ChAT and p75 immunoreactivity (Steininger et al 1993, Sobreviela et al 1994). Similar results were obtained in experiments of in situ hybridization: ChAT and TrkA mRNAs show an overlapping pattern of distribution in the basal forebrain region during the entire postnatal period (Li et al 1995). Moreover, the temporal patterns of expression of both mRNAs are very similar, suggesting a regulation by the same factors. TrkA and ChAT start to be detected at P0. A dramatic increase in labeling for both TrkA and ChAT is observed in the first postnatal week up to P11 and remains sustained during the third and fourth week of age. The labeling decreases thereafter and reaches a level of expression similar to that seen in the adult at P30 (Li et al 1995). Cholinergic neurons of the basal forebrain respond to NGF by increasing the level of ChAT activity (Mobley et al 1985), as well as ChAT and TrkA mRNA (Li et al 1995). The trophic action of NGF on basal forebrain neurons is well documented. Intraventricular injection of NGF can prevent the death of axotomized septal neurons (Hefti 1986, Koliatsos et al 1991). Conflicting results were reported for BDNF in preventing loss of cholinergic neuronal population in response to various injuries (Lapchak and Hefti 1992,Widmer et al 1993, Morse et al 1993, Skup et al 1994). NGF is also able to reverse the age-dependent atrophy of BFB cholinergic neurons, to improve behavioral performance and to reverse cognitive impairment in aged rats (Fischer et al 1987, Martinez-Serrano et al 1996). By using anti-NGF antibodies it was observed that deprivation of NGF induces a decrease in the number of ChAT-positive neurons in the BFB (Vantini et al 1989, Molnar et al 1998), as well as a reduction in their cell size (Li et al 1995, Molnar et al 1998). Similar, but more severe effects have been obtained by blocking TrkA receptor with a monoclonal antibody (Cattaneo et al 1999). However, the sensitivity of BFB cholinergic neurons to NGF seems transient and the effects of NGF depletion are almost completely recovered in few weeks (Molnar et al 1998). The disruption of the NGF gene in transgenic mice produces a lethal phenotype in the early postnatal period, nevertheless in CNS, basal forebrain cholinergic neurons differentiate and continue to express phenotypic markers for the brief life span of the null mutant mice (Crowley et al 1994). These data suggest that NGF is playing a key role in phenotype maintenance rather than representing a survival factor for BFB cholinergic neurons. Milder genetic manipulation, such as disruption of a single allele of the NGF gene, shows behavioral deficits accompanied by both shrinkage and loss of septal cells

expressing cholinergic markers and by a decrease in cholinergic innervation of the hippocampus (Chen et al 1997). Similarly, transgenic mice expressing a recombinant version of a neutralizing anti-NGF monoclonal antibody after the fourth week of life, reveal a strong reduction of ChAT positive neurons, as well as a strong reduction in the number of TrkA and p75 immunoreactive cells in the BFB of adult animals (Ruberti et al 2000). Also in this case spatial memory impairment was observed. Thus, in contrast with the previous findings it seems that the incomplete block of NGF, during early development, leads BFB cholinergic neurons to maintain their survival-dependence on NGF throughout life. Very recently, comparable results were shown for homozygous BDNF-deficient mice. During postnatal development, cholinergic hippocampal innervation arborizes to a lesser extent than in controls and has reduced levels of acetylcholinesterase suggesting that also BDNF could be critical for postnatal development and maturation of cholinergic forebrain neurons (Ward and Hagg 2000).

1.6 Plasticity and neurotrophins

A large body of evidence suggest that neurotrophins are involved in modulation of synaptic transmission and in synaptic plasticity: i) the expression and partially the release of NT is activity dependent, ii) NT are able to regulate several ionic currents and consequently membrane excitability, iii) these trophic factors are able to influence the release of several types of neurotransmitters.

The regulation of NT mRNA expression by neuronal activity was demonstrated in neuronal cultures of rat hippocampus and cerebellum. Glutamate (through both NMDA and non-NMDA receptors) causes upregulation of NT mRNA expression, while GABA causes downregulation. Potassium depolarization and kainic acid-mediated increases in BDNF and NGF mRNAs are reduced by a calcium channel blocker in a dose-dependent manner (Lu et al 1991, Thoenen et al 1991, Bessho et al 1993, Berninger et al 1995). In addition, in hippocampus and cerebral cortex both NGF and BDNF mRNA expressions are increased following induction of epileptiform activity (Ernfors et al 1991, Isackson et al 1991, Dugich-Djordievic et al 1992). In contrast, similar experiments cause a reduced expression of NT-3 mRNA (Castren et al 1993). More physiological stimuli, such as high frequency stimulation in

hippocampus (Patterson et al 1992, Castren et al 1993), or even better, light and sensory stimulation can also regulate NGF, BDNF and NT-3 mRNA levels in the visual cortex (Schoups et al 1995) and somatosensory cortex (Rocamora et al 1996), respectively.

In peripheral tissues, NT are secreted by the constitutive calcium-independent pathway (Barth et al 1984). In the CNS they appear to be released by both the constitutive and the activity-dependent pathway, with differences among different NT. Blöchl and Thoenen (1995, 1996) reported that potassium-induced depolarization, glutamate and acetylcholine induce the secretion of NGF from hippocampal slices and dissociated hippocampal neurons. While recently, Murphy and collaborators (Mowla et al 1999, Farhadi et al 2000) claimed that both NGF and NT-3 are released constitutively and that the regulated secretory pathway releases only BDNF. Despite these conflicting results there is a merging concept of a regulated secretion of neurotrophins, possibly linked to neuronal activity. The constitutive release pathway could subserve the trophic action of these compounds.

Modulation of membrane excitability is a key factor in controlling neuronal plasticity. The effects of NT in the regulation of intrinsic electrical excitability have been studied since the seventies (Greene and Tischler 1976), focusing on NGF-mediated enhancement of voltage-gated sodium (D'Arcangelo et al 1993, Toledo-Aral et al 1995), calcium (Levine et al 1995a), and potassium (Sharma et al 1993, Holm et al 1997, Bowlby et al 1997) currents. Indeed, it was found that each NT induces a unique pattern of expression of ionic currents despite similar activation of initial signal transduction events. In particular, while NGF appears to increase equally the density of all channel types, BDNF increases excitability upregulating calcium and sodium channels, conversely NT-3 decreases excitability elevating potassium channel expression (Lesser et al 1997).

In addition to differentially modulate the expression of voltage gated currents, the NT appear to regulate the release of neurotransmitter from presynaptic terminals. BDNF and NT-4 enhance spontaneous synaptic activity in dissociated cultures of hippocampal neurons (Lessman et al 1994, Levine et al 1995b, Li et al 1998), while NT-3 potentiates spontaneous activity in cultured cortical neurons (kim et al 1994). In synaptosomal preparations of both hippocampus and visual cortex, BDNF and NGF increase the potassium-induced release of acetylcholine and glutamate (Knipper et al 1994, Sala et al 1998). In slice preparations, BDNF and NT-3 potentiate synaptic transmission at the Schaffer collateral/CA1 synapses (Kang and Schuman 1995), and both BDNF and NGF potentiate excitatory transmission in visual cortex

(Carmignoto et al 1997). These findings are compatible with either a retrograde (Bhattacharyya et al 1997) or an anterograde (Altar and DiStefano 1998) transport of neurotrophic factors, thus increasing the complexity of feedback and feedforward loops by which NT can contribute to specific forms of synaptic plasticity.

Besides biochemical changes, the NT are capable of inducing structural modifications in axonal and dendritic arbors with a change in the number and locations of functional synapses. NT application to organotypic cortical slices (Mc Allister et al 1995) or removal of endogenous neurotrophic factors by immunoadhesins (Mc Allister et al 1997) can rapidly change the length and complexity of dendrites of pyramidal neurons. Each cortical layer responds to different subsets of NT and each NT elicits a unique pattern of dendritic changes. Interestingly, blockade of NGF-TrkA signaling induces a significant reduction both in number and size of cholinergic contacts (Debeir et al 1999).

So far, evidence for a fast action of NT in modulating neuronal excitability has been widely supported, but how far is the contribution of neurotrophic factors to long lasting changes in synaptic transmission? The high level of expression in hippocampus and the activity dependent synthesis and release of BDNF in neurons led several researcher to investigate the possible involvement of this neurotrophic factor in LTP and LTD (Malenka 1994). Studies with BDNF knockout mice shows that the lack of endogenous BDNF drastically impairs LTP in the hippocampus (Korte et al 1995, Patterson et al 1996). In addition, both re-expression of the BDNF gene mediated by adenovirus (Korte et al 1996) or treatment with recombinant BDNF (Patterson et al 1996) are able to restore LTP in a few hours. Moreover, acute depletion of BDNF by antibodies or immunoadhesins impairs LTP in hippocampus (Figurov et al 1996, Kang et al 1997). In visual cortex, BDNF enhances LTP and prevents the induction of LTD by low-frequency stimulation (Akaneya et al 1996, 1997, Huber et al 1998). The merging role of BDNF is consistent with a permissive action of this factor on the potentiation of synaptic transmission (Figurov et al 1996) or according to the BCM algorithm that BDNF shifted the modification threshold to a lower frequency (Huber et al 1998).

NGF was extensively investigated as regulator of membrane excitability but, paradoxically, data concerning its possible role as neuromodulator of synaptic plasticity are substantially lacking. Indeed, it was reported that in hippocampal slices, perfusion of NGF blocks the expression of LTP (Tancredi et al 1993). In contrast, several other groups reported no effects of NGF in modulating synaptic plasticity both in hippocampus and visual cortex (Kang

and Schuman 1995, Figurov et al 1996, Akaneya et al 1997). NGF was the first neurotrophic factor whose effects on the visual cortical plasticity were examined. During the critical period, NGF injection into the lateral ventricle of rats (Domenici et al 1991, Maffei et al 1992) or TrkA activation (Pizzorusso et al 1999) largely prevents the dramatic change in ocular preference of visual cortical neurons in response to monocular deprivation. These results suggest that when NGF level in the visual cortex increases the degree of plasticity of visual cortical neurons is reduced and, consequently, monocular deprivation is no longer effective. Successive experiments showed that sequestering freely diffusible NGF by antibodies prolongs the length of critical period, therefore extending the period of sensitivity to monocular deprivation (Domenici et al 1994a). Thus, complementary data support the idea that a high level of NGF blocks plasticity when cortical network is highly plastic, while a low level of NGF re-induces plasticity when the cortical network has lost this property. Nevertheless, it is conceivable that NGF is one of many factors relevant to the definition of plasticity.

1.7 Aims

The aim of this project is to define the role of the Nerve-Growth Factor and the cholinergic system in long lasting modifications of synaptic strengthening in the neocortex. In order to achieve this result I used the rat and the mouse visual cortex as experimental models. Indeed it was taken advantage, as it will be shown in the results section, of some aspects that are usually considered limitations in using the visual cortex as experimental preparation to investigate synaptic plasticity. Firstly, it was fundamental the interposition of thalamocortical fibers and the extracortical modulatory inputs (particularly the basal forebrain cholinergic fibers). Secondly, it was exploited the feature of the rat visual cortex to express a synaptic plasticity that is developmentally regulated. In these experimental models, either the Long-Term Potentiation or the Long-Term Depression of synaptic transmission have been induced by experimental paradigms such as High Frequency Stimulation (HFS) and Low Frequency Stimulation (LFS) of the underlying WM, respectively.

In the work presented here, a mouse model genetically modified to produce an anti-NGF antibody, was used to block a trophic factor known to be necessary for survival and

differentiation of the cholinergic neuronal population. Consequently, it was investigated the indirect long-term effects of NGF deprivation in the expression of cortical synaptic plasticity. At the same time, the rat visual cortex was used to define the modulatory role of the cholinergic system in synaptic plasticity, by using both pharmacological tools and cytotoxic lesion of the basal forebrain neurons. Moreover, by using the latter experimental preparation it was explored the possible role of NGF as neuromodulator of synaptic plasticity, that is an acute effect besides its classical role as trophic factor.

2.0 MATERIAL AND METHODS

2.1 Generation of anti-NGF transgenic mice

A detailed description of the production of anti-NGF transgenic mice has been described by Ruberti et al. (2000). Briefly, the variable regions of the light and heavy chains of αD11 were cloned using the RACE method, and reassembled with the respective constant regions of the human immunoglobulin to obtain the chimeric antibody αD11 (Ruberti et al 1993). The constructs were placed under the transcriptional control of the ubiquitous human cytomegalovirus (CMV) early region promoter [-601 to –16; (Boshart et al 1985)]. The transcriptional units of the light and heavy chain genes of the chimeric αD11 antibody were digested from the plasmids pcDNneo/VKαD11HuCK (light chain) and pcDNAI-neo/VHαD11HuCγ (heavy chain) using KpNI-ApaLI and Kpni-XbaI, respectively. The fragments were separately injected in the 1-cell fertilized B6SJL mouse eggs (DNX Corp., USA), to obtain lines of mice expressing the light or the heavy chain. The analysis of genomic DNA and RNA was performed as described before (Piccioli et al 1995). Mice expressing the functional anti-NGF antibody (anti-NGF mice) were obtained by crossing mice expressing the light chain (CMV-VKαD11) with mice expressing the heavy chain (CMV-VHαD11).

2.2 Basal forebrain lesion

Disruption of the rat basal forebrain (BFB) region, of 10 days old rats, was achieved by unilateral injection of 0.16 M quisqualic acid (250 nl) dissolved in 0.1 M phosphate-buffered saline (PBS, pH = 7.4) solution. The injection was made by using a glass micropipette at the stereotaxic coordinates of 0.5 mm posterior to the bregma, 2.7 mm from the midline, 7.4 mm from the pial surface for the nucleus basalis (NB), and 0.8 mm anterior to the bregma, 0.8 mm from the midline, 7.7 mm from the pial surface for the diagonal band of Broca (DBBh). Following the method previously described (Siciliano et al 1997), each infusion lasted 3 min., and additional 3 min were allowed for diffusion before the micropipette was removed. The

electrophysiological recordings were carried out one week later, in order to allow the degeneration of BFB neurons and their cortex-projecting fibers, by cytotoxic effect of quisqualic acid.

2.3 Slice Preparation

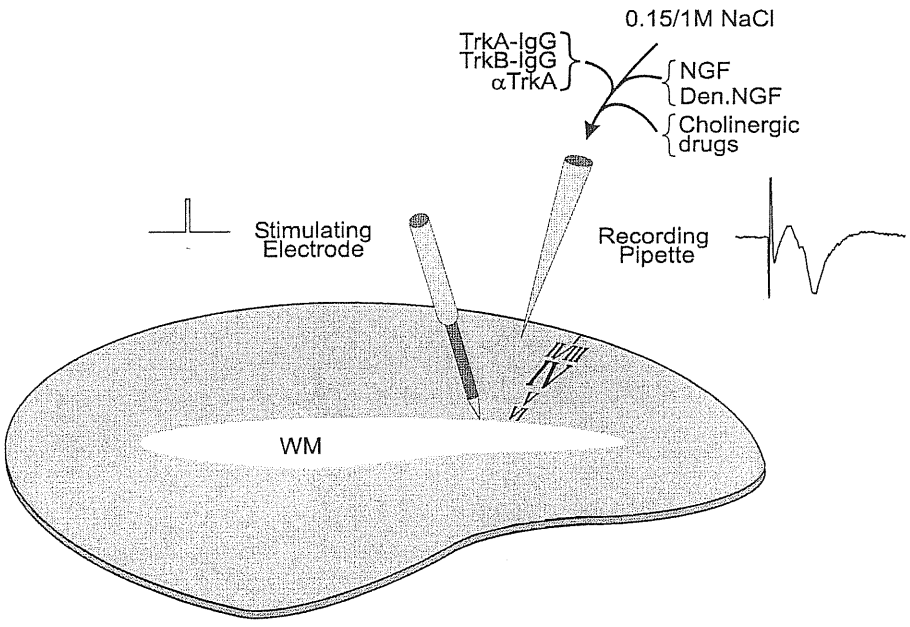
Primary visual cortex slices were prepared from Wistar rats at postnatal days 16-18 or 30-35, and from six months old mice. Animals were deeply anesthetized by urethane intraperitoneal injection and then decapitated. The brain was rapidly removed and 400 μm thick coronal sections of the occipital poles were sliced by a vibratome. All steps were performed in ice cold ACSF solution (rat: 126 mM NaCl, 3.5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose, mouse: 119 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 11 mM glucose) bubbled with 95% O_2/5% CO_2. Prior to recording, slices were stored for at least 1 hr in a recovery chamber containing oxygenated ACSF solution, at room temperature. During electrophysiological recordings, slices were perfused at 4 ml/min. with oxygenated ACSF, at 33 ± 1 °C.

2.4 Electrophysiological Recordings

Extracellular field potentials (FP) were evoked by a tungsten concentric bipolar stimulating electrode placed at the White Matter/layer VI border. The recording electrode was placed in layer II/III. High Frequency Stimulation (three trains of 100 pulses at 100 Hz, 10 s interval) was used to induce Long-Term Potentiation, whereas Long-Term Depression was evoked by Low Frequency Stimulation (900 pulses at 1 Hz). Exogenous NGF and heat inactivated NGF (Den.NGF, 80 °C for 10 min.) were administered at 100 ng/ml. To block the action of endogenous NGF a neutralizing monoclonal antibody MNAC13 against the TrkA receptor (αTrkA, 5 μg/ml) or immunoadhesins (TrkA-IgG, 1 μg/ml; TrkB-IgG, 1 μg/ml) were used. To check possible involvement of the cholinergic system the agonists of cholinergic receptors, muscarine, nicotine or acetylcholine (10 and 100 μM), the antagonist, atropine (100

μM), and the inhibitor of acetylcholinesterase, edrophonium (100 μM) were used. **NGF, immunoadhesins, αTrkA, and cholinergic drugs were dissolved at the concentrations indicated above in 1 M (or 0.15 M for mice preparation) NaCl solution and delivered through the recording pipette**, starting 25 to 35 minutes before applying the HFS or LFS protocols. As a general rule, ten-fold higher concentrations of drugs then the ones commonly employed in bath application experiments were used. To evaluate the diffusion of compounds from pipette tip, biocytin (either 0.5% in 0.15 M NaCl or 0.1% in 1 M NaCl) was added to the recording solution in some experiments.

Changes in the amplitude of FP mirror changes in the slope of the negative potentials and correlate with variations in the magnitude of a monosynaptic current sink (Mitzdorf, 1985). Thus, the amplitude of the negative field potential in layer II/III was used as a measure of the evoked population excitatory current. Baseline responses were obtained with a stimulation intensity that yielded 50-60% of maximal amplitude. All FP had peak latency from stimulation ranging from 5 to 7 ms (4.5-5.5 ms in mice), and maximal amplitude of at least – 0.6 mV. FP amplitude was monitored every 15 s and averaged every four responses (every 20 s and averaged every three responses in mice). LTP amplitude was measured, for each kind of treatment, by averaging three minutes of monitored FP centered on 60 minutes since protocol administration. Values were expressed as mean ± S.E.M. percentage change relative to their mean baseline amplitude. Statistical comparison between control and treated slices was done by applying the non-parametric statistical Rank Sum Test. Differences were considered significant when $p \leq 0.05$.



2.5 Immunohistochemistry

Four hundred µm thick rat brain slices treated with biocytin were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 6 hours at 4 °C, and cryoprotected overnight at 4 °C in 20% sucrose in PBS. 50 µm-thick sections were cut from the brain slices using a sliding microtome, and were then washed in PBS. Sections were incubated in PBS + 2% H_2O_2 for 20 minutes at room temperature then washed 3 times in PBS (pH 7.4). Non-specific binding sites were blocked with 30 minutes incubation in PBS + 0.05% Triton + 5% normal goat serum (NGS). After 3 washes in PBS sections were incubated in ABC kit alkaline phosphatase conjugated, 1:100 in PBS, for 1 hour at room temperature. After 2 washes in PBS and 2 washes in pH 9.5 buffered solution (100 mM TrisCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$), sections were incubated in the same solution + NBT (Nitrobluetetrazolium, 3.375 mg in 10 ml; stock solution is 75 mg in 1 ml 70% dimethylformamide) + BCIP (Bromocloroindolilphosphate, 1.75 mg in 10 ml; stock solution is 50 mg in 1 ml 100% dimethylformamide) for no more than 10 minutes. Reaction was stopped by washing sections with PBS. Sections were mounted on gelatinized slides. Once dried, slides were dehydrated with 100% methanol, 50% methanol + 50% xylene, 100% xylene and finally mounted in DPX medium.

Visual cortex and BFB slices from rats treated with quisqualic acid, and visual cortex slices from anti-NGF mice and age-matched control mice, were labeled with a monoclonal antibody recognizing choline acetyltransferase (Umbriaco et al 1994). Rat BFB and 400 µm thick slices (previously used for electrophysiological recordings), of both rats and mice, were fixed and cryoprotected as described above, and were then cut in 40 µm thick sections. Immunohistochemistry was performed as described by Molnar et al (1998) but PBS (pH 7.4) was used instead of 0.1 M PB (pH 7.4) and developing buffer was 0.04% 3.3'-diaminobenzidine tetrahydrocloride, 0.2% nickel ammonium sulphate, 0.2% D(+)-glucose, 0.02% glucose oxidase in 0.1 M Tris-Cl buffer (pH 7.5). Sections were dehydrated and mounted as described above.

3.1 Diffusion of locally released compounds

To assess the maximal spreading of delivered compounds, biocytin was released locally through the recording pipette. Neurons are able to take up biocytin applied extracellularly and can convey it via anterograde and retrograde transport. Thus, biocytin represents a useful tool to label individual neurons and to trace neural connections (McDonald, 1992). Biocytin was applied to slices of rat visual cortex through the recording pipette and an electrophysiological experiment was mimicked to assess the extent of substance diffusion from the recording site. The intensity of labeling was proportional to the concentration of biocytin loaded into the pipette (Fig. 3.1A-D). At the lowest concentration used (0.1%, Fig. 3.1C, D), just a few neurons were labeled, thus supporting the idea of a spatially restricted action of compounds delivered through the recording pipette.

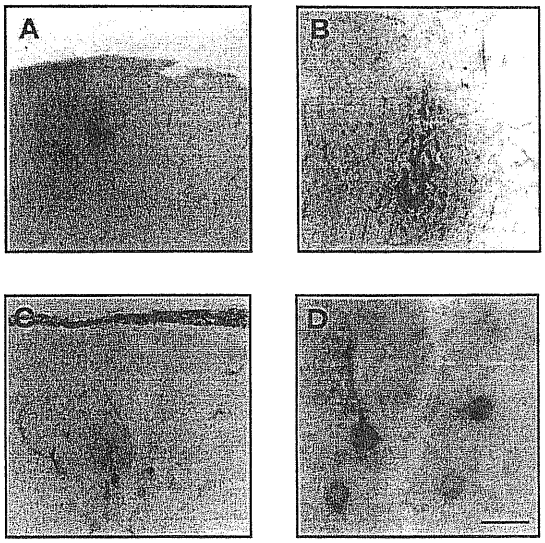


Fig. 3.1 Biocytin labels neurons surrounding recording pipette

A, B) Labeling of neurons by uptake of 0.5% biocytin in 0.15 M NaCl solution. Scale bar in (D) is 400 μm for (A) and 100 μm for (B).

C, D) Labeling of neurons by uptake of 0.1% biocytin in 1 M NaCl solution. Scale bar in (D) is 80 μm for (C) and 25 μm for (D).

3.2 Cholinergic system modulates synaptic plasticity

3.21 BFB lesion impairs LTP

The role of BFB projection in modulating synaptic transmission was assessed in experiments where the basal forebrain region was disrupted by quisqualic acid-induced cytotoxicity. The quisqualic acid injection was

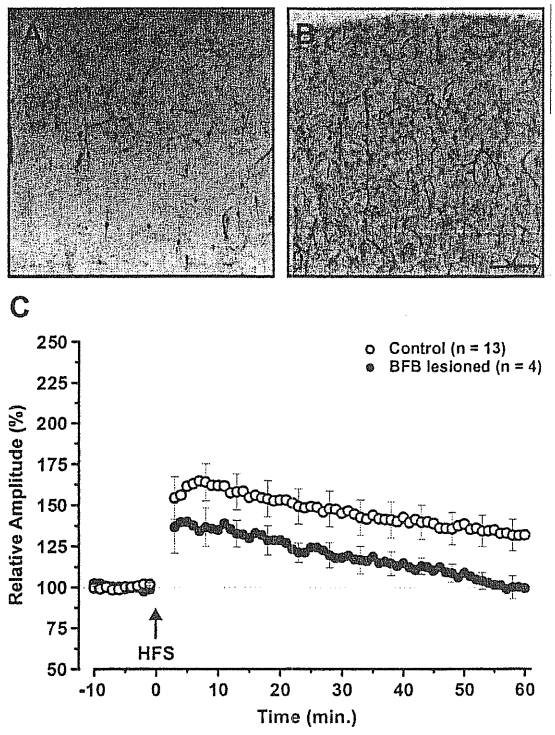


Fig. 3.21 BFB lesion impairs LTP

A; B) ChAT immunostaining reveals a strong reduction of cortical cholinergic neuropil in BFB lesioned rats (A) as compared with control animals (B). Bar = 100 μm.

C) In BFB lesioned rats (●), both induction and maintenance of LTP are impaired as compared to control animals (○).

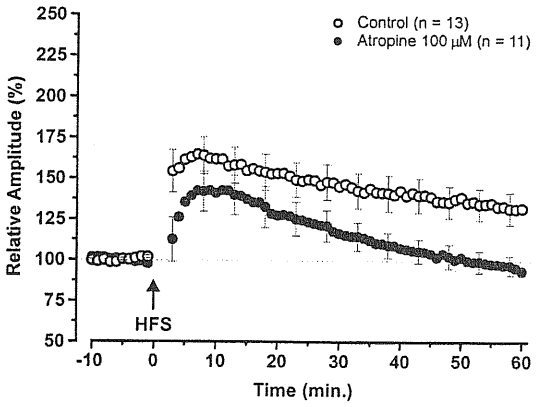
performed at P10 by stereotaxic injections of the quisqualic acid unilaterally in basal forebrain cholinergic region. In particular, injections were performed in two nuclei, the horizontal limb of the diagonal band of Broca and the nucleus basalis, which project to the visual cortex (Mesulam et al 1983, Rye et al 1984). Following this protocol, it was previously shown that the cholinergic innervation of visual cortex was reduced up to 70% as assessed by ChAT immunohistochemistry (Siciliano et al 1997). The electrophysiological recordings were made one week later, to allow the degeneration of BFB neurons. After electrophysiological recordings, slices were fixed and processed for ChAT immunohistochemistry. Fig. 3.21 shows slices processed for ChAT-immunoreactivity from control untreated rats (Fig. 3.21A) and from BFB lesioned animals (Fig. 3.21B); it is clear that ChAT immunolabeling is severely reduced in the visual cortex ipsilateral to the side of quisqualic acid injection, as previously reported (Siciliano et al 1997).

In visual cortex slices from control untreated rats, a stable LTP was reliably elicited by HFS (Fig. 3.21C, after 60 min., mean FP = 133 ± 10%, n = 13, 11 rats). In contrast, in BFB-lesioned animals both induction and maintenance of potentiation were affected. In particular, 45-50 minutes after tetanic stimulation, FP amplitude was not different from baseline values (Fig. 3.21C; after 50 min., mean FP = 105 ± 5%; after 60 min., mean FP = 99.0 ± 6.0%; n = 4, 4 rats; p <= 0.001).

Thus, it appears that the presence of BFB innervation is necessary to have a full expression of LTP in the visual cortex, otherwise only a transitory strengthening of the synaptic transmission can be induced.

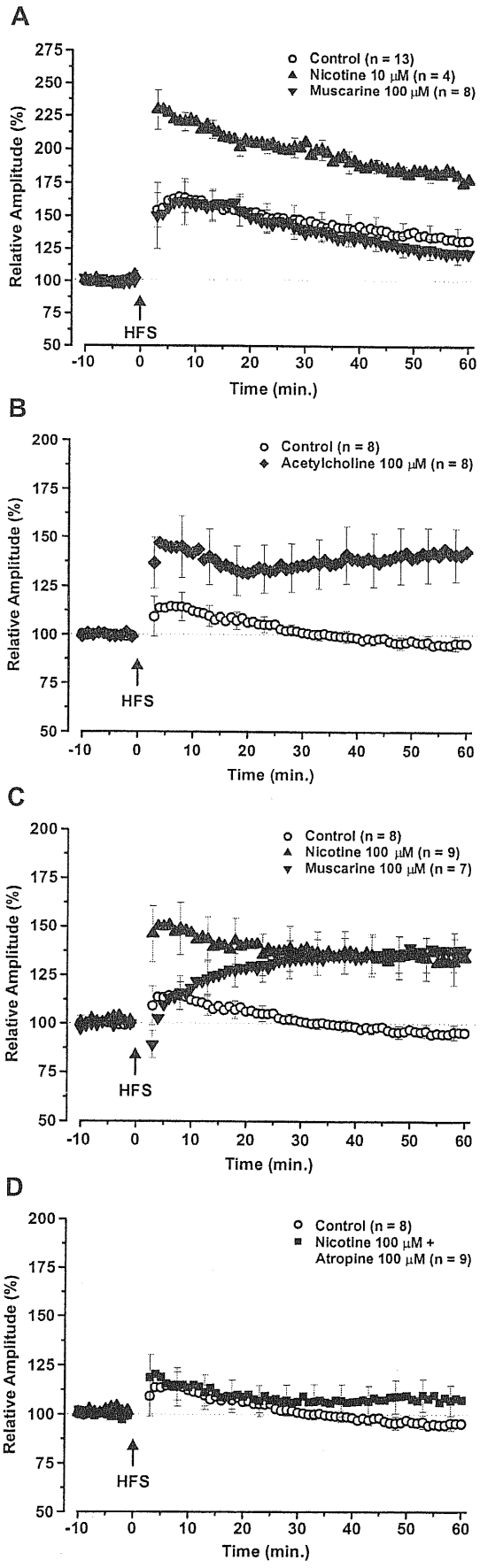
3.22 Antagonists of cholinergic receptors block LTP

The role of the cholinergic system in modulating synaptic strengthening was studied by using pharmacological tools in rat visual cortex slices where cholinergic afferents are preserved. Antagonists of either the muscarinic or the nicotinic component of the cholinergic transmission were released through the recording pipette. Blockade of muscarinic receptors by 100 μM atropine produced an alteration of LTP similar to that observed in basal forebrain lesion experiments. A transient potentiation was induced by HFS, with the amplitude of FP returning to baseline levels after 35-40 minutes (Fig. 3.22; after 40 min., mean FP = 108 ± 7%; after 60 min., mean FP = 94.5 ± 5.9%; n = 11, 4 rats; p <= 0.001). The effect of



3.22 Antagonist of cholinergic transmission blocks LTP

A) At P16-18, blockade of mAChR, by 100 μM atropine (●), significantly impairs LTP as compared to control slices (O).



3.23 Activation of either muscarinic or nicotinic receptors differentially affect LTP

A) At P16-18, 100 μM muscarine (▼) does not alter LTP as compared to controls (O), while 10 μM nicotine (▲) causes a dramatic increase in FP amplitude in response to HFS.

B) At P30-35, 100 μM ACh (◆) promptly rescues LTP as compared to control slices (O).

C) At P30-35, either 100 μM muscarine (▼) or 100 μM nicotine (▲) rescue LTP as compared to controls (O). However, the two treatments differ in the post-tetanic phase, in their response to HFS.

D) At P30-35, concomitant activation of nAChR, by 100 μM nicotine, and blockade of mAChR, by 100 μM muscarine (■), does not rescue significantly LTP, as compared to controls (O).

atropine on LTP was less pronounced when a lower dose was used (20 μM, data not shown). Preliminary results by using nicotinic receptor antagonists suggest that nicotinic receptors are necessary not only in the maintenance of potentiation but also in the induction phase of LTP (data not shown).

Taken together, the results from lesion and pharmacological experiments suggest that the disruption of cholinergic transmission impairs the expression of LTP induced by HFS of the WM.

3.23 Activation of muscarinic or nicotinic receptors differentially affect LTP

At P16-18, when LTP was reliably elicited by HFS, the exogenous application of 100 μM muscarine did not alter the expression and maintenance of LTP as compared to control slices (Fig. 3.23A; after 60 min., mean FP = 124 ± 7%; n = 8, 4 rats). A different result was obtained when low concentration of nicotine was used. 10 μM nicotine induced a dramatic increase of FP amplitude, after HFS, that more than doubled the amplitude of baseline during the post-tetanic phase (Fig. 3.23A; after 60 min., mean FP = 177 ± 4%; n = 4, 2 rats; $p <= 0.001$).

To assess the effectiveness of cholinergic transmission as permissive factor in LTP expression 30-35 days old rats were used, an age at which LTP is no longer inducible. In fact, in control slices a post-tetanic potentiation of small amplitude was observed, that disappeared completely after 10-15 minutes (Fig. 3.23B; after 60 min., mean FP = 94.6 ± 3.7%; n = 8, 8 rats). In contrast, local application of 100 μM acetylcholine rescued a significant and stable potentiation in response to HFS (Fig. 3.23B; after 60 min., mean FP = 143 ± 15%; n = 8, 5 rats; p <= 0.001); similar results were obtained also with 10 μM ACh (data not shown). Data analysis reveals that activation of both nicotinic and muscarinic receptors were able to rescue LTP at the same extent (Fig. 3.23C; 100 μM muscarine: after 60 min., mean FP = 137 ± 10%; n = 7, 4 rats; p <= 0.001; and 100 μM nicotine: after 60 min., mean FP = 135 ± 11%; n = 9, 7 rats; p <= 0.001, respectively). However, a striking difference between the two treatments exists, when the first 10 minutes after HFS are considered. In this time range, while the amplitude of FP in the presence of nicotine promptly reached values equivalent to those obtained by using acetylcholine, the timecourse of the muscarine-dependent potentiation followed a slower kinetics.

These data strongly suggest that nicotinic receptors are involved in induction and possibly in maintenance of LTP, while the muscarinic receptors affect mainly the maintenance of synaptic potentiation. Indeed, in experiments where nicotinic receptors were stimulated by 100 μM nicotine and muscarinic receptors were concomitantly blocked by 100 μM atropine, HFS failed to induce LTP (Fig. 3.23D; after 60 min., mean FP = 108 ± 9%; n = 9, 5 rats), indicating that the activation of muscarinic receptors is necessary to induce a significant and stable potentiation.

3.3 Other possible modulators of synaptic plasticity

Beside the BFB cholinergic system, other modulatory systems are thoughts to play an important role in modulating cortical synaptic plasticity. These systems are both intracortical and extracortical. Among these, the intracortical GABAergic system has been shown to play an important role in determining the critical period for monocular deprivation (Hensch et al 1998a, Huang et al 1999) and in synaptic plasticity (Artola and singer 1987, Huang et al 1999). In particular, concerning synaptic plasticity, GABAergic transmission is thought to play an inhibitory role in LTP expression. In fact, it was reported that blockade of GABAergic transmission has a facilitatory effect in the induction of LTP (Artola and Singer 1987, Huang et al 1999), on the contrary, the increase in GABAergic transmission blocks the expression of LTP (Huang et al 1999). The delivery, through the recording pipette, of the antagonists of $GABA_A$ and $GABA_B$ receptors, 200 μM bicuculline and 50 μM CGP 55845A (CGP) respectively, was unable to rescue potentiation (Fig. 3.3A; after 60 min., mean FP = 86.2 ± 5.2%; n = 4; 2 rats) in all tested slices at P30-35. This result suggests that a localized relieve of GABAergic

inhibition is not able to allow the re-expression of LTP at the age considered, in contrast to what observed in ACh treated slices or in experiments with bath application of bicuculline (Artola and Singer 1987).

The extracortical adrenergic system, arising in locus coeruleus, was reported to affect both synaptic (Bröcher 1992, Skrebitsky and Chepkova 1998) and in vivo visual cortical (Bear and Singer 1986, Imamura and Kasamatsu 1989, Siciliano et al 1999) plasticity. To investigate the effect of such

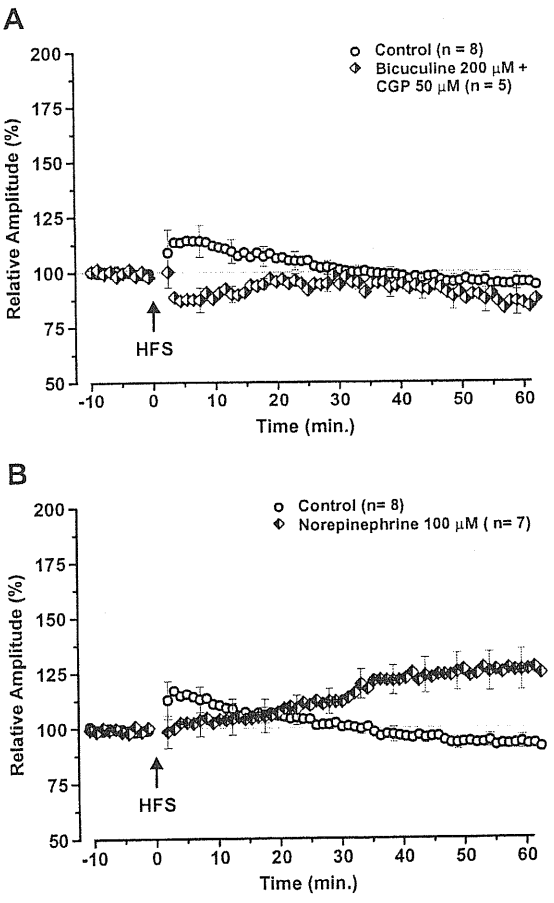


Fig. 3.3 Other possible modulators of synaptic plasticity

A) At P30-35, control slices (O) do not show any significant potentiation in response to HFS. Similarly, local delivery of $GABA_{A/B}$ receptor antagonists (◆) is not able to rescue LTP at this age.

B) At P30-35, local application of the adrenergic receptors agonist, norepinephrine (◆), rescues LTP. However, it takes about 25 minutes, since HFS, to become significant, as compared to control slices (O).

System on LTP the endogenous neurotransmitter, norepinephrine was locally released. At 100 μM concentration, norepinephrine was able to induce a significant LTP in response to HFS (Fig. 3.3B; after 60 min mean FP = 125 ± 8%; n = 7; 3 rats; p <= 0.001). However, the onset of this potentiation followed a very slow kinetic taking about 25 minutes to reach a significant value with respect to the baseline. Thus, even if norepinephrine can effectively promote the rescue of LTP at P30-35, the lack of post-tetanic potentiation suggests that the underlying mechanisms are "different" from those mediating the LTP normally expressed during an early stage of postnatal development (P16-18). It has to be noticed that, in contrast with results observed by using norepinephrine and bicuculline, acetylcholine administered with the same criteria was able to rescue a normal LTP at P30-35, thus suggesting that cholinergic transmission is per se sufficient to induce and to maintain LTP at an age when it is no longer expressed.

3.4 NGF-mediated cholinergic deficit in anti-NGF mice

3.41 LTP impairment in anti-NGF mice

The use of transgenic mice producing anti-NGF antibodies has allowed to study the effects of long-term NGF deprivation on the expression of cortical synaptic plasticity. The phenotypic characterization of these animals showed that they were characterized by: i) a

delayed expression of recombinant antibodies that allows to overcome the deleterious effects of NGF deprivation during the early postnatal development (Capsoni et al 2000a; Ruberti et al 2000). Antibodies are detectable after P45 and allow a 50% reduction of free NGF levels in the brain and, in adulthood they exhibit several neurodegenerative markers and behavioral defects (Ruberti et al 2000; Capsoni et al 2000b); ii) a strong impairment of the cholinergic system as shown by the loss of cholinergic neurons in BFB (Ruberti et al 2000) and a decreased innervation of cerebral cortex as reported in the present thesis (Fig. 3.42A).

The expression of long term forms of synaptic potentiation in the mouse visual cortex was shown to be maximally expressed during the critical period and being down regulated by about its end (Huang et al 1999).

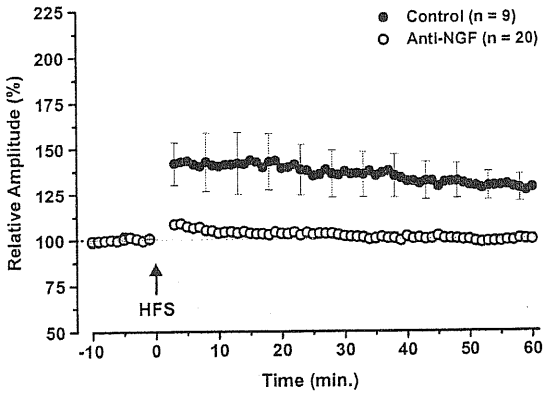


Fig. 3.41 LTP impairment in anti-NGF mice

A) Anti-NGF transgenic mice slices (O) do not show any significant potentiation in response to HFS. Conversely, slices from control animals (●) show significant potentiation.

Preliminary data, obtained in Domenici's laboratory, confirmed these results showing that LTP peaks during the middle of the critical period for monocular deprivation, almost disappearing around the end of such period.

Beyond this observation, new results are reported in this thesis, which show that high-frequency stimulation of the WM/layer VI border induced a significant and stable LTP (Fig. 3.41; after 60 min. mean FP = 128 ± 7%; n = 9; 6 mice) in six months old control mice. In contrast, matched-age anti-NGF mice slices did not express any significant potentiation in response to tetanic stimulation (Fig. 3.41; after 60 min. mean FP = 102 ± 3%; n = 20; 10 mice; p <= 0.001). These results match the findings reported in the experiments where the cholinergic transmission was blocked by both lesion of BFB (see paragraphs 3.21) and local application of ACh receptor antagonists (see paragraphs 3.22).

3.42 Cholinomimetic drugs rescue LTP in anti-NGF mice

Adult anti-NGF mice exhibit a strong reduction in the number of cholinergic neurons in BFB (Ruberti et al 2000). In the present work, this observation was extended to visual cortex slices previously used for electrophysiological recordings. A strong reduction of ChAT immunostaining in the

neuropil of the visual cortex slices from anti-NGF mice (Fig. 3.42A) was found as compared to control slices (Fig. 3.42B). Nevertheless, residual ChAT immunoreactivity is preserved suggesting that spared cholinergic fibers are still present in the visual cortex in anti-NGF mice.

It was then decided to determine whether the local application of acetylcholine was able to rescue strengthening of synaptic transmission in anti-NGF cortex slices. Local delivery of 10 μM ACh produced a small but significant potentiation (Fig. 3.42C; after 60 min. mean FP = 115 ± 7%; n = 6; 6 mice; p <= 0.02), while 100 μM ACh allowed the expression of a nearly maximal potentiation (Fig. 3.42C; after 60 min. mean FP = 173 ± 10%; n = 5; 4 mice; p <= 0.001). A similar result was obtained by inhibiting acetylcholinesterase, with 100 μM edrophonium, increasing in this way the availability of endogenous acetylcholine (Fig. 3.42D; after 60 min. mean FP = 130 ± 12%; n = 5; 5 mice; p <= 0.001). These findings suggest that LTP impairment in anti-NGF mice can be rescued completely by increasing ACh-mediated transmission, thus mimicking the LTP rescue by ACh, muscarinic, and nicotinic agonists observed in P30-35 aged rats (see paragraphs 3.23).

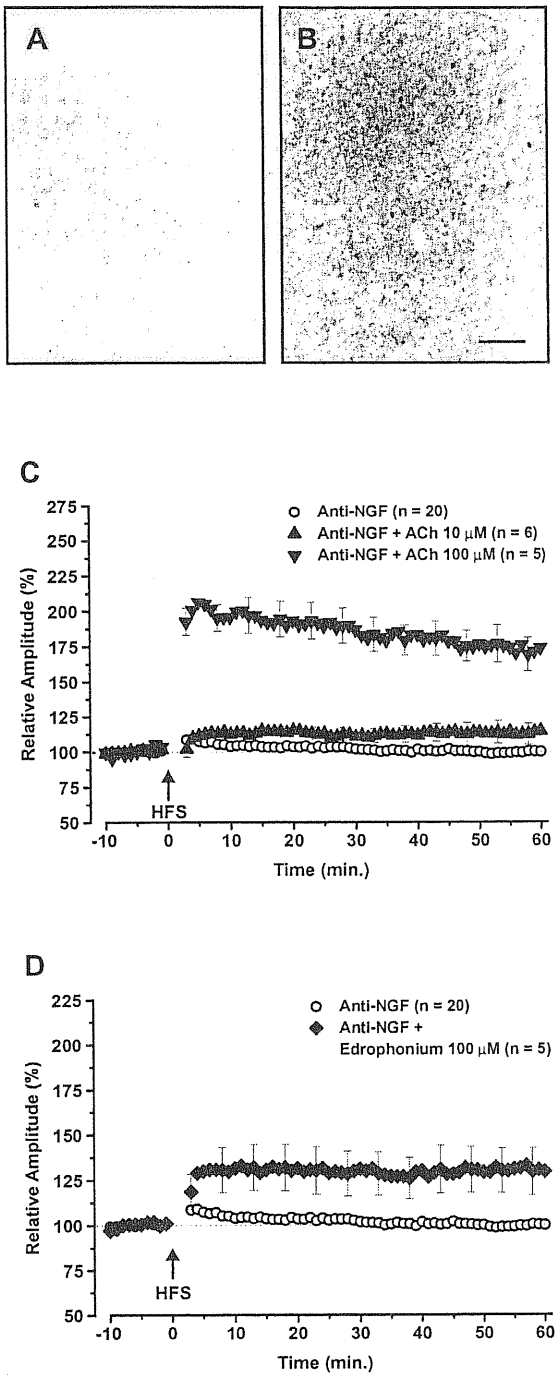


Fig. 3.42 Cholinomimetic drugs rescue LTP in anti-NGF mice

A; B) ChAT immunostaining shows a strong reduction of cortical cholinergic neuropil in anti-NGF mice (A) as compared with control animals (B). Bar = 100 μm.

C) Both 10 μM ACh (▲) and 100 μM ACh (▼) rescue LTP in a dose dependent manner as compared to untreated anti-NGF slices (O).

D) Blockade of acetylcholinesterase with 100 μM edrophonium (◆) rescues a significant LTP as compared to untreated anti-NGF slices (O).

3.5 NGF as modulator of synaptic plasticity

3.51 Exogenous supply of NGF inhibits LTP

Previous studies reported that NGF represents an essential factor for neuronal plasticity. However, the studies reported till now have been focused on in vivo cortical plasticity (Gu 1995, Cellerino and Maffei 1996). In these studies, the effects of agonists and antagonists of NGF action on visual cortical plasticity were assessed by using long treatments, hence making difficult to discriminate between the trophic and other possible actions of NGF. In fact, the question whether NGF can act as a modulator of synaptic plasticity such as LTP and LTD was unanswered. The possible role of NGF in synaptic plasticity was studied in rat visual cortex slices, which contain the cholinergic afferents. In particular, were examined the effect of local acute supply of NGF, and compounds blocking the NGF signaling, on LTP expression.

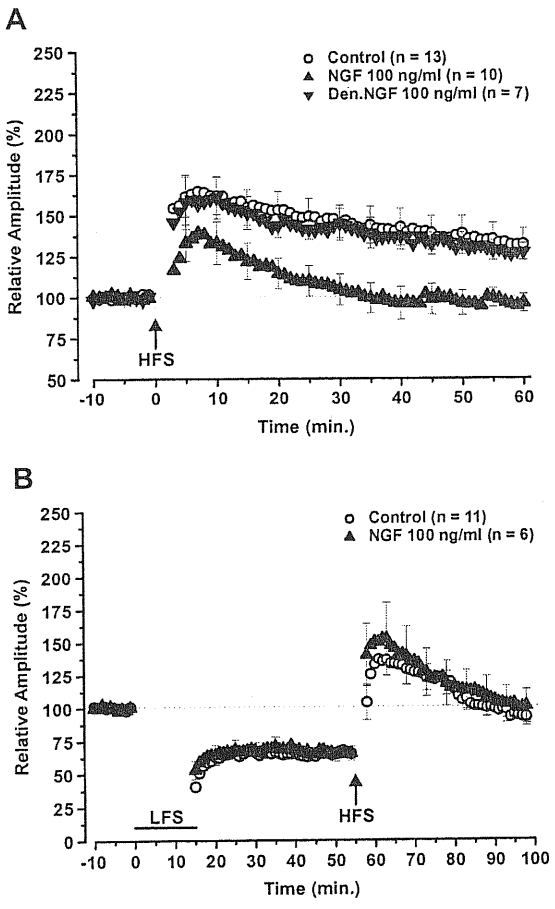


Fig. 3.51 Exogenous supply of NGF affects LTP

A) At P16-18, application of 100 ng/ml NGF (▲) is able to block potentiation as compared to control slices (O). 100 ng/ml of heat inactivated NGF (▼) is unable to prevent LTP expression.

B) At P16-18, administration of 100 ng/ml NGF (▲) does not affect either LTD and potentiation of previously depressed synapses as compared to controls (O).

In the presence of local release of 100 ng/ml NGF, the potentiation was blocked (Fig. 3.51A); in fact, 20-25 minutes after tetanic stimulation the FP amplitude was not significantly different from the baseline condition (after 25 min. mean FP = 108 ± 9.2%, after 60 min mean FP = 95.6 ± 5.6%; n = 10; 5 rats; p <= 0.001). While at P16-18, a consistent LTP was induced in response to HFS (Fig. 3.51A; after 60 min., mean FP = 133 ± 10%; n = 13; 11 rats) as previously described. It has to be noticed that the concentration of 100 ng/ml in the pipette should correspond to a nearly physiological concentration range of NGF in the surrounding tissue. In addition, NGF did not change the basic properties of field potentials, namely their amplitude, shape and dependence on stimulation rate.

In order to check the specificity of the inhibition of potentiation by NGF, slices were treated with locally delivered Den.NGF. It was tested on PC12 cells for potential residual activity immediately before slice application (Pesavento et al 2000). Results reported in figure 3.51A clearly show that LTP was not altered by local delivery of Den.NGF (100

ng/ml; after 60 min. mean FP = 127 ± 6%, n = 7; 4 rats).

In a further set of experiments, it was shown that the NGF effect was restricted to LTP, and did not extend to other forms of synaptic plasticity. Stable LTD was induced by low frequency stimulation of WM in all control slices, at P16-18. Data reported in Fig. 3.51B indicate that the amplitude of LTD was not significantly different between control and 100 ng/ml NGF treated slices (after 40 min. since LFS, mean FP = 66.3 ± 3.7%, n = 11; 9 rats; mean FP = 65.1 ± 4.9%, n = 6; 4 rats, respectively).

In a previous paper (Sermasi et al 1999a), it was reported that it is possible to potentiate previously depressed synapses at an early stage of postnatal development (P16-18) when visual cortical circuitry is still immature. To test if NGF would inhibit this form of synaptic plasticity, the amplitude of potentiation after tetanic stimulation of previously depressed synapses was compared. From Fig. 3.51B it appears that HFS of previously depressed synapses reliably elicited potentiation, in both NGF-treated and untreated slices without significant differences.

3.52 LTP dependence on endogenous NGF level

The evidence that exogenous NGF application is able to block LTP at P16-18 prompted me to investigate the role of endogenous NGF on this form of synaptic plasticity. To this purpose, a soluble form of TrkA receptor engineered as an immunoadhesin (TrkA-IgG) was used. With 1 μg/ml TrkA-IgG in the pipette filling solution, spontaneous bursts of activity (afterdischarge) during the HFS protocol was observed, a clear indication of neuronal hyper-excitability. Despite this effect, FP was maximally potentiated only in the post-tetanic phase, decreasing in 25-30 min. to LTP values recorded in control slices (Fig. 3.52A; after 60 min. mean FP = 135 ± 19%; n = 8; 4 rats). The concentration used is known to prevent NGF dependent differentiation of PC12 cells (Pesavento et al 2000), and it is several orders of magnitude greater than the concentration of cortical endogenous NGF (Whittemore et al 1986).

Having first established that neutralization of endogenous NGF at P16-18 did not affect LTP, then the potential role of endogenous NGF at P30-35 was examined, when LTP is no longer inducible. Blockade of endogenous NGF by 1 μg/ml TrkA-IgG, was able to rescue LTP following the HFS protocol (Fig. 3.52B; after 60 min. mean FP = 122 ± 9%; n = 10; 5 rats; p <= 0.001), as compared to control slices (Fig. 3.52B; after 60 min. mean FP = 94.6 ± 3.7%; n = 8; 8 rats). Since it was shown that high concentration of TrkA-IgG, can cross react with BDNF (Pesavento et al 2000), it was important to check whether the re-appearance of LTP was due to blockade of other related neurotrophins, in addition to NGF. To answer this question, TrkB-IgG was

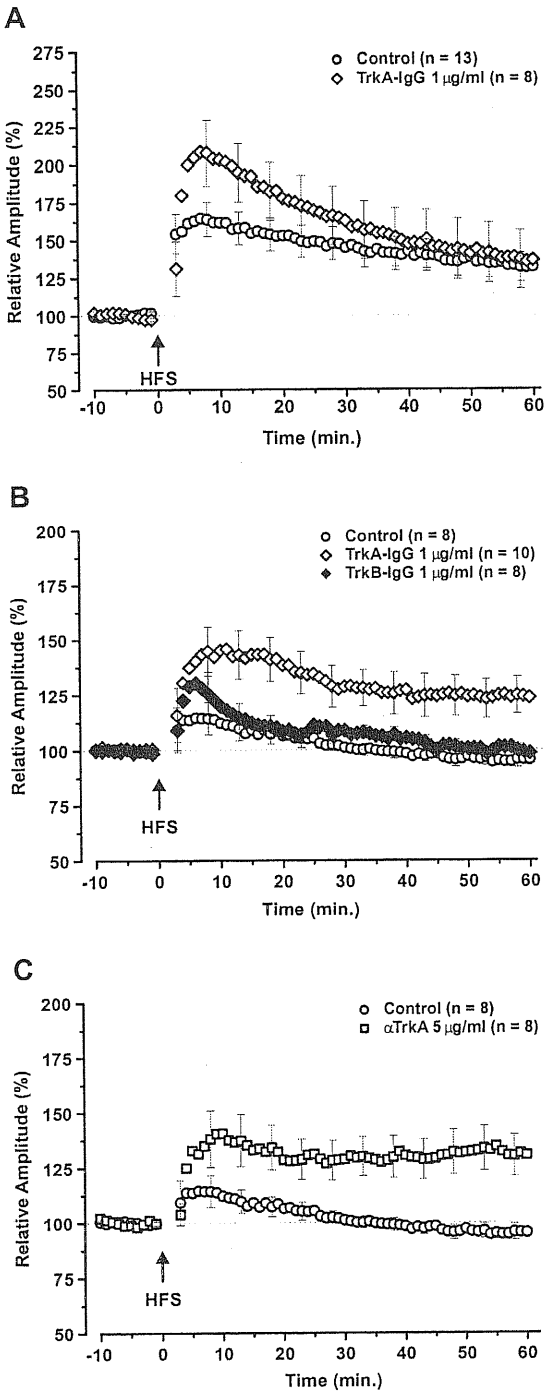


Fig. 3.42 LTP dependence on endogenous NGF level

A) At P16-18, local depletion of endogenous NGF, by administration of 1 µg/ml TrkA-IgG (◇) does not alter significantly LTP as compared to control slices (○).

B) At P30-35, blockade of endogenous NGF, by local application of 1 µg/ml TrkA-IgG (◇), rescues the ability of slices to be potentiated as compared to controls (○). Conversely, blockade of endogenous BDNF and NT-4, by 1 µg/ml TrkB-IgG (◆), does not enable LTP to be re-expressed.

C) At P30-35, blockade of TrkA receptor, by local administration of 5 µg/ml αTrkA (□), allows re-expression of LTP, as well as TrkA-IgG, in response to HFS protocol as compared to controls (○).

used, an immunoadhesin that binds and sequesters endogenous BDNF and NT-4. Fig. 3.52B shows that locally delivered 1 µg/ml TrkB-IgG was not able to re-induce LTP at P30-35 (after 60 min. mean FP = 98.9 ± 3.1%; n = 8; 5 rats).

In a second group of experiments, the role of TrkA on LTP at P30-35 was examined. To this purpose, a new monoclonal antibody MNAC13 was used, which has been raised against human TrkA receptor (αTrkA) expressed in balb/c 3T3 cells. It recognizes the TrkA receptor with a high degree of specificity (Cattaneo et al 1999). αTrkA antibodies included into the pipette filling solution at 5 µg/ml concentration induced the re-appearance of LTP (Fig. 3.52C; after 60 min. mean FP = 131 ± 10%; n = 8; 4 rats; p <= 0.001) in response to HFS, in analogy to what was found using TrkA-IgG.

3.6 Interaction between the cholinergic system and NGF

A large body of evidence indicates that the TrkA receptor is present in the visual cortex, and is maximally expressed on BFB cholinergic neurons (Steininger et al 1993, Sobreviela et al 1994, Li et al 1995, Molnar et al 1998), including the cholinergic terminals in the visual cortex (Pizzorusso et al 1999). Hence, the possible involvement of the cholinergic system in NGF dependence of LTP was examined.

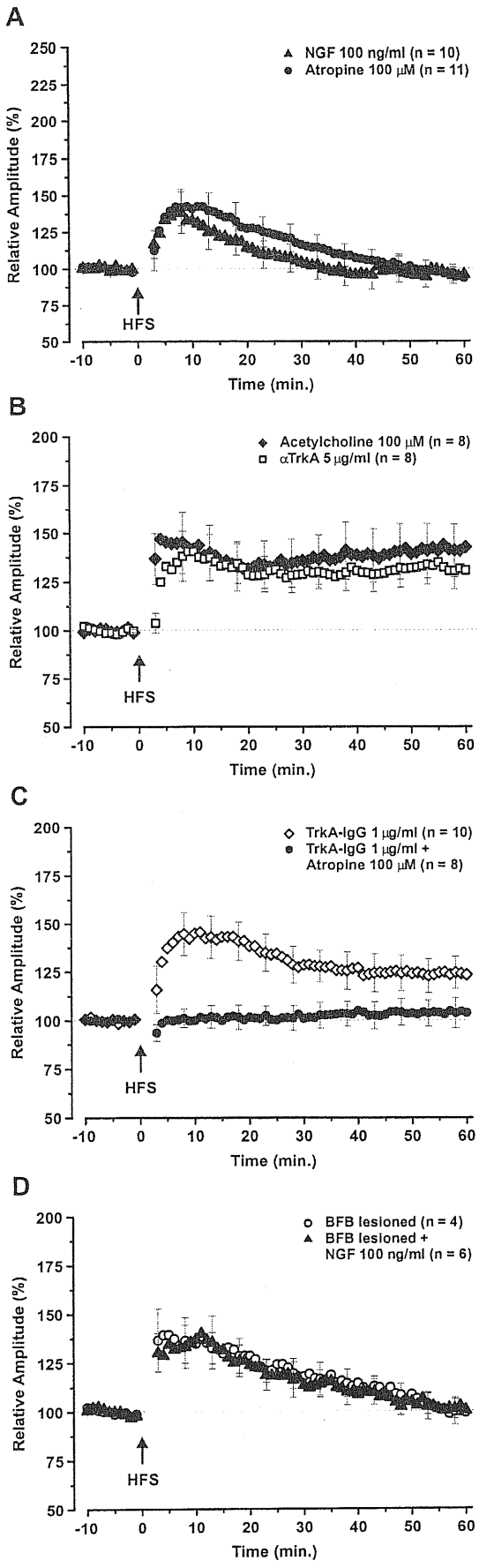


Fig. 3.6 Interaction between cholinergic system and NGF

A) At P16-18, local application of 100 µM mAChR antagonist, atropine (●), mimics the effect of 100 ng/ml NGF (▲) application in inhibiting potentiation.

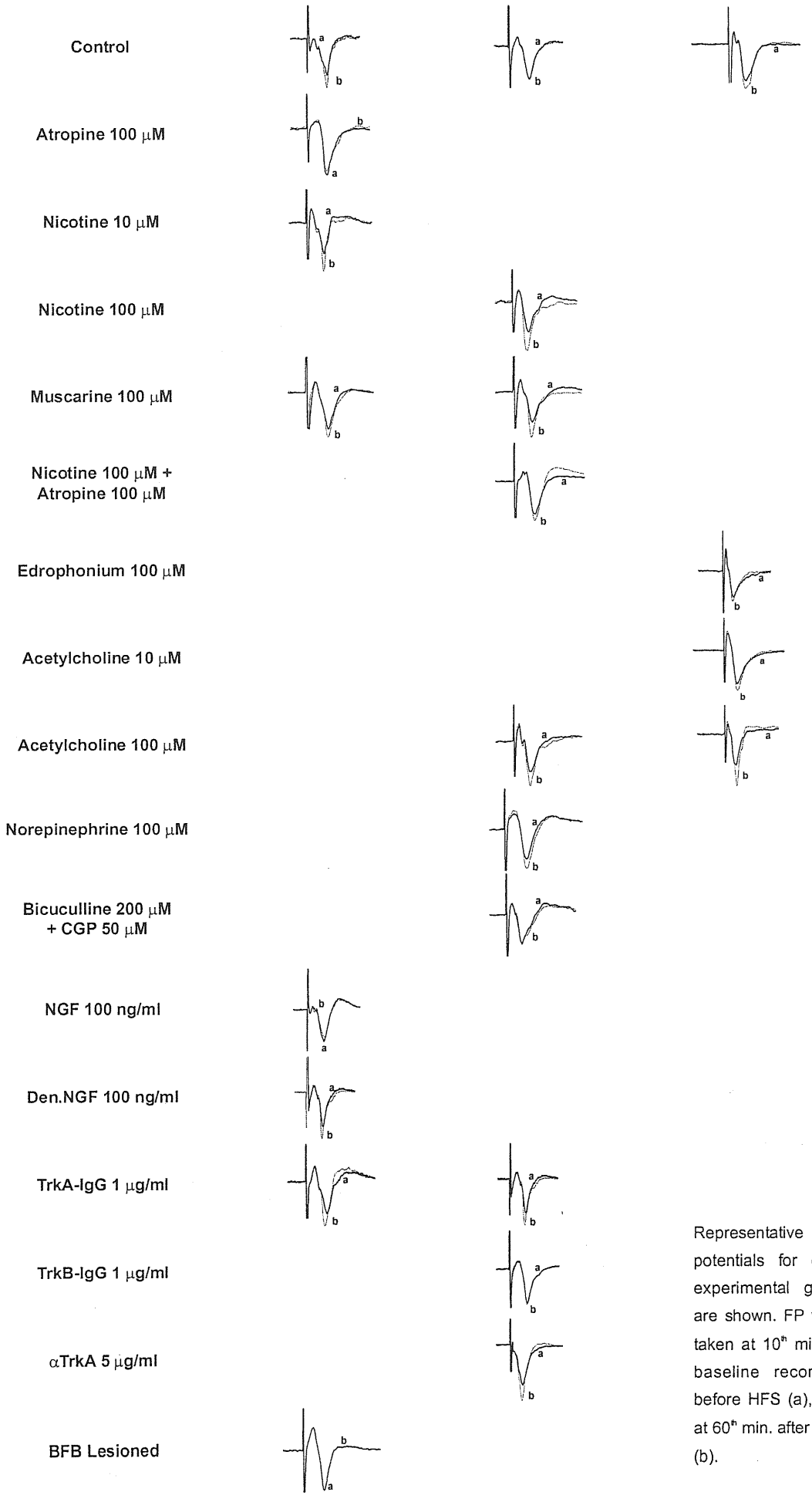
When the response to HFS in NGF- and atropine-treated slices were compared at P16-18, it was clear that the effects elicited by the two treatments were similar with almost perfect overlapping curves describing the potentiation timecourse (Fig. 3.6A). In both cases, a transient post-tetanic potentiation returned to baseline levels in about 25-30 minutes. Thus, an increase of NGF levels (i.e. an increase of TrkA activation), as well as blockade of cholinergic transmission, inhibits LTP. At P30-35, in both acetylcholine and αTrkA treated slices, LTP was readily and significantly re-expressed with the same timecourse (Fig. 3.6B). This suggests that both stimulation of cholinergic system and blockade of TrkA signaling have a permissive effect on LTP expression. Particularly interesting was the observation that LTP could not be re-induced when 100 µM atropine was locally supplied together with 1 µg/ml TrkA-IgG (Fig. 3.6C, after 60 min. mean FP = 104 ± 8%; n = 8; 5 rats). These data are consistent with an interaction between NGF-TrkA signaling and the cholinergic system in regulating LTP.

B) At P30-35, blockade of TrkA receptor, by local administration of 5 µg/ml αTrkA (□), allows re-expression of LTP in response to HFS protocol, as well as 100 µM ACh (◆).

C) At P30-35, 100 µM atropine (●) is able to prevent TrkA-IgG-mediated potentiation (◇).

D) Identical timecourse of posttetanic FP amplitude was seen in 100 ng/ml NGF (▲) and untreated (○) BFB lesioned rat slices.

To further support this possibility, it was taken advantage of visual cortical slices deprived of BFB projecting fibers (see paragraph 3.21). The aim was to ascertain whether NGF, in absence of its main target represented by the cholinergic fibers, was still capable of inhibiting LTP. At P16-18, the experiments on LTP in the presence of local application of NGF were repeated, this time cortex slices from the hemisphere ipsilateral to the BFB lesion and therefore largely deprived of cholinergic input (see Fig 3.21A). As expected, no differences in FP amplitude timecourse were observed between NGF treated and untreated slices (Fig. 3.6D) of BFB lesioned animals. This result suggests that LTP is no longer sensitive to NGF when the visual cortex is deprived of the cholinergic input.



Representative field potentials for each experimental group are shown. FP were taken at 10th min. of baseline recording before HFS (a), and at 60th min. after HFS (b).

4.0 DISCUSSION

Different forms of long term changes in synaptic efficacy are differentially regulated during postnatal development of rat visual cortex. LTP is maximally expressed soon after eye opening, at P15, and is progressively down regulated thereafter, completely disappearing after P30 (Kirkwood et al 1995). Synapses are also capable of reversing a given long-term change in synaptic efficacy (bidirectional plasticity) at P16-18 but this property is lost one-week later (Sermasi et al 1999a). Finally, LTD is expressed throughout life, although its amplitude is reduced in adult animals (Sermasi et al 1999b). In this study, it was attempted to identify possible factors modulating the expression of LTP in visual cortex, both during rat postnatal development and mouse adulthood.

4.1 Methodological considerations

4.11 Local delivery of compounds

An important feature of the present work was the delivery of drugs through the recording pipette filling solution. This novel approach allowed to avoid a general, indiscriminate stimulation of the entire cortical network, and to restrict the action of substances to a small area surrounding the pipette tip. Reported effects of drugs in previous works (Tancredi et al 1993, Bröcher et al 1992, Sokolov and Kleschevnikov 1995), led to estimate a 10 to 100 fold dilution, close to the pipette, when substances dissolved in the pipette filling solution were delivered into the slice. According to this estimation, as general rule, concentrations 10 fold higher than those found to be effective when the same compounds were added to perfusion medium were employed.

In an attempt to estimate the range of diffusion of substances coming out from the pipette tip, it was made use of different biocytin concentrations locally applied through the recording pipette. The lower molecular weight of biocytin presumably causes a faster diffusion, as compared to proteins, such as NGF and immunoadhesins. However, this is probably compensated for by a less effective uptake due to the interaction of biocytin with low affinity

aminoacid receptors. The reported results show that, at the lowest concentration used (which is still, at least, a hundred fold higher than the concentration of proteins/drugs employed in the experiments), just a few neurons were labeled, thus supporting the idea of a spatially restricted action of substances delivered following this method. In general, it is conceivable that endogenous neurotransmitters and neuromodulators are not released in massive concentrations over wide areas in normal physiological conditions, such as instead happens in perfusion experiments. Rather, it is more likely that the release of such substances, in the extracellular space, is restricted both in time and in space, as well as in their amount. Probably, this is particularly true for NT, which are powerful substances with hormone-like properties.

Further support to the idea of a circumscribed and mild action of substances released from the pipette comes from experiments in which the agonist of nicotinic receptor class, nicotine, was employed. It is known that nicotinic receptors rapidly desensitize when challenged with endogenous or exogenous agonists. In the experimental conditions used, the diffusion of nicotine from the pipette lasted for at least 25 minutes before application of the HFS protocol. Nevertheless, a significant effect of nicotinic stimulation was observed in response to tetanic stimulation (see paragraph 3.23), suggesting that the local concentration of nicotine was lower than the desensitizing level for nicotinic receptors.

4.12 LTP and LTD induction

One of the major weaknesses of most studies concerning synaptic plasticity is represented by the stimulation protocol used to induce strengthening or weakening of synaptic transmission. As matter of fact, it could be objected that synapses in the brain would never be challenged with patterns of stimuli such as HFS and LFS. This seems particularly true for the LFS protocol, because it is difficult to imagine that any natural sensory stimulus could last for 15 minutes with a constant intensity (neuronal response would almost certainly undergo adaptation). The HFS protocol can be considered a very intense stimulus, possibly much stronger than any "physiological" sensory input. However, two considerations justify the use of this kind of induction protocol: i) the average potentiation in control slices was well below the maximal inducible strengthening of field potentials (see Fig. 3.23A and 3.41/42C); ii) in the great majority of slices, FP potentiation lasted for at least one hour after HFS protocol, suggesting that no gross damage was induced. These observations, are partially supported by recent data

(Reinagel et al 1999) showing that cat LGN relay neurons can fire bursts of frequency as high as 90 Hz even if with low probability.

In the attempt to find a more "physiological" way of inducing modifications in synaptic strengthening, several authors started to use the so-called theta bursts stimulation (TBS). This protocol is based on the fact that in hippocampus, both *in vivo* and *in vitro,* oscillations in the range of theta frequencies (3-12 Hz), can be induced by several stimuli (Bland 1986). The same kind of theta oscillations was described in neocortical slices (Lukatch and Mc Iver 1997). For instance, blocking GABA transmission and activating the cholinergic receptors at the same time induces theta oscillations, and the neocortical oscillation generator is localized in the superficial layers, and the peak amplitude of oscillations occurs in layers II/III. However, it has to be recognized that the TBS protocols used are, only on theoretical ground, related to the theta oscillatory phenomenon. In any case, the TBS protocol was not as effective as the HFS protocol in inducing LTP in the experimental preparation used. Recently, Huerta and Lisman (1995) reported that a very brief burst of stimuli at high frequency can lead to strengthening or weakening of synaptic transmission depending on whether the burst is in phase or out of phase with carbachol-induced theta oscillations, i. e. with the activation of cholinergic receptors. Thus, it can be hypothesized that, when the sensory input is in phase with the cholinergic modulatory input, synapses are strengthened, while when these two inputs are not synchronized the synaptic transmission is either unmodified or weakened depending on the degree of the mismatch. In other words, synapses might behave as coincidence detectors.

The experimental preparation used was probably critical in allowing us to observe such striking results on the effects of the cholinergic system in modulating synaptic plasticity. In the neocortex, the thalamocortical fibers and the BFB cholinergic fibers are intermingled where they ascend from the underlying white matter toward the pial surface. Thus, when white matter is stimulated, there is a concomitant activation of both the thalamic input and the cholinergic input, a coincidence that synapses are built to detect.

4.2 The cholinergic system and synaptic plasticity

It is well known that cholinergic transmission has a role in modulating memory and attentional processes (Wenk 1997, Baxter and Chiba 1999), and cortical plasticity in sensory cortices (Bear and Singer 1986, Baskerville et al 1997, Kilgard and Merzenich 1998). In the present work, it was dealt with the question whether the cholinergic system may act as a modulator of cortical synaptic plasticity. In hippocampus, disruption of cholinergic projecting fibers by several methods appears to have no effect on the expression of LTP (Kleschevnikov et al 1994, Jouvenceau et al 1996), while pharmacological activation or inhibition of the cholinergic transmission gives conflicting results (Williams and Johnston 1988, Blitzer et al 1990, Sokolov and Kleschevnikov 1995). In the visual cortex, it was reported that cholinergic agonists enhance LTP, but only in conjunction with agonists of adrenergic receptors (Bröcher et al 1992).

To assess the influence of the cholinergic system in modulating LTP in the visual cortex a rodent model was used, in which the BFB was lesioned. This animal model was previously used to assess the effects of cholinergic input deprivation on the postnatal development of the visual cortex. The lesion of BFB neurons projecting to the visual cortex, namely nuclei of diagonal band of Broca and nucleus basalis (Mesulam et al 1983, Butcher and Semba 1989, Sefton and Dreher 1995) was performed by stereotaxic injections of quisqualic acid, which at high concentration has a cytotoxic effect. Following this procedure it has been previously reported that ChAT immunoreactivity and ChAT activity were strongly reduced in the rat visual cortex (Siciliano et al 1997). In the present work, previous results were confirmed by showing that after quisqualic acid injections ChAT immunoreactivity was strongly affected, almost disappearing. As reported in paragraph 3.21, cytotoxic lesion of the BFB, by quisqualic acid injection, caused the impairment of LTP in rat visual cortex slices. This result was a first indication that BFB innervation is essential for the expression of synaptic potentiation. However, since the cytotoxic effect of quisqualic acid is non-specific for the cholinergic neurons it was not possible to exclude that other BFB populations projecting to the visual cortex, such as GABAergic neurons (Gritti et al 1997), could be lesioned as well. To exclude such possibility future experiments have to move towards two directions: i) testing the possibility to rescue LTP impairment induced by BFB lesions with a local application of ACh in slices deprived of

cholinergic input; ii) producing selective lesions of BFB cholinergic neurons sparing other neuronal populations such as the GABAergic neurons projecting to the visual cortex.

To clarify whether the impairment of synaptic potentiation was due to deficits in cholinergic transmission a pharmacological approach was used. This kind of approach is complicated by the presence of different subtypes of cholinergic receptors expressed throughout the neocortex. There are five different subtypes of muscarinic cholinergic receptors (M_{1-5}) coupled to different second messengers, and distributed both at pre- and postsynaptic sites (Caulfield and Birdsall, 1998). It is generally accepted that M_1 receptors are located at postsynaptic sites (Mrzljak et al 1993, Levey 1996). The localization of M_2 receptors is less clearly defined. Traditionally, they are localized to presynaptic boutons acting as negative feedback auto-receptors. However, electron microscopy studies identified M_2 receptors also on postsynaptic pyramidal and non-pyramidal neurons (Rouse et al 1997, Mrzljak et al 1998). In addition, Auerbach and Segal (1996) described a form of LTP dependent on activation of M_2 postsynaptic receptors in hippocampus. A similar complex scenario appears for the nicotinic receptor class. Two receptor subtypes seem mainly expressed in the neocortex, the $\alpha4\beta2$ subtype, which account for more than 90% of nicotinic binding sites, and the $\alpha7$ subtype that is characterized by a high permeability to calcium ions (Mc Gehee and Role 1995). So far, the nAChR are considered to be located at presynaptic terminals where they modulate synaptic transmission. More recently, it has been suggested that nicotinic receptors are expressed also at the postsynaptic level (Jones et al 1999). Such difficulties in discriminating presynaptic versus postsynaptic effects and the poor selectivity of pharmacological tools acting on different muscarinic receptor subtypes, as well as the substantial lack of specific drugs for the different nicotinic receptor subtypes, led to the use of the endogenous neurotransmitter ACh and the agonist/antagonist recognizing all subclasses of nicotinic and muscarinic receptors. It was found that blockade of cholinergic receptors in layer II/III, by atropine, affects LTP at P16-18. Conversely, activation of cholinergic receptors by local application of the endogenous neurotransmitter ACh promptly rescued LTP at P30-35. Also muscarine and nicotine rescued LTP at this age but with a different kinetic. Nicotine treatment mimicked the effect of ACh on LTP both in the onset and in the maintenance phase, while in the presence of muscarine the onset of LTP was much slower. Muscarine and nicotine administration modulates differently LTP expression at P16-18. At this age, while muscarine did not alter significantly the LTP expression as compared to controls, nicotine allowed the expression of a nearly saturating LTP.

The observed differences between stimulation of nicotinic receptors versus the muscarinic receptors suggest that nicotinic receptor activation can play a major role in the induction phase of synaptic strengthening, while the metabotropic (muscarinic) receptors can exert a specific role in maintenance of potentiation. Following this suggestion, the activation of nAChR in the presence of an inhibitor of mAChR should induce a post-tetanic potentiation with the FP recovering baseline values in a short time. In other words, in these experimental conditions, it should be possible to induce but not to maintain LTP. In contrast, it was found that the concomitant activation of nAChR and inhibition of mAChR did not induce a clear post-tetanic potentiation at P30-35, indicating that the activation of the muscarinic component, by endogenous ACh, is necessary in order to have a full nicotinic-mediated LTP induction.

Taken together these results support the idea that the cholinergic system is able to modulate LTP in the visual cortex. In particular, it was shown that cholinergic transmission is both necessary and sufficient for a full expression of LTP. Moreover, these findings suggest that nicotinic and muscarinic receptors play slightly different roles in the induction and maintenance of potentiation. The results, concerning muscarinic receptors, reported in the present work are in agreement with previous data obtained in *in vivo* visual cortical plasticity. Indeed, it was reported that blockade of muscarinic receptors in developing visual cortex was able to prevent the effects of monocular deprivation (Gu and Singer 1993). Thus, although possible discrepancies between *in vivo and in vitro* experiments may exist, these data indicate that cholinergic system controls neuronal plasticity in the visual cortex.

The maturation and functional neuroanatomy of the BFB cholinergic system fits the requirements necessary for playing a key role in cortical synaptic plasticity during development. Rat BFB cholinergic neurons and ChAT enzyme complete their maturation by the end of the second postnatal week, i. e. when the animals open their eyes (Dinopoulos et al 1989, Gould et al 1991). All muscarinic (Weiner and Brann 1989, Levey et al 1991, Mrzljak et al 1993, Schliebs et al 1994, Hohmann et al 1995, Aubert et al 1996, Tigges et al 1997) and nicotinic (Clarke et al 1985, Swanson et al 1987, Bravo and Karten 1992, Sargent 1993, Dominguez del Toro et al 1994, Broide et al 1995, Nakayama et al 1995, Aubert et al 1996) receptors described are present in layers II/III, where all the drugs tested were locally applied. In addition, all these receptors reach their adult pattern of distribution and level of expression between P15 and P35, i.e. coincident with the time window of the critical period (Aubert et al 1996). Only M_2 receptors start to be expressed significantly after P7 and their levels continue to increase at least up to

P60 (Aubert et al 1996). This delayed and continued increase of the M_2 receptor, in conjunction with its presumed role as mediator of negative feedback signals, could account for the down-regulation of LTP in the developing rat visual cortex.

Beyond cholinergic system, the extracortical adrenergic system, arising in locus coeruleus, was also reported to affect both synaptic (Bröcher 1992, Skrebitsky and Chepkova 1998) and in vivo visual cortical (Bear 1986, Imamura and Kasamatsu 1989, Siciliano et al 1999) plasticity. Experiments performed at P30-35 showed a rescue of LTP as well as it was seen with cholinergic agonists. However, the timecourse of LTP onset appeared to be very slow and the potentiation became significant only 25 minutes after HFS. This peculiar timecourse suggests that the mechanisms underlying the LTP induced by norepinephrine are different from those responsible for LTP expression at P16-18 and those mediated by the cholinergic system at P30-35. Certainly, it would be of great interest to investigate deeper the role of the adrenergic system in modulation of synaptic plasticity and its possible interaction with other modulatory systems.

4.3 Anti–NGF mice and synaptic plasticity

NGF is expressed in BFB cholinergic neurons (Lauterborn et al 1991, Saporito and Carswell 1995) and in the regions of nervous system innervated by the magnocellular component of BFB nuclei (Korsching et al 1985, Large et al 1986, Whittemore et al 1986), including the visual cortex. In addition, BFB cholinergic neurons are sensitive to NGF and reduce their ChAT expression when endogenous NGF is blocked (Steininger et al 1993, Sobreviela et al 1994, Li et al 1995, Molnar et al 1998). In particular, cholinergic terminals are reduced in the cortical areas in response to a functional blockade of endogenous NGF (Debeir et al 1999).

A dysfunction of the central cholinergic system is postulated to be responsible for plastic changes leading to cognitive and learning deficits (Wenk 1997). In the present thesis, it was shown that the integrity of the BFB cholinergic system is an essential requirement for the expression of LTP in the developing rat visual cortex (see paragraph 4.2). Beyond these effects in developing visual cortex, it was of great interest to know whether cholinergic system could play a role in adult forms of synaptic plasticity. To this aim, it would be helpful to employ animal

models where the integrity of BFB cholinergic neurons is lost, such as happen in NGF-deprived animals, and consequently the expression of synaptic plasticity in the neocortex should be altered. However, homozygous transgenic mice in whom the NGF gene has been knocked out do not survive more than 3 weeks (Crowley et al 1994), preventing the analysis of NGF deprivation in adult animals. Heterozygous mice from the same line survive until adulthood but they show only a mild phenotype due to an ineffective block of free NGF (Chen et al 1997). To overcome this problem, advantage of a transgenic mouse (Ruberti et al 2000) expressing a neutralizing anti-NGF antibody was taken. As reported in the results section, in anti-NGF mice the level of antibodies increases after birth up to two months, reaching a stable level afterwards. It has been estimated that antibodies (Ruberti et al 2000) block 50% of total NGF. Previous studies confirmed at neuroanatomical level the effectiveness of such NGF deprivation both in the peripheral and central nervous system (Capsoni et al 2000a, b, Ruberti et al 2000). ChAT expression was dramatically decreased in BFB and hippocampus too (Ruberti et al 2000).

In the present work, it was provided evidence that cholinergic innervation is reduced also in the visual cortex (see Fig. 3.42A, B). It has to be remarked that, although strongly reduced, a few cholinergic fibers are still present in the visual cortex of anti-NGF mice suggesting that a residual cortical cholinergic innervation resists to NGF deprivation.

To analyze the effects of NGF deprivation on synaptic plasticity mice slices containing the visual cortex were used. As reported in the results section, LTP elicited by HFS of white matter is expressed during the critical period for monocular deprivation (Huang et al 1999, paragraph 3.41), as well as in adulthood. As expected, it was found that in anti-NGF mice there was an impairment of LTP as compared to control animals; moreover, this deficit could be completely rescued by either exogenous application of ACh or blockade of AChE (that is increasing the availability of endogenous ACh in the extracellular space). In fact, the evidence that blockade of AChE is sufficient to restore LTP is in accordance with immunohistochemical data showing a residual cholinergic cortical innervation in anti-NGF mice.

Thus, it appears that the cholinergic system is an important modulator of synaptic plasticity both during development and in adulthood. It could be objected that chronic deprivation of NGF caused a widespread degeneration of the cortex and this would be the reason why LTP is impaired. Indeed, it was reported that at this age, in addition to a cholinergic deficit, the anti-NGF mice exhibit a complete compendium of neurodegenerative markers, as well as extensive neuronal loss throughout the cortex (Ruberti et al 2000, Capsoni et al 2000a,

b). However, two considerations favor the idea of a predominant role of degeneration of the cholinergic system in impairing LTP. At first, synaptic transmission in transgenic mice did not appear to be affected by NGF deprivation; indeed, field potentials did not differ in their amplitude, latency and timecourse from age-matched controls. Secondly, the ability of cholinomimetic drugs, namely ACh and edrophonium, to restore LTP in visual cortical slices of anti-NGF mice indicate that at this stage of the neurodegenerative process, the cholinergic system appears to be the most affected and to be the most likely candidate for mediating the alterations observed on synaptic plasticity.

4.4 NGF and synaptic plasticity

Neurotrophins have been proposed to modulate activity-dependent development and plasticity of visual cortex (Gu 1995, Thoenen 1995, Bonhoeffer 1996, Cellerino and Maffei 1996). In particular, NGF has been shown to prevent the geniculocortical alterations induced by monocular deprivation performed during a restricted time window of postnatal development (Domenici et al 1991, Maffei et al 1992). However, the role of NGF in modulating synaptic efficacy changes is largely undefined. In the previous paragraph, it has been reported that a chronic deprivation of NGF, as that observed in anti-NGF mice, alters the expression of an important form of synaptic plasticity, namely LTP. However, the fact that NGF was chronically deprived had the consequence to reduce the cortical cholinergic innervation, which in turn was responsible for LTP alteration. An important question is whether acute changes in NGF cortical level, not interfering with cortical cholinergic innervation, may modulate the efficiency of synaptic transmission, in particular LTP.

NGF is synthesized in the neocortex and it is maximally expressed in layers II/III (Lauterborn et al 1994, Nishio et al 1994). In hippocampal slices, it has been reported that NGF blocks the expression of LTP (Tancredi et al 1993). In contrast, other groups have reported no effects of NGF in modulating synaptic plasticity (Kang and Schuman 1995, Figurov et al 1996, Akaneya et al 1997).

In this thesis it was demonstrated that, at an early stage of postnatal development (P16-18), a local supply of NGF in layer II/III affected LTP elicited by a tetanic stimulation of white

matter in the rat visual cortex. In particular, synaptic potentiation could not be maintained longer than 20-25 min. The NGF effect on potentiation was specific, since control experiments with biologically inactive NGF left LTP amplitude completely normal. Thus, NGF seems to play an inhibitory role in LTP elicited by tetanic stimulation of the white matter. This NGF effect on synaptic plasticity is restricted to LTP. Indeed, NGF affected neither LTD nor LTP of previously depressed synapses elicited by stimulation of WM; it may well be that these two forms of synaptic plasticity rely on other neurotrophins and/or different factors and mechanisms (Mc Allister et al 1999).

In a previous paper (Akaneya et al 1997) dedicated to the role of neurotrophins in visual cortex synaptic plasticity, it was reported that exogenously supplied NGF is not able to induce modifications of LTP elicited by stimulation of layer IV. Possible discrepancies could depend upon experimental differences; indeed, in that study, NGF was dissolved in the perfusion medium, while in my experiments it was locally applied. In addition, stimulation of layer IV induces activation of a different type of intracortical circuitry with respect to stimulation of WM (Kirkwood and Bear 1994).

At P16-18 blockade of endogenous NGF achieved by local supply of soluble forms of TrkA receptor (TrkA-IgG) often induced spontaneous bursts of field potentials during tetanic stimulation but did not affect LTP expression. These results suggest that basal endogenous NGF levels, at this stage of postnatal development, allow for maximal expression of LTP (Kirkwood et al 1995). When NGF increases under the action of physiological stimuli or because it is exogenously supplied LTP amplitude is dramatically reduced. Endogenous NGF blockade did not change LTP amplitude probably because at this time, HFS of WM occludes furthers strengthening of synaptic responses.

In keeping with the hypothesis that LTP expression depends on NGF level, it was found that blocking endogenous NGF by local supply of TrkA-IgG induced the re-appearance of LTP, at an age when this form of synaptic plasticity is normally no longer expressed (P30-35). In addition, it was demonstrated that this effect is mediated by TrkA receptors; indeed, blockade of TrkA receptors, by neutralizing αTrkA, produced the same effect of endogenous NGF blockade, i.e. re-induction of LTP expression following tetanic stimulation.

It is well known that during postnatal development visual cortical neurons are malleable to manipulations of visual experience. A classical example is the loss of responsiveness to stimulation of an eye that has been deprived of vision. In early experiments, it was established

that an exogenous supply of neurotrophins, and in particular NGF, prevents the physiological and morphological consequences of monocular deprivation during the critical period (Domenici et al 1991, Maffei et al 1992). More recent data indicate that TrkA mediates NGF effects; indeed, activation of TrkA by polyclonal antibodies is able to prevent the effects of monocular deprivation (Pizzorusso et al 1999). In the present thesis it was shown that a local supply of NGF inhibits LTP at an early stage of postnatal development. Thus, it can be hypothesized that when NGF concentration increases, synaptic strengthening is inhibited, the degree of plasticity of visual cortical neurons is reduced, and, consequently sensory deprivation is no longer effective. Sequester of freely diffusible NGF and/or blockade of TrkA receptors is able to re-induce LTP at an age when it is normally absent. These results suggest that NGF effects on LTP are relevant to the length of critical period for monocular deprivation. Interestingly, NGF chronic blockade, by intraventricular implants of hybridoma cells producing inhibiting antibodies to NGF, delays the end of critical period for monocular deprivation (Domenici et al 1994a). Although results obtained *in vitro* and *in vivo* point to similar conclusions, one should consider possible discrepancies arising from: i) different mechanisms underlying synaptic plasticity with the property of LTP and monocular deprivation effects; ii) acute and local versus chronic and systemic NGF/TrkA treatments.

In the last few years, it has been reported that, in addition to NGF, also BDNF is able to modulate postnatal development and plasticity in the visual cortex. An exogenous supply of BDNF affects the development of ocular dominance columns (Cabelli et al 1995) and enhances LTP in the visual cortex (Akaneya et al 1997). In addition, transgenic mice overexpressing BDNF in early postnatal development show a fast maturation of the functional properties of visual cortical neurons (Huang et al 1999). Interestingly, the maturation of the GABAergic circuitry is also accelerated (Huang et al 1999) and this could account for the modulation of visual cortical plasticity (Hensch et al 1998a) and possibly the length of the critical period.

4.5 Interplay between NGF and the cholinergic system in modulating LTP

NGF action on LTP can be mediated through TrkA receptors, the low affinity p75 receptor or a combination of both. LTP rescue, at an age when this plastic property is normally

lost, is mediated by TrkA receptors as using specific monoclonal antibody against this receptor showed it. TrkA receptors are present in the visual cortex (Pizzorusso et al 1999), with a fiber-like pattern of distribution. TrkA receptors are also widely expressed in cholinergic neurons of BFB nuclei (Steininger et al 1993, Sobreviela et al 1994, Li et al 1995, Molnar et al 1998), which send a well described cholinergic projection to many cortical areas, including visual cortex (Korsching et al 1985, Large et al 1986, Whittemore et al 1986). Thus, it is conceivable that TrkA receptors, localized on BFB cholinergic fibers innervating visual cortex, are the potential target for NGF neuromodulatory action.

At P30-35, it was shown that blockade of NGF-TrkA interaction, as well as activation of cholinergic receptors, was able to rescue LTP, while blockade of mAChR could not do so (data not shown). In similar experiments, TrkA-IgG immunoadhesin and the antagonist of muscarinic receptors, atropine, were used at the same time. Simultaneous local delivery of these compounds prevented the restoration of LTP in response to HFS, at P30-35. Consistently, at P16-18, both exogenous NGF supply and the blockade of muscarinic receptors by atropine impaired LTP in the same fashion, and exogenous NGF supplied to slices of visual cortex of BFB lesioned animals did not induce any significant change in LTP as compared to the untreated slices. This observation is consistent with the idea that degeneration of BFB cholinergic projecting fibers disrupts also the target for NGF, i.e. the TrkA receptors on cholinergic terminals suggesting that LTP dependence on NGF is mediated by the cholinergic system.

It is important to point out that, so far: i) it has been widely reported that TrkA receptors are expressed by BFB cholinergic neurons and not by intrinsic cortical neurons; ii) NGF has never been reported to directly modulate synaptic transmission in the cortex, by acting as a classical neurotransmitter. Thus, if it is assumed that NGF acts as a neuromodulator (hence occupying a hierarchically higher level with respect to the neurotransmitter ACh) at the postsynaptic level, the blocking effect of mAChR antagonist on LTP rescue, when endogenous NGF is blocked by TrkA-IgG, would not occur. Indeed, it was observed that blockade of mAChR inhibited completely LTP-rescue mediated by TrkA-IgG (see Fig. 3.6C) and consequently, I suggest that NGF acts as neuromodulator of BFB cholinergic transmission.

In the rat visual cortex, NGF is expressed in neurons of layers II/III and the adult pattern is reached by the end of the third postnatal week (Lauterborn et al 1994, Nishio et al 1994). In addition, NGF is expressed throughout the course of life in the LGN, which projects to the visual

cortex (Lauterborn et al 1994). NGF and its receptor TrkA are synthesized in BFB cholinergic neurons and expressed also in the visual cortex (Steininger et al 1993, Sobreviela et al 1994, Li et al 1995, Molnar et al 1998, Pizzorusso et al 1999). These neuroanatomical data fit perfectly with the possible neural circuitry mediating NGF action on LTP (see Fig. 4.5). NGF released from thalamocortical fibers could mediate a negative feedforward signal, while cortical neurons and the BFB cholinergic fibers could release NGF to convey a negative feedback signal. All these signals would stimulate TrkA receptors present on BFB cholinergic terminals and possibly down-regulate acetylcholine release.

However, it cannot be completely ruled out the alternative possibility that the cholinergic system and NGF signaling control mutually independent mechanisms acting on LTP expression and leading to analogous effects. *In vitro* experiments on single cells should help to understand the type and mechanism of interaction between NGF and the cholinergic system and their effects on LTP. Indeed, it has been reported that NGF has the ability to change synaptic transmission in visual cortex, probably, acting at pre-synaptic sites (Carmignoto et al 1997).

Although the exact site(s) and mechanism of interaction between NGF and the cholinergic system remain to be determined, a few obvious possibilities can be considered. In our experimental conditions, NGF could have a modulatory down-regulating effect on the cholinergic system. NGF could reduce ACh release simply by binding TrkA receptors on BFB cortical fibers, in this way activating an intracellular second messenger cascade interfering with ACh release and/or turnover. This hypothesis is difficult to reconcile with findings showing that NGF increases ACh release in synaptosomes (Knipper et al 1994, Sala et al 1998) and in the whole cortex (Maysinger et al 1992). In addition to these results, there is an extensive literature reporting a NGF-mediated increase of ChAT, in agreement with the idea that NGF induces an up-regulation of the cholinergic system. All these findings suggest that local delivery of NGF might lead to an increase of ACh release in visual cortex slices. However, it has to be considered that in most of these works the time-scale of NGF action is probably pertaining to a trophic action of NGF rather than a neuromodulatory effect. At the postsynaptic site, ACh would activate both excitatory and inhibitory neurons. Indeed, muscarinic receptors are expressed in pyramidal cells as well as in different subsets of GABAergic interneurons (Mrzljak et al 1993, Kawaguchi 1997, Xiang et al 1998). It would be of great interest to know whether NGF action is mediated by muscarinic activation of GABAergic interneurons. Recent evidence indicates that GABA cortical network is involved in the type of visual cortical plasticity tested by monocular

deprivation. Knockout mice for a GAD isoform, an enzyme responsible for GABA synthesis, are characterized by a reduced level of available endogenous GABA. In these mice, monocular deprivation is ineffective unless a GABA agonist is supplied, in this way restoring the capacity of visual cortex to respond to monocular deprivation (Hensch et al 1998a). These results suggest that in absence of GABA there is a loss of cortical plasticity. From works in cortical slices it is known that GABA influences the expression of LTP. For example, in rat visual cortical slices, LTP can easily elicited by stimulation of white matter during early postnatal development, while this property is progressively reduced as development proceeds. However, reducing GABA action by GABA antagonists restores the capacity to elicit a significant LTP even at late ages (Artola and Singer 1987, Huang et al 1999). The hypothesis has been raised that the endogenous level of GABA is essential to allow LTP expression; when GABA inhibition is weaken LTP can be easily elicited. These observations are not easily reconciled with the above reported results in knockout mice for GAD. Indeed, if LTP has a central role in ocular dominance shift during the critical period an increased inhibitory tone instead of a reduction would be expected to facilitate LTP. This discrepancy between in vivo and in vitro studies prompted me to test the effects of GABA in my experimental system (i.e. local delivery of GABA receptor antagonists at a concentration ten times higher than that commonly used in perfusion experiments). As reported in the results section, GABA blockade performed at P30-35, when LTP is no more expressed, was unable to rescue any potentiation of FP in response to HFS. In contrast, it was reported that both NGF blockade and ACh supply were able to rescue a normal LTP at P30-35. Thus, these results indirectly suggest that NGF and ACh action on LTP are not mediated by the cortical GABAergic system.

Finally, NGF could act directly on postsynaptic pyramidal neurons to regulate intracellular signals underlying synaptic potentiation, possibly interfering with the cholinergic modulated intracellular pathway. A few laboratories have reported that TrkA mRNA is expressed in visual cortex, although at low level (Valenzuela et al 1993, Cellerino and Maffei 1996) and an action of NGF in intrinsic neurons has been documented in organotypic cultures of developing visual cortex (Mc Allister et al 1995). However, these data are not supported by electrophysiological evidence of a direct action of NGF on cortical neurons.

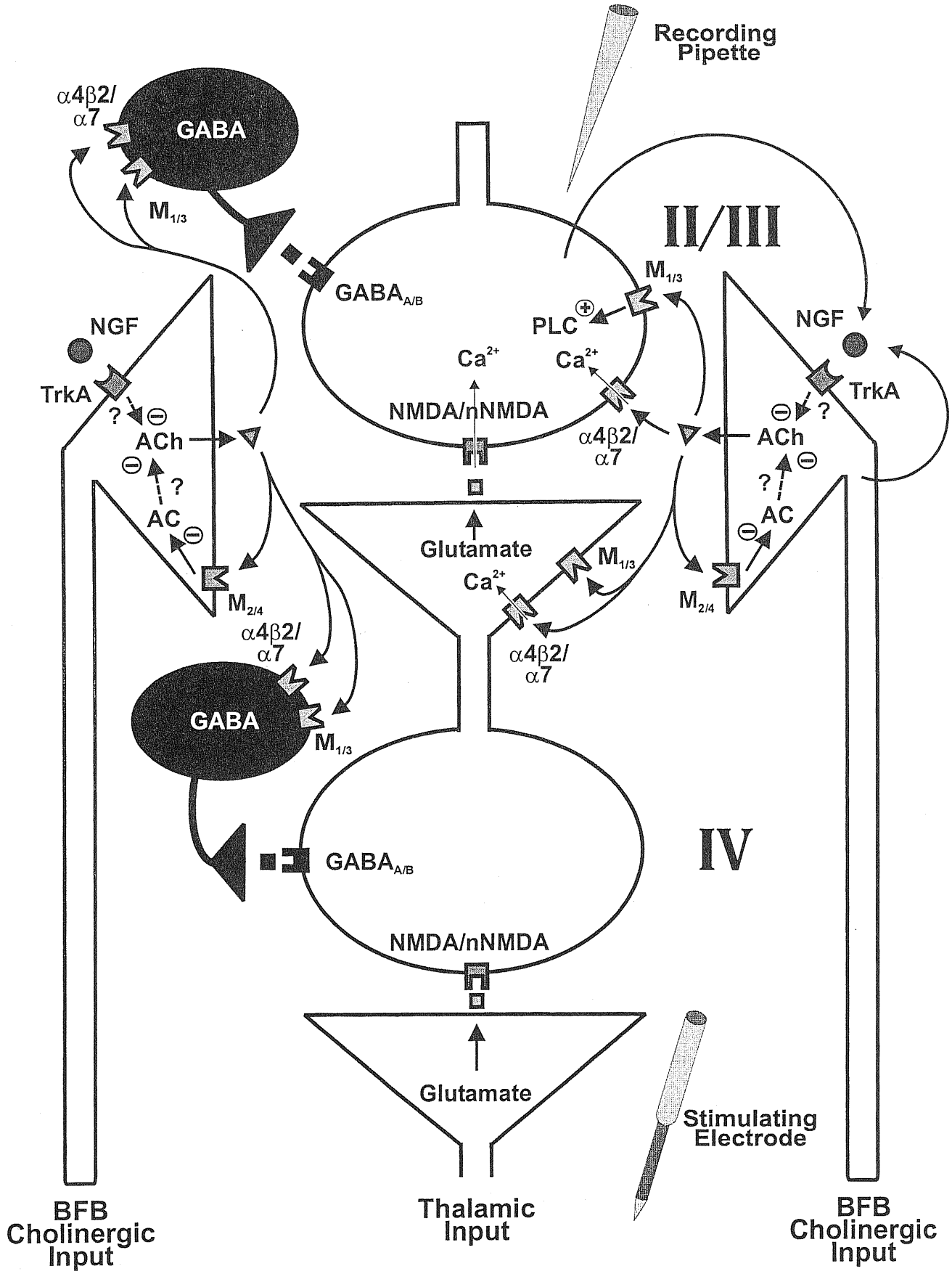


Fig. 4.5 Simplified scheme of the possible neural circuitry mediating NGF action on LTP

5.0 CONCLUSIONS

The aim of this thesis was to evaluate the role of the cholinergic system arising in the basal forebrain and of Nerve-Growth Factor in modulating cortical synaptic plasticity. It was demonstrated that, in the rat visual cortex during postnatal development, either blockade of cholinergic transmission or stimulation of NGF-TrkA interaction impairs LTP. Conversely, stimulation of both nAChR and/or mAChR, as well as blocking NGF-TrkA interaction increases LTP during the critical period and rescues LTP when it is normally absent. Moreover, adult mice deprived of NGF show both a deficit of cholinergic innervation and an impairment of LTP in the visual cortex. Treatment with cholinomimetic drugs can readily rescue this impairment. Finally, the results obtained strongly suggest that the effect of NGF, in modulating Long-Term Potentiation in the visual cortex, is mediated by the BFB cholinergic system.

In conclusion, I claim that the cholinergic system and NGF are effective modulators of cortical synaptic plasticity.

In the last few years, BDNF, among NT, gained increasing attention as a modulator of cortical synaptic plasticity. Here, it is reported that NGF also possesses the ability to modulate changes in synaptic strength. BDNF was also demonstrated to be an important trophic factor for the maturation of the intracortical GABAergic system during postnatal development of visual cortex. Similarly, NGF is a well-documented trophic factor for the BFB cholinergic neuronal population. Thus, both BDNF and NGF exert fast neuromodulatory actions and long-term effects as trophic factors, depending on the time scale considered. The cellular mechanisms underlying these two apparently unrelated effects probably rely on the same signal transduction pathway (triggered by binding to Trk receptor) and diverge in the downstream metabolic steps. The different fast and long-term effects could depend on the release pattern of NT, where the constitutive release pathway would ensure a tonic level of NT that would act as trophic factor. Conversely, the activity-dependent release pathway would lead to a phasic delivery of NT responsible for modulation of synaptic strengthening.

Thus, BDNF and NGF regulate activity-dependent postnatal development and plasticity in the visual cortex. Although common mechanisms in the ability of different neurotrophins to

modulate plastic phenomena cannot be excluded, it is likely that NGF and BDNF may have two separate types and loci of effects.

I suggest that BDNF may modulate activity-dependent postnatal development and plasticity by acting on inhibitory interneurons in the visual cortex, while NGF action appears to focus on the BFB cholinergic system projecting to the visual cortex.

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