OK

ISAS - INTERNATIONAL SCHOOL
FOR ADVANCED STUDIES

INFLUENCE OF SENSORY EXPERIENCE, NERVE GROWTH FACTOR AND ACETYLCHOLINE IN THE REGULATION OF GFAP IN THE MOUSE VISUAL CORTEX.

Thesis submitted for the degree of "Doctor Philosophiae"

Candidate:
Luigi Corvetti

Supervisor
Luciano Domenici

SISSA - SCUOLA
INTERNAZIONALE
SUPERIORE
STUDI AVANZATI
TRIESTE
Via Beirut 2-4

TRIESTE

Part of this thesis has been published in

Postnatal development of GFAP in mouse visual cortex is not affected by light deprivation. (2003) **Corvetti L**, Capsoni S, Cattaneo A and Domenici L. Glia 41:404-414.

TABLE OF CONTENTS

ABBREVIATIONS

AC = Adenilate cyclase

ACh = Acetylcholine

AChE = Acetylcholinesterase

BDNF = Brain derived neurotrophic factor

BFB = Basal forebrain

cAMP = Cyclic AMP

CNS = Central nervous system

E = Embrionic

GFAP = Glial fibrillary acidic protein

IP_3 = Inositol 1,4,5-trisphosphate

IR = Immunoreactive

M1-5 = Muscarinic subunit 1-5 of the acetylcholine receptor

mAb = Monoclonal antibody

mAChR = Muscarinic acetylcholine receptor

MD = Monocularly deprived

nAChR = Nicotinic acetylcholine receptor

NGF = Nerve growth factor

P = Postnatal

PLC = Phospholipase C

PNS = Peripheral nervous system

RC2 = Radial cell 2

TrkA = Tyrosine kinase A

VAChT = Acetylcholine transporter

VC = Visual cortex

ABSTRACT

Glial fibrillary acidic protein (GFAP) is a member of the class of the intermediate filaments highly expressed in astrocytes, in which it contributes to form cytoskeleton. Its expression is modulated by functional changes of cellular environment and it is altered during pathological events. In the present study GFAP expression was investigated in visual cortex of mouse. The study has been performed in visual cortex since this area is mostly used to study activity-dependent plasticity, cell phenotype differentiation and maintenance. Mammalian visual cortex is immature at birth and develops gradually during defined postnatal temporal windows. In the present work, I studied the maturation of astrocytes in developing mouse visual cortex (VC). Cellular distribution and level of glial fibrillary acidic protein (GFAP) were analyzed by immunohistochemistry and western blot analysis. Experiments were performed at different postnatal ages: postnatal day 12 (P12), before eye opening, P24, corresponding roughly to the peak of the critical period for monocular deprivation (MD); and P60, after the end of the critical period. At P12, GFAP-immunoreactive (IR) astrocytes were distributed throughout all cortical layers. At P24, there was a prominent localization of GFAP immunoreactivity in layers I, II and VI, while cortical layers III, IV and V contained no longer GFAP-IR cells. No differences were found in GFAP immunoreactivity between P24 and P60. Western blot analysis revealed a reduction of GFAP expression in the VC at P24 with respect to P12 and no significant difference between P60 and P24. These results show that GFAP expression is modulated during early postnatal development, its maturation is rapid, reaching and adult like status before P60. To know whether visual experience influences the maturation pattern of GFAP expression, mice were deprived of visual experience (dark rearing) from P12 to P24. It is well known that visual experience represents the driving force of visual cortex maturation and, in particular, of neural cells. I found that dark rearing did not change the distribution and the expression of GFAP. The results indicate that maturation of GFAP expression occurs early in postnatal development in mouse VC. GFAP development is not regulated by light, since GFAP expression is not affected by visual deprivation. The conclusion is that different genetic and epigenetic factors regulate maturation of neurons and astrocytes. After this first result I moved to investigate factors and mechanisms controlling astrocytes

phenotype and in particular GFAP expression. To this aim I moved well out of the end of maturation period for GFAP; all experiments were conducted in P60 mice. The first investigated factor has been Nerve Growth Factor (NGF). NGF belongs to the family of trophic proteins (neurotrophins) it regulates the survival and differentiation of developing neurons and contributes to the maintenance of specific functions, in particular class of neurons, during adult life. Effects of NGF in developing and adult visual system have been observed, on neurons, by several investigators. In the present study, I investigated the consequence of NGF deprivation on GFAP immunopositive astrocytes in adulthood. To this aim, I analyzed visual cortex of 2-month-old transgenic mice, expressing a recombinant version of the neutralizing anti-NGF monoclonal antibody (mAb αD11). I found a reduction of GFAP-immunoreactive astrocytes in visual cortex of anti-NGF transgenic mice without a concomitant reduction of the total number of astrocytes, as assessed by immunoistochemistry for S-100, another marker of mature astrocytes. Western blot analysis revealed a decrease of GFAP amount in visual cortex. Similar results were obtained in area CA1 of hippocampus indicating that the effects of NGF deprivation were not limited to the visual cortex. To discard the possibility of unspecific effects due to insertion of transgene, the endogenous NGF was also blocked by using intraventricular implants of hybridoma cells releasing mAb αD11. This treatment induced reduction of GFAP expression. To account for a possible indirect effect of NGF on GFAP expression, acetylcholine, a neurotransmitter active on neurons and astrocytes, was investigated. It is known that NGF is fundamental for survival of cholinergic neurons also during adult life. In the transgenic mouse, I found a reduction of cholinergic neurons of the BF. It is known that cholinergic neurons of the BF project to the cortex, thus raising the possibility that NGF effects on GFAP are mediated by the cholinergic system. To validate the cholinergic hypothesis, a mild dose of vesamicol was used to block the release of acetylcholine (ACh) in wild type mice. ACh reduction induced a decrease of GFAP immunoreactivity and a modification of astrocytic shape comparable to that provoked by NGF deprivation. Previous results showed that experimental reduction of ACh or alteration of cholinergic system induced an increase of GFAP-IR astrocytes instead of a reduction. To test whether GFAP was sensitive to ACh in a dose dependent manner, a higher dose of vesamicol was injected to provoke a sustained ACh reduction. I found that animals treated with a higher dose of vesamicol were

characterized by an increase of GFAP immunoreactivity and a gliotic reaction of astrocytes. These results suggest that astrocytes are sensitive to acetylcholine in a dose dependent manner.

Altogether the results indicate that:

i) GFAP expression in mouse visual cortex, is regulated during the development reaching the adult-like status before P60;

ii) light deprivation, does not affect GFAP maturation;

iii) NGF deprivation induces a down regulation of GFAP expression and a reduction of cholinergic neurons;

iv) Reduction of ACh release mimics the effect of NGF deprivation on GFAP expression, suggesting that the action of NGF on astrocytes might be mediated through the cholinergic system;

v) Effects induced by ACh deprivation are dose dependent. Indeed, mild and strong reduction of ACh release leads to two opposite action, i.e. to decrease and increase of GFAP immunoreactivity, respectively. I suggest that acetylcholine and NFG act synergistically to create a bridge between neurons and astrocytes permitting them to react to an environmental change and/or perturbation.

INTRODUCTION

1.1 Astrocytes: Historical Overview and Classification

In the mammalian brain glial cells occupy 50% of the total brain volume (Peters et al., 1991; Laming et al., 1998). Evidence accumulated during the last twenty years indicate that they are not only mere supportive elements, but that they are essential to understand how the brain works and integrates different signals (Araque, 2001). Glial cells are divided into two main classes: Macroglia and microglia. Astrocytes are part of macroglial, which includes astrocytes and olygodendrocytes, in the central nervous system (CNS), and schwann cells in the peripheral nervous system (PNS). The main aim of the present thesis is to contribute to elucidate some of the factors that control phenotype and functions of a particular subclass of macroglial cells, namely astrocytes, in the mouse brain.

Rudolf Virchow (1846) was the first to describe a large population of cells with different structural characteristics compared to neurons, in vertebrate brain. He called these cells "*Nervenkitt*" to indicate their main role to serve as connective tissue in which neurons are embedded. Virchow intuition, of a new class of cells present in the brain and distinct from neurons, posed the basis for a more comprehensive view of the brain as formed by different cell types actively interacting to set the adaptive response to changes in the environment. Successively, Camillo Golgi (1885), the pioneer of the morphological identification of cell types in the nervous system of vertebrates, provided the basis for a modern investigation of glial morphology using the technique of silver staining. In particular, metallic impregnation techniques allowed the identification of glial processes distinct from neuronal processes.

Golgi's studies on glial cells were continued by Santiago Ramon y Cajal (1909). Taking advantage of the improvement of the metallic impregnation technique used by Golgi, he described different types of astrocytes, classifying them into three main subgroups: radial glia, fibrous astrocytes and protoplasmic astrocytes. This classification was very important and it has currently been used.

Radial glia.

Radial glial cell (Fig. 1.1a) are a ubiquitous cell type, present in the developing central nervous system (CNS) of vertebrates. Radial glial cells have a bipolar soma whose axis is perpendicular with respect to the plane of the growing tissue. The bipolar soma is located close to the proliferative ventricular zone and it extends long radial processes. Using Rio-Hortega impregnation, astrocytic processes appear as thin,

unbranched fibers, forming elongated filaments spanning the entire cerebral wall (Kettenmann and Ransom, 1995). More recently, a specific antibody for radial glia, radial cell 2 (RC2) (Misson et al., 1988), allowed the identification of two main forms of radial glial cells in mammals: bipolar radial glia, which spans the whole thickness of the cerebral wall and monopolar radial glia, which appears later during development. During the prenatal period these cells loose the descending processes and present only a single process or multiple processes arising from their apical pole and terminating in endfoot varicosities at the pial surface. The entire radial glia network is not static and its configuration changes during development to accommodate the extensive increases in thickness and surface of the growing brain (Misson et al., 1988; Kadhim et al., 1988; Gadisseux et al., 1992). Radial glia plays an important role in many areas of the CNS, such as the cerebral cortex (Woodhams et al., 1981), the cerebellum (Edmondson and Hatten, 1987) and the hippocampus (Woodhams et al., 1981; Gasser and Hatten, 1990). During development, neurons use a scaffold of radial glia to reach the final site in a functionally mature organization (Rakic, 1981; Hatten, 1999) (Fig. 1.1 b). While radial glia persists in some vertebrate like amphibia, reptilian and birds, it almost completely disappears in the mammalian nervous system with the exception of cerebellum, where they persist as Bergman glia.

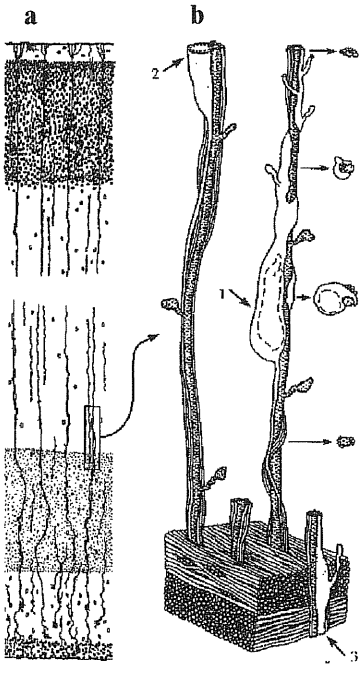


Fig. 1.1 Neurons migrating along radial glia during development. (a) Camera lucida drawing of the occipital lobe of a monkey fetus at mid-gestation. Radial glia fibers run from the ventricular zone to below to the surface of the developing cortex above. (b) Three-dimensional recinstruction of migrating neurons (in white)
(from Nicholls)

Fibrous Astrocytes. Using metallic impregnation, fibrous astrocytes (Fig. 1.2a) appear as star-shaped cells with long and poorly ramified processes that radiate in all directions. They are abundant in the white matter, in which they run among myelinated fibers and spread throughout the entire white matter. Often, fibers push on far from their cell body entering the gray matter, without reaching the pial surface. Sometimes these cells get in contact with blood vessels, through endfeet attached to vascular endothelium.

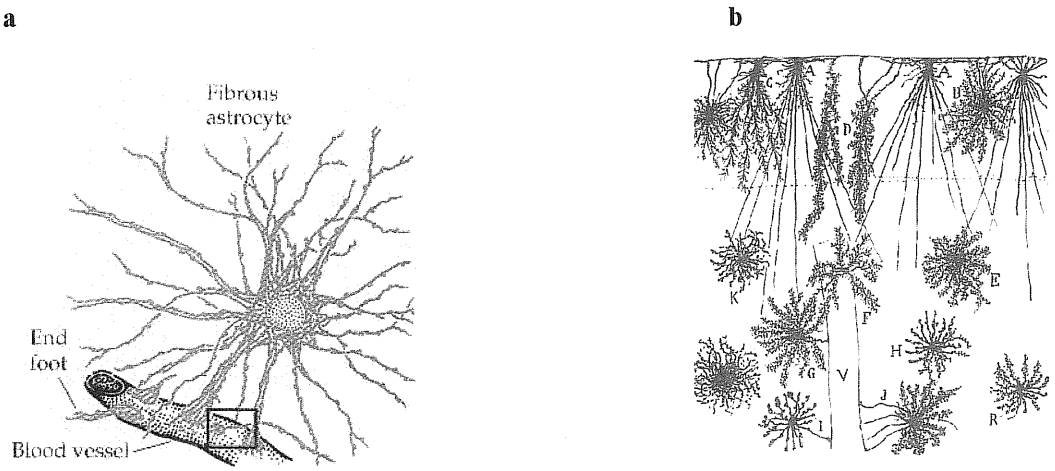


Fig. 1.2 Silver impregnation. (a) Fybrous astrocyte in the white matter contacting a blood vessel. (b) Protoplasmic astrocytes in superficial layer of the human infant cerebral cortex.
A,B,C,D astrocytes in the plexiform layer; E,F,G,H, astrocytes in layer 2 and 3; astrocytes with vascular end feet; V, blood vessel. (From Cajal)

Protoplasmic Astrocytes. Cells with short processes, called protoplasmic astrocytes, are prevalent in the gray matter. Unlike fibrous astrocytes of the white matter, they have short, branched processes. In the cerebral cortex, it is possible to observe bushed astrocytes that take contact with the pia or blood vessels. In addition, there are astrocytes around neuronal cell bodies, dendrites and synapses. Furthermore, protoplasmic astrocytes at the white matter border show a fibrous shape, suggesting, to the first anatomists, the idea that their morphology changed according to their laminar position. In the second half of the past century, development of electron microscopy, allowed the investigation of the ultrastructural organization of astrocytes (Fig. 1.3). It has been reported that astrocytes can be intimately associated with the synapse, literally enwrapping many pre- and post-synaptic terminals. In the hippocampus, 57% of the axon-spine interfaces are associated with astrocytes

(Ventura et al., 1999). In the cerebellum there are eight Bergmann glia for each Purkinje cell, and each Bergmann cell ensheats between 2142 and 6358 synapses (Reichenbach et al., 1995). It is likely that this close physical relationship provides an opportunity for functional interactions between astrocytes and neurons.

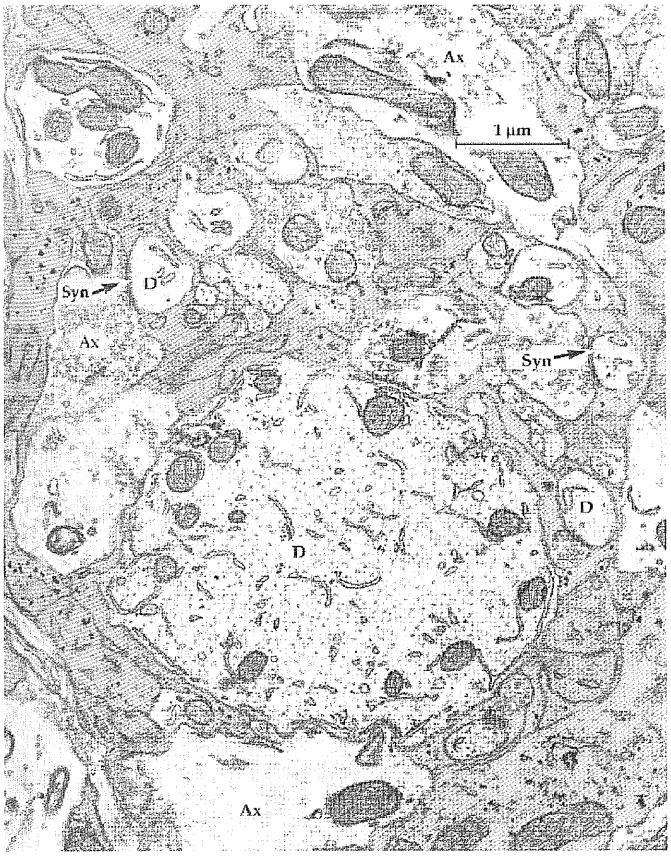


Fig. 1.3. Electron microphotography of Neurons and astrocytes processes in rat cerebellum. Glial cells are in blue. (D) Dendrites, (Ax) Axons. (Syn) Synapse.
It is clear the close association between neurons and astrocytes

1.2 FUNCTION OF ASTROCYTES.

Histochemical observations conducted by the first anatomists formed the ground for the first hypothesis about the function of astrocytes. Virchow was the first to propose that astrocytes represent a sort of connective tissue. Successively, Golgi noted the close contact of glial processes with blood vessels and neurons, and he speculated that astrocytes could serve to supply nutrients to nerve cells. His (1889) proposed that embryonic glial cells guide the migration of developing neurons. Lugaro, in 1907, suggested that astrocytes could buffer the interstitial space to ensure the correct neuronal function. In 1909, Ramon y Cajal rejected the Golgi's nutrition idea, raising a new theory called the "Isolation theory". He observed that glial cells are abundant in regions of the gray matter where neuronal dendrites are present in great number such as the molecular layers of the cerebellum and the molecular layer of the cerebral cortex and hippocampus. He speculated that the high number of

astrocytes in these regions could regulate neuron-neuron contacts, preventing the diffusion of neurotransmitters far from the release sites thus contributing to restrict their area of action *"..There is in fact, a real need to avoid, insofar possible, the indiscriminate spread of neuronal fluxes from any processes to its neighbors for fear or psychological confusion and generalized response"* (Cayal SR, 1909).

Some of the reported studies advanced the idea that astrocytes have important functional and structural roles beyond the passive supportive initially prposed by Virchow. Nevertheless, astrocytes have been largely neglected and neurons have long been thought to represent the sole information-processing elements of the CNS. However, the anatomical proximity of neuroglia to neuronal cells (Fig. 1.4) makes these cells particularly suitable for active roles in neural information processing, and different neuroscientist agree in supporting a key role for glia in brain physiology and pathology.

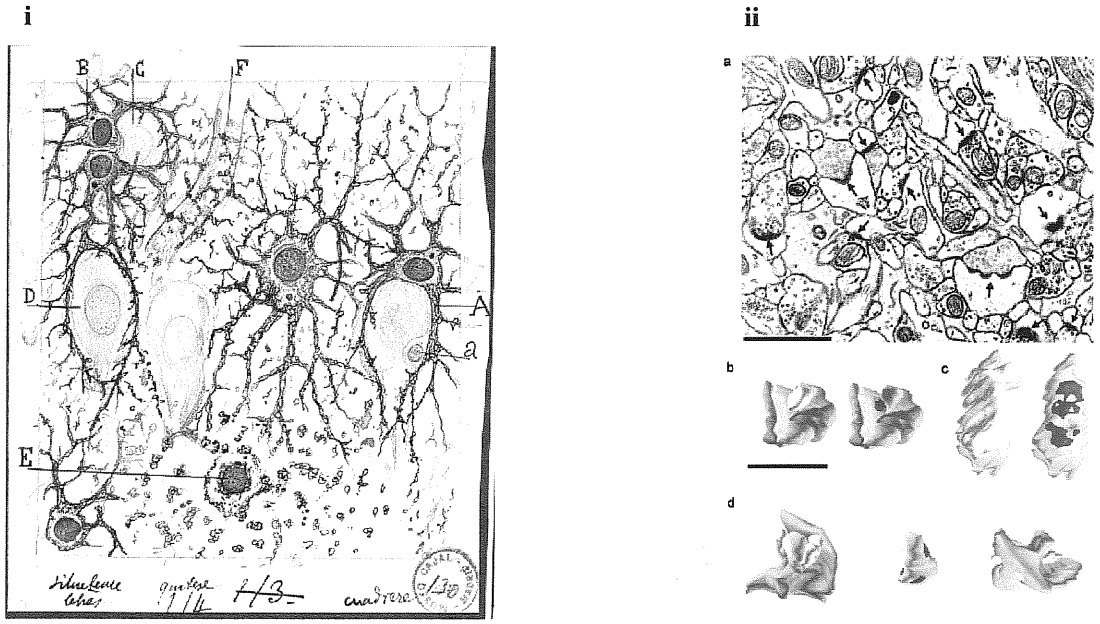


Fig.1.4 Stratum radiatum of hippocampal area CA1Adult man autopsied three hours after death. Draw from a Chloride of gold impregnation (i) (*A*) big astrocyte embracing a pyramidal neuron. (*B*) twin astrocytes forming a nest around a cell (*C*), while one of them sends two branches forming another nest (*D*). (*E*) cell with signs of autolysis" (1). (Figure reproduced from original drawing of Legado Cajal). **Astrocytic profiles and 3D reconstructions illustrating their relationships to synapses (ii).** *a*, Astrocytic profiles are illustrated (*blue*) on a single thin section in the vicinity of 11 synapses (*arrows*). Three synapses have astrocytic profiles at their perimeters (*arrowheads*). *b–d*, 3D reconstructions illustrate astrocytic profiles (*blue*), boutons (*green*), spines (*gray*), and PSDs (*red*). Astrocytic profiles surround (*b*) 50% of the perimeter of this macular synapse and (*c*) 3% of the perimeter of a perforated synapse, both occurring on SSBs. In *d*, three synapses occur with a single presynaptic bouton, called a multiple synapse bouton, and a single astrocytic process surrounds 75, 64, or 100% of the perimeter of each synapse from *left* to *right*, respectively. Scale bars: 1 mm (shown in *b* for *b–d*). (From Ventura and Harris, 1999)

This hypothesis is supported by data showing that astrocytes can integrate neuronal inputs and modulate synaptic plasticity (Kang et al., 1998). Studies in cell cultures and slices have demonstrated that the release of glutamate by neurons induces a transient elevation of calcium in astrocytes, resulting in calcium waves that spread away from the site of glutamate release through astrocytic gap junctions (Rouach et al., 2000). These results suggest that astrocytes contribute to form a network mediating long-range signalling in the CNS. More recently, it has been reported that, in addition to glutamate, other neurotransmitters such as noradrenaline, GABA, and acetylcholine induce elevation of calcium in astrocytes (Araque et al., 2002). In addition, glutamate stored by astrocytes is released in vesicles when they are stimulated by neurotransmitters, using a mechanism similar to the neuronal secretory process. (Jeftinija et al., 1997; Volknandt, 2002). Since astrocytes surround many of the synaptic connections in the CNS, released glutamate exerts a feedback signal for synaptic neuronal terminals resulting in modulation of synaptic transmission. Thus, astrocytes may control synaptic transmission and efficacy, coupling together several synaptic neuronal terminals that are contacted by excited astrocytes, in this way sculpting the neuronal circuitry involved in activity-dependent plasticity. These results support the Cajal hypothesis in which astrocytes are described as essential elements integrating neuronal inputs in the brain.

Astrocytes functions of include not only their role in nutrition and formation of a barrier between the nervous system and the blood/cerebrospinal fluid (blood brain barrier, BBB), guidance for neuronal migration, and integration of neuronal signals, but also the fundamental role in response to neurodegeneration and, in general in pathophysiological processes. In adult CNS, astrocytes react, to injury, trauma, neurodiseases, genetic disorders, or chemical insults, in a characteristic way. Reactive astrocytes display phenotypic modifications that include increase in nuclear diameter, glutamine synthetase and glycogen, high oxidoreductive enzymatic activity and increased syntesis of cytoskeletal proteins such as glial fibrillary acidic protein (GFAP) and vimentin (Steward et al., 1993; Eng et al., 1992). Pathological accumulation of GFAP has been used as a marker of gliosis in several experimental models including cerebral cryogenic lesions (Amaducci et al, 1981), stab wounds (Latov et al., 1979; Mathewson and Berry, 1985), experimental allergic encephalomyelitis (Smith et al., 1983; Aquino et al., 1988), toxic lesions (Brock and O'Callaghan 1987; Rataboul et al 1989) and ischemia (Cheung et al., 1999). Increase

of GFAP mRNA expression has been observed in Alzheimer's disease (Beach et al., 1989; Delacourte, 1990), Creutzfeldt-Jacob disease (Manuelidis et al., 1987), and mechanical lesions of the rat cerebral cortex (Condorelli et al., 1990). The obtained results point to GFAP as a sensitive and early marker of neurotoxicity.

It is thought that astrogliosis is important in controlling the molecular and ionic contents of the extracellular space after injury in the CNS. In addition, reactive astrocytes are important in removing myelin and cellular debris from the injured site and have an active role in insulating damaged regions of the nervous system from the extracellular environment. Besides these positive effects, strong gliosis can produce dangerous effects. In fact, reactive astrocytes release factors which could prevent re-myelination and axonal regeneration (Ridet et al., 1997; Rudge and Silver, 1990; Spillmanm et al., 1986). Concerning the molecular mechanisms inducing gliosis after neuronal damage, it is known that after injury several molecules, such as cytokines, neurotransmitters, neurotrophic factors and ions, are released in the extracellular space. The consequent altered composition of the extracellular space may induce neuronal sufferance and gliotic response.

1.3 Origin and Development of Astrocytes.

Recent data converge to the hypothesis that, in the CNS, neurons and astrocytes are originated from a common progenitor, namely radial glia (Voigt, 1989; Bentivoglio and Mazzarello, 1999; Malatesta et al., 2000; Alvarez-Buylla et al., 2001; Götz et al., 2002). In general, glial differentiation is slower than neurogenesis and when the latter is over the glial cell population continue to expand (Jacobson, 1991). Mature glial cells maintain their capacity to proliferate throughout the life span as shown by autoradiography after a pulse of [^{3}H]thymidine (Korr et al., 1973; 1975).

As mentioned before, radial glia is characterized by long fibers, leaving the ventricular surface and spanning the entire gray matter. These cells not only provide a mechanical support, acting as guiding cables for neuronal migration, but also play an active role in brain trophism and general metabolism. In non-mammalian nervous systems radial glia persists during adulthood, while in the mammalian brain, they disappear early during postnatal development with the exception of the cerebellum (Bovolenta et al., 1984). Radial glia appears early in the embryonic development. Using the monoclonal antibody RC2, they were detected in the pseudostratified

neuroepithelium of the mouse neural tube at 9 days of embryonic development (E9) (Misson et al, 1988). Concerning the use of this antibody it should be mentioned that, despite it is frequently used to label mouse radial glial cells in all parts of the nervous system, during embryonic and early postnatal life, the antigen recognized by this antibody still needs to be identified. Recently (Chanas-Sacre et al., 2000) it has been shown that the RC2 antibody recognizes a developmentally regulated intermediate filament associated protein (IFAP) expressed, by cells of the glial and myogenic lineages. Thus it seems not the best marker for glial cells. However, increasing evidence suggests that radial glia acts as progenitors of astrocytes. First of all, mature astrocytes start to be detected only after radial glia disappearance, during the perinatal period after completion of neuronal migration (Choi and Lapham, 1978; Cameron and Rakic, 1991; Misson et al, 1991). Moreover, radial glia and astrocytes share common antigens such as GFAP, a classical marker for mature astrocytes (Levit and Rakic, 1980; Stichel et al., 1991) as well as the astrocyte specific glutamate transporter (GLAST) (Shibata et al, 1997) and the brain lipid binding protein (BLBP) (Feng et al, 1994), two specific astrocytic markers. Another finding which support this idea is the presence of cells displaying a transitional shape between bipolar radial glia and multipolar astrocytes (Pixley and McKay, 1984; Marin-Padilla, 1995), which, assume mature astrocytic phenotype when cultured (Cullican et al, 1990). Particularly relevant to this issue is the result obtained when a fluorescent tracer was injected under the pial surface of newborn ferrets, at a time when virtually no mature astrocytes were present. The dye was found in mature astrocytes several weeks later (Voigter, 1989). Recent studies also highlighted the importance of radial glia in neurogenesis (Malatesta et al., 2000; Noctor, 2001), showing radial that radial glia comprise a subpopulation that generate neurons only, and a subpopulation that generates only glial cells. The different classes of macroglial cells present in the CNS, i.e. astocytes and oligodendrocytes, originate from glial cells in the ventricular and subventricular zones and later migrate into the white and gray matter. The process of maturation of glial cells, and in particular of astrocytes, is characterized by the presence of different transitional steps, characterized by typical markers. Astrocytes express two different proteins forming intermediate filaments, namely vimentin and GFAP, during early stage of development. Vimentin is expressed in radial glia and immature astrocytes during the motile phase of astrocytes. GFAP is expressed mainly in mature astrocytes although can be expressed in transitional cells together with

vimentin. Another marker for mature astrocytes is the calcium binding protein, S-100, expressed in two forms: S-100α and S-100 β (Moore, 1965; Ludwin et al., 1976; Cocchia, 1981).

1.4 ASTROCYTIC MARKERS.

Most studies aiming to unveil the morphological features of astroglial cells were performed using heavy metal impregnation techniques. Although these techniques allowed a precise description of the cell distribution and structure, they did not give much information on the functional organization of astrocytes. Furthermore, since particular proteins are temporally and spatially segregated, classical histological techniques do not allow to appreciate dynamic processes. Modern immunohistochemistry and molecular biology lead to a more complete knowledge of this protein, allowing both to observe dynamic transformations of astrocytes during life and to study relationships among astrocytes themselves and other neural cells. So far, several astrocyte specific molecules have been identified. Most of them are distinctive of astrocytes, while others are shared with neurons or other glial cells, even though some of them have different functions in astrocytes than in other cells. Since the number of protein expressed by astrocytes is high, it is beyond the scope of the present thesis to describe the functions and roles of all of them. Since the aim of the present thesis is to investigate factors and mechanisms modulating astrocytic phenotype I focused my attention on GFAP.

1.5 GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP)

Gial fibrillary acidic protein (GFAP) is a member of the family of the intermediate filament (IF) proteins, with which share the common structure, consisting of a non-α-helical N-terminal head domain, a central α-helical rod domain, and a C-terminal head domain (Fig. 1.5a) (Weber and Geisler, 1985). The head regions of porcine GFAP is composed of 35 aminoacid residues (Geisler and Weber, 1986) and has a β-turn structure, it contains eight arginine residues that characterize this highly basic region (Inagaki et al., 1990; Feinstein et al., 1992). Phosphorylation and dephosphorylation of specific aminoacids residues in the head region are involved in the regulation of GFAP assembly. The rod region is composed of 345 aminoacid residues, contaning long tandem repeats of seven aminoacid sequences called "heptad

repeats". This region is essential for the packaging of the α-helical coil and which is important for the formation of 10 nm filaments. The tail region of about 50 aminoacids residues has a globular structure and may be involved in the interactions between IF and other proteins. Under physiological conditions, the majority of GFAP proteins assemble to form 10 nm filaments in vitro (Geisler and Weber, 1983). They built up in three steps (Fig. 1.5b): i) as a firs step, two GFAP molecules gather to form a parallel α-helical coiled-coil dimer structure, ii) two dimers form anti-parallel filaments composed as tetramer and iii) the protofilament assembles to constitute a 10 nm filament structure (Soellner et al, 1985; Shoeman and Traub et al., 1993; Stewart et al., 1989).

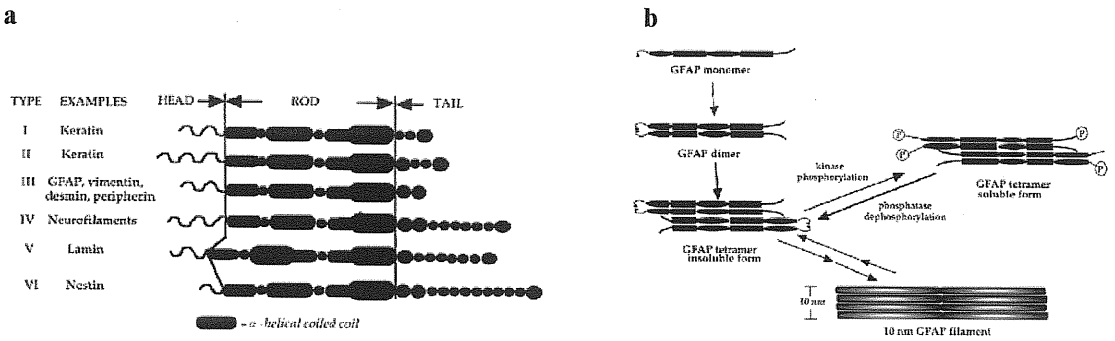


Fig. 1.5. **(a) Classification and structure of the IF family of proteins**. On the basis of amino acid sequence homologies, the IFs can be subdivided into six principal types as shown. Whereas the head and tail domains of the IF subtypes vary considerably and provide the basis, in part, for their tissue-specific expression, there is considerable homology within the central rod domain, which is composed of interconnected protein segments forming alpha-helical coils. Glial fibrillary acidic protein is a Type III IF along with vimentin, desmin, and peripherin. **(b) Regulation of GFAP assembly and disassembly.** (For explanations see the text)

In vitro reconstitution studies revealed that assembly and morphology of GFAP filaments are affected by various physical factors, such as GFAP concentration, pH, divalent cations, ionic strength, aminoacids, and temperature (Inagaki et al., 1990; Herrmann et al., 1993; Nakamura et al., 1991). The suitable pH range for in vitro correct assembly is around pH 7 (Inagaki et al., 1990). At lower pH, they form an unusual ribbon like filament, wider than 10 nm. Divalent cations, such as Mg^{++} and Ca^{++} greatly accelerate GFAP assembly. However, in high concentrations, they induce the formation of ribbon-like GFAP filaments (Tanaka et al., 1989). Althought most of the GFAP is in the polymerized state with few free tetramers (Soellner et al., 1985), recent studies revealed that they dynamically shift between assembled and disassembled states (Nakamura et al., 1991; Vikstrom et al., 1989). Thus GFAP

undergoes dynamic changes between the two phases (Nakamura et al., 1991). Phosphorylation and dephosphorylation of the GFAP head region have been shown to have a critical role for the maintenance and reconstruction of GFAP (Inagaki et al., 1990; Inagaki et al., 1987). When aminoacid residues in the head region are phosphorylated, GFAP filaments disassemble into a soluble form (Inagaki et al., 1990). The head region is positively charged due to the many arginine residues present within the region. This is important for assembly of GFAP filament. When the head region is removed from trombin digestion (Matsuoka et al., 1992) or the arginine residues are eliminated (Inagaki et al., 1989) GFAP molecules cannot assemble regularly. Thus, the mechanism of GFAP assembly regulation may relate to regulation of the electric charge in the head domain by phosphorylation. Sites of phosphorylation of 2^{nd} messenger dependent protein kinases have been identified in the head region (Inagaki et al., 1990; Nakamura et al., 1992). Four serines (Ser-8, Ser-13, Ser-17 and Ser-34) and threonine (Thr-7) are phosphorylated by different protein kinases; cyclic-AMP dependent protein kinase (A kinase), Ca^{++} calmodulin-dependent kinase II (CaM kinase II) and C kinase. "A kinase" phosphorylates Thr-7, Ser-8, Ser-13 and Ser-34 of the head region. "C kinase" phosphorylates Ser-8, Ser-13 and Ser-34. "CaM kinase" phosphorylates Ser-13, Ser-17, Ser-34 and Ser-389.

Experiments *in vitro* have shown that the GFAP revealed both a fast-decaying pool (half-life: 18 h) and a more stable form (half-life: 18 h) (Chiu and Golman 1984), in contrast GFAP turnover *in vivo* is much slower (DeArmond et al., 1983, 1986).

GFAP is an aqueous insoluble molecule, characterized by species-specific amino acid sequence and molecular weight, being 48,000 KD in the mouse, 49,000 in human 50,000 in bovine and 51,000 in the rat (Eng, 1980; DeArmond 1983). The gene encoding for GFAP is located on chromosome 11 in mouse, (Bernier et al, 1988; Boyer et al, 1991), and on chromosome 17q21 in humans (Bongcam-Rudloff et al., 1991; Kumanishi et al., 1992). The GFAP is composed of nine exons distributed over about 10 kb of DNA and yield a mature mRNA of about 3 kb. The coding sequences for the three species are highly homologues. GFAP is heavily expressed in a subpopulation of mature astrocytes and, in low amount, in radial glia of primates (Levit and Rakic, 1980) and rodents (Stichel et al., 1991), where it forms bundles within the cell body and processes. In mammalian CNS, GFAP expression shows strong regional differences and it is developmentally regulated. During development,

GFAP has been associated with maturation of astrocytes (Eng, 1994; Raff et al., 1979).

GFAP in developing CNS

Studies conducted in the developing nervous system demonstrated that GFAP expression is developmentally and regionally regulated. Employing Northern blot analysis and *in situ* hibridization, GFAP mRNA expression was analyzed in different mouse brain regions at different developmental stages. GFAP expression was first observed at E20 within the germinal zone of the fourth ventricle in the cerebellum. In hindbrain and midbrain, GFAP mRNA is first detected at E20 as well its expression increasing and reaching adult-like level around P15 (Landry et al., 1990). In forebrain high level of expression were detected. In hippocampus GFAP mRNA is expressed at low levels at P1, and reaches a peak of expression at P15 and then decreases (P60) (Landry et al., 1990; Tardy et al., 1989). In aged animals, GFAP expression is strongly increased compared to adult, as revealed by analysis of both mRNA and protein in different species. Analysis of GFAP mRNA (Goss et al., 1990; Kohama et al., 1995) in the mouse and rat brain reveals a generalized increase starting from 18 month of age. An age related increase in the intensity of GFAP immunoreactivity was found in aged mice (O' Callagan and miller, 1991; Kohama et al., 1995) and in subcortical white matter of monkeys (Sloane et al., 2000). In both monkey (Sloane et al., 2000) and mouse (Kohama et al., 1994) it was not associated with an increase in the number of GFAP posiytive astrocytes. Developmental analysis of the protein expression in cortical astrocytes reveals that protein localization and protein content corresponds to mRNA level. In the rat visual cortex, GFAP expression is localized to radial glial fibers at early stages of postnatal development, disappearing towards the end of the third postnatal week. Staining of astrocytes in the white matter and in cortical layers I and VI reaches an adult-like density at postnatal days 8 and 20, respectively. A progressively later development of GFAP-positive astrocytes is observed in cortical layers II-V where it is complete between postnatal days 47 and 50. In the adult rat visual cortex GFAP-positive astrocytes are especially localized to layers VI, moderate in layers II/III and V and I and nearly absent in layer IV and lower layer III (Stichel et al., 1991). In cat visual cortex, GFAP protein expression is detected at P7 in small cells with long processes extending radially, sometimes reaching the pial surface. These cells are reminiscent of radial glia and they are

reduced in number later developmental stages. One month after birth, radial glia is confined to the superficial cortical layers and many GFAP-positive stellate cells are found throughout all cortical layers. Radial glia completely disappears after five weeks. At this age GFAP-IR cells are present in all cortical layers, including layer IV. Intensity of staining and density of cells increases until seven weeks of postnatal age, when it assumes an adult like pattern (Muller, 1992). Thus, the cortical organization of GFAP in cat visual cortex strongly differs from that observed in rat suggesting the presence of species differences. Few data are available on the expression of GFAP in the mouse brain and until now results on mouse neocortex are lacking. Experiments that I performed during my Ph.D. course aimed to identify, at least in part, molecules involved in the bidirectional communication among neurons and astrocytes. Thus, the first step of my work was the characterization of GFAP postnatal development in mouse visual cortex. For that I used immunohistochemistry to describe the distribution of GFAP positive astrocytes and western blot analysis to correlate cellular distribution with endogenous protein content.

GFAP knock out mice: role of GFAP in pathological disease in central nervous system.

During the last years several investigators have suggested that GFAP might be important for CNS functions although its precise role has not yet been clarified. One can speculate that GFAP might be important in maintaining the correct shape and cytoarchitecture of astrocytes and that its overexpression and migh be essential to induce the typical hypertrophy of the cell body and process, during the gliotic process, which characterize the response of astrocytes to injury. Studies with GFAP mutant mice tried to afford the functional role of GFAP astrocytes. Several mutants GFAP mice have been developed during the last years and their phenotype has been investigated both in physiological and pathological conditions, both *in vivo* and *in vitro*. Pekny et al. (1995) showed that mice lacking GFAP develop normally, reach adulthood and reproduce, without any apparent abnormality in the structural architecture of the CNS, behaviour, motility, and cognitive functions such as learning and memory (Pekny et al., 1998). Other investigators showed a suppression of long-term potentiation (LTP) after ischemic insult (Tanaka et al., 2002), and an increase of cortical infarct volume (Nawashiro et al., 2000) in GFAP-null mice, indicating that the absence of GFAP promoted a high susceptibility to cerebral ischemia.

One of the proposed roles of astrocytes is to interact with specialized endothelial cells of the blood vessels in the construction of constituting the BBB. The major function of the BBB is to shield the brain from molecules and ions, while simultaneously providing an access to nutrients and aminoacids. An *in vitro* model of BBB showed that, astrocytes explanted from brain tissue of GFAP null mice failed to induce the production of a normal BBB when cocultured with wild type endothelial cells (Pekny et.al. 1998).

As described previously, GFAP overexpression is a hallmark of gliosis, and thus GFAP knock out mice are expected to be characterized by an abnormal gliosis in response to injury. Indeed, GFAP null mice are characterized by a normal gliotic reaction to injury, as shown by S-100β increase (Tanaka et al., 2002), suggesting that gliosis can occour also without GFAP presence. On the other hand, other authors (Pekny et al., 1999) showed an abnormal reaction to injury in mice lacking both GFAP and vimentin. These mice develop less dense scars frequently accompanied by bleeding. This latter finding shows that these two cytoskeletal proteins interact with each other to promote a correct gliotic reaction after pathological events in the CNS. Altogether, reported data suggest a fundamental role for GFAP in pathological and physiological processes occurring in the mammalian brain.

Factors modulating GFAP expression

As described before, the expression of GFAP, both at the mRNA and protein level, is developmentally regulated. It has been reported a rapid increase of GFAP during postnatal development followed by a rapid phase of reduction. The age in which the steady state is reached varies according to the animal specie. In aged animals, it has been reported that GFAP tend to increase, although the exact meaning of the change is still unknown. The protein amount, in astrocytes, varyies in parallel with mRNA expression, indicating that GFAP increase is not due to a merely GFAP accumulation, but that its synthesis is not constant during the development. Bulk of evidence indicates that GFAP expression can be modulated by several chemical and physical factors.

Sensory experience

It is well documented that sensory experience modulates neuronal plasticity in the mammalian CNS. In the rat visual cortex, for example, neuronal functional properties such as orientation selectivity, receptive field size, and ocular dominance are largely immature soon after eye opening (postnatal day 14-15) reaching progressively adult properties (Fagiolini et al., 1994). Visual experience is a necessary requirement for maturation of cortical neuronal connections that subserve visual function. In the rat (Fagiolini et al., 1994), as well as in other species (Timney et al., 1978; Wiesel, 1982), visual deprivation (dark rearing) from birth slows down the maturation of neuronal functional properties and maintains the cortical neuronal circuitry plastic (Timney et al., 1978; Mower et al., 1981; Cynader, 1983). Factors and mechanisms regulating maturation of visual cortical neurons have been extensively investigated. Studies conducted *in vivo,* on cat and rat, showed that maturation of GFAP expression is sensitive to manipulations of visual experience (Muller, 1990; Hawrylak, 1995). It is known that a complex environment provokes an increase of the surface of GFAP-IR astrocytes in the visual cortex of young rats, (Jones et al., 1996). A longer exposure to a complex environment results in an increase in the number of GFAP positive cells, beyond the surface, showing experience-mediated astrocytic plasticity as a two-stages event. The first stage consists in astrocytes hypertrophy and the second involves proliferation or delayed death of astrocytes. These changes may be related to brain information processing, since astrocytes are known to be modulators of synaptic activity and may possibly serve as regulators of synaptic density (Sirevaag and Greenough, 1991). An impoverished environment resulting in a poor sensory experience affects to some extent astrocytic plasticity. Monocular deprivation in young rats provokes a decrease of cell surface density of astrocytic processes in lamina IV of visual cortex, but not in other cortical layers and not in adult rats (Hawrylak and Greenough, 1995). However, other laboratories did not succeed in confirming the effects of monocular deprivation on astrocytes.

In the cat visual cortex, long periods of dark rearing from birth provoke a reduced expression of GFAP immunoreactivity, without affecting the density of astrocytic cell bodies (Muller, 1990). In the rat visual cortex dark rearing from birth provokes a reduction of GFAP amount, as measured by enzyme-linked immunosorbent assay (Gabbott et al., 1986). In contrast with the reported studies,

more drastic procedures involving deprivation of visual input such as the enucleation induces an increase in GFAP immunoreactivity in lateral geniculate nucleus (LGN) (Canady et al., 1994). Similar results were obtained with intraocular injections of tetrodotoxin which blocks the spiking activity of retinal ganglion cells projecting to LGN (Canady et al., 1994). The idea mutuated by the reported studies is that changes of neuronal activity dinamically regulate the astrocytic phenotype. However, the mass of data acquired by several laboratories was obtained using different experimental protocols and different mammalian species leading sometimes to contradictory results, which do not allow to draw definitive conclusions. Later, in this thesis I will report original data obtained on studying the influence of light on mouse visual cortex using a short protocol of dark rearing.

Calcium-induced GFAP regulation

The ability of calcium to induce gliosis was observed both *in vitro* and *in vivo*. It is know that acute cerebral ischemia induces acidification of the cerebro spinal fluid (CSF) (Crockard et al 1987). Acidification of the culture medium, to simulate brain damage, induces strong increase of GFAP immunoreactivity, which can be attenuated by treating cultures with nifedipine, a calcium channel antagonist (Oh et al., 1995). Thus suggesting that extracellular acidosis may produce a rapid increase of GFAP immunoreactivity in astrocytes, possibly by increasing intracellular levels of free Ca^{2+} ions.

In rat spinal cord lesion astrogliosis is reduced by treating lesioned animals with L-type calcium channels blockers (Klepper et al., 1995; Du et al., 1999), or by inhibiting μ-calpain, a calcium dependent protease (Du et al., 1999). These data show the ability of calcium ions to modulate astrocytic response to traumatic events occurring within the CNS.

Proinflammatory cytokines

Proinflammatory cytokines are involved in mechanisms of inflammation. Inside CNS they are also able to induce gliotic response. Their presence in the CNS was classically associated with damage of the BBB, as consequence of inflammation, and with the penetration of immunocompetent cells, from the systemic circulation, into

the brain. However, it was shown that some of these molecules are produced, in basal conditions, by glial cells and neurons and they contribute to induce inflammation and immune response within the CNS. These cytokines include interferon-gamma (IFN-γ), interleukin-1 (IL-1), tumor necrosis factor alpha and beta (TNF-α and TNF-β), interleukin-6 (IL-6), transforming growth factor beta (TGF-β) and the colony stimulating factors (CsFs).

IFN-γ is produced by cells of the immune system but not by neural cells. It is detectable in CNS only after the disruption of BBB provoked by injury. Studies conducted in model of ischemic injury, *in vitro* and *in vivo,* have shown that this cytokine is able to induce a marked increase of glial reactivity (Yong et al., 1991).

In physiological condition, both **IL-1α** and **IL-1 β** are expressed by neurons but not by astrocytes, and their expression increases after neuronal damage both in neurons (Minami et al., 1991; Liu et al., 1993) and in astrocytes (da Cunha et al., 1993). Data from astrocytic pure cultures, demonstrate a mitogenic effect of IL-1 (Giulian and Lachman, 1985). Injection of IL-1β into the brain provokes a marked astrogliosis (Giulian et al., 1988), suggesting an active role of this cytokine in modulating astrocytic response to pathological events.

Like IL-1, **TNF-α** is not expressed by astrocytes in physiological conditions but its expression and that of its receptor are induced by toxic agents (Lieberman et al., 1989; Chung and Benveniste, 1990). TNF-α has been reported to have a mitogenic effect on bovine astrocytic cultures (Selmaj et al., 1990) and to modulate GFAP expression, inducing a reduction of both the protein and the mRNA level (Selmaj et al., 1991).

IL-6 also has a mitogenic effect on astrocytic cultures (Selmaj et al., 1990). It is not produced by astrocytes in physiological conditions but its expression can be induced by many factors including other cytokines.

In several experimental models of pathological events, one cytokine can induce a response by neural cells in a direct way and in an indirect fashion, activating the expression of other cytokines or growth factors, which act together on cellular biology, in a synergic or antagonistic manner. Beside the ability of cytokines to stimulate synthesis of each other, they are also able to influence the synthesis of different diffusible molecules. Neurotrophic factors of the nerve growth factor family interact with cytokines in a complex way. In hypoxic conditions, IL-1 β modulates the

expression of NGF by neurons promoting recovery from cell damage (Di Loreto et al., 2000). In addition, activation of IL-1 receptors induces NGF secretion from cultured rat cortical astrocytes (Juric and Carman-Krzan, 2001) and the effect is magnified when IL-1 acts synergistically with TNF-α (Gadient et al., 1990). IL-6, present in the CSF of brain-injured patients, is able to induce overexpression of NGF in cultured mouse astrocyte (Kossmann et al., 1996), and the same effect is observed with IL-4 (Brodie et al., 1998). An interaction between neurotrophins and cytokines occurs in several pathological conditions, and virtually all members of the neurotrophin family can be modulated by cytokines.

1.6 NERVE GROWTH FACTOR (NGF)

NGF is the prototype of a family of trophic proteins (neurotrophins), which regulate the survival and differentiation of developing neurons and contributes to the maintenance of their specific functions during adult life (for review see Levi-Montalcini, 1987; Barde, 1990). Neurotrophins characterized up to now are nerve growth factor (NGF; Levi-Montalcini, 1951; Cohen, 1960), brain-derived neurotrophic factor (BDNF; Barde *et al.*, 1982; Leibrock *et al.*, 1989; Ernfors *et al.*, 1990a), neurotrophin-3 (NT-3; Ernfors *et al.*, 1990b; Jones and Reichardt, 1990; Kaisho *et al.*, 1990), and neurotrophin-4/5 (NT-4/5; Berkemeier *et al.*, 1991; Hallböök *et al.*, 1991).

Classical actions of NGF include trophic, differentiative and tropic modulation of neurons (Levi-Montalcini, 1987). More recently, NGF and the other neurotrophins have been involved in modulation of synaptic transmission (Thoenen, 1995).

The biological action of neurotrophins is mediated by specific receptors present on the plasma membrane of neurons as well as of other cell types. Various types of neurotrophin receptors have been characterized: a low-affinity receptor (p75) able to bind all neurotrophins and a group of so called high-affinity receptors specific for each neurotrophin (Fig. 1.6). The high-affinity receptors are tyrosine kinase-type receptors (Trk) and they have been identified as: TrkA (Kaplan *et al.*, 1991a,b; Klein *et al.*, 1991), TrkB (Klein *et al.*, 1990a,b; Middlemas *et al.*, 1991) and TrkC (Cordon-Cardo *et al.*, 1991; Lamballe *et al.*, 1991). They bind NGF, BDNF and NT-4/5 (with apparent equal affinity), NT-3 respectively; a low level of cross-reactivity of NT-3 with TrkA and TrkB has also been reported (Squinto *et al.*, 1991).

p75 was initially described as "the" NGF receptor (Chao *et al.*, 1986; Johnson et al., 1986; Radeke *et al.*, 1987): it is a transmembrane receptor with a large extracellular portion containing four negatively charged, cysteine-rich domains and a cytoplasmic domain which is highly conserved among species and devoided of any homology with protein kinases. The intracellular domain contains a so called death domain, involved in signaling pathways modulating apoptosis (for review see Wallach, 1997; Barrett, 2000). There is homology between p75 receptor and the tumor necrosis factor (TNF) receptor superfamily (Banner *et al.*, 1993). Interaction of neurotrophins with p75 may be important for increasing the specific activation of the Trk receptors (Benedetti *et al.*, 1993; Clary and Reichardt, 1994; Chao and Hempstead, 1995).

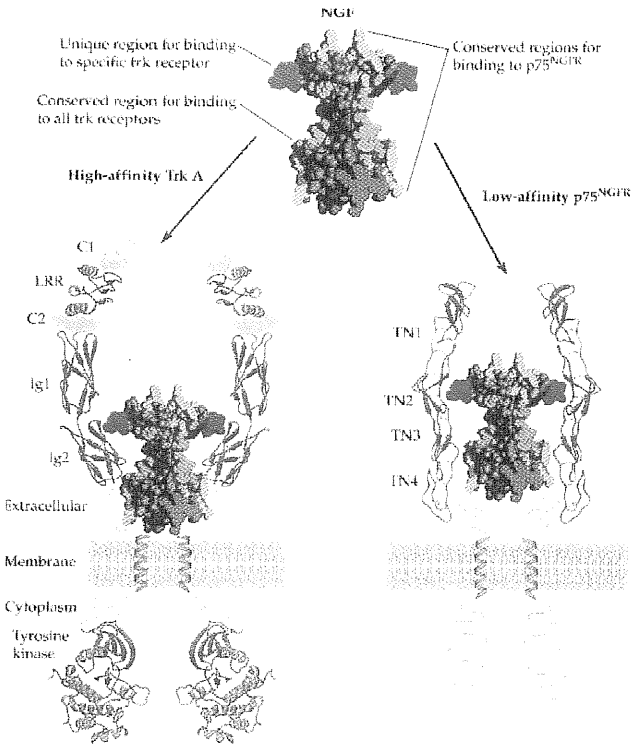


Fig. 1.6 High- (TrkA) and low- (P75) affinity NGF receptors.
NGF dimer binds to two the Immunoglobulin like-2 domain of the TrkA receptors (Ig-1) trough conserved regions common for the bindi to all Trk receptors, (in yellow) and the region specific for the TrkA receptor (in red). Region highly conserve in all neurotrophins (in blu) mediate the binding to the P75 receptor.
Ig1-2 Immunoglobulin like domain
LRR Leucine-repeat domain
C 12 cysteine-rich cluster
Tn1-4 Tumor necrosis factor like domai
(From Nicholls)

The extracellular portion of Trk receptors possess two cysteine-rich clusters, one of which is located in the amino-terminal region and is separated from the other by three consecutive leucine repeat motifs, and two IgG–related domains in the membrane-proximal region. The second IgG-like domain determines the specificity of

Trk receptors for neurotrophins (Urfer *et al.*, 1998). The other domains may be required for efficient expression, *in vivo* folding and traslocation across the membrane. Moreover, the first IgG-like domain may contribute to the formation of a stable signal, transducing complex by controlling receptor dimerization leading to a more efficient autophosphorylation (Urfer *et al.*, 1998). It is conceivable that Trk receptors carry cell adhesion molecules (CAM) binding domains, as CAMs are thought to play an important role in the development and maintenance of the nervous system by promoting morphological plasticity and maintaining stable contacts between cells (for a review see Goodman and Shatz, 1993). A candidate for this function is one of the leucine-rich motives, since all leucine-rich repeat containing proteins appear to be involved in protein-protein interactions and at least half of them take part in signal transduction pathways (Kobe and Deisenhofer, 1994). Finally, functional studies have reported that neurotrophins bound to Trk receptors are internalized and retrogradely transported to the cell body (DiStefano *et al.*, 1992). Hence, an alternative function for the domains not involved in the binding might involve attachment to transport proteins within the cell (Urfer *et al.*, 1998).

The cytoplasmic region of Trk receptors contains a catalytic tyrosine-kinase domain, a small insert and a short cytoplasmic tail. When bound to their specific neurotrophins, Trk receptors dimerize increasing their transphosphorylation activity and trigger a cascade of intracellular events (for review see Barbacid, 1994; Heumann, 1994). TrkB and TrkC also exist in a truncated form, lacking the intracellular domain. These truncated forms might have the function of modulating the local availability of active neurotrophins (Biffo *et al.*, 1995).

Activated Trk receptors induce a rapid increase of the phosphorylation of certain cellular substrates and a subsequent activation of several intracellular pathways. In particular, these are: 1) the pathway induced by the activation of phospholipase C leading to activation of protein kinase C and Ca-calmodulin-sensitive kinases; 2) the pathway mediated by p21-ras, leading to the activation of the MEK kinase cascade; 3) the pathway induced by phosphoinositide 3 kinase (PI 3 kinase) (for review see Heumann, 1994; Patapoutian and Reichardt, 2001). In general, tyrosine phosphorylation events result in downstream regulation of cellular proteins responsible for cell shaping and survival, and for activating the gene transcriptional machinery that controls growth, migration, morphology and survival of neurons.

NGF was the first neurotrophin to be identified. It is required for the development and survival of sympathetic neurons of the paravertebral chain in the PNS (Levi-Montalcini and Booker 1960). More recently, it has been shown that a neuronal subpopulation of the PNS involving in processing nociceptive information, is either redirected to a propioceptive 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, has been observed that blockade of endogenous NGF prevents both heat and mechanical hyperalgesia (Lewin et al 1994, Woolf et al 1994), and conversely exogenous supply of NGF can cause increased sensitivity to painful stimuli (Lewin et al 1994).

Using both mRNA in situ hybridization (Lauterborn et al 1994) and immunohistochemistry (Nishio et al 1994) it has been shown that NGF is mainly expressed in the cerebral cortex, 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 until 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, the labeling being restricted mainly to layers II-III and V-VI (Lauterborn et al 1994). Consistently, NGF immunoreactivity in adult rat is mainly restricted 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).

Concerning the cell types expressing NGF if much is known about the action of NGF on neurons, its role on glial phenotype has been less investigated

NGF and Astrocytes

Cultured astrocytes are able to synthesize NGF (Furukawa et al., 1986; Yamakuni et al., 1987; Condorelli et al., 1995) in physiological conditions, and its expression is developmentally regulated (Schwartz and Nishiyama, 1994). NGF production increases in response to pathological conditions. High levels of NGF are present on *in vitro* models of reactive astrocytes (McMillian et al., 1994; Wu et al., 1998) as well as after treatment with molecules closely associated with pathological processes, namely Dopamine (Furukawa et al., 1987) and its D_1/D_2 receptor agonist, apomorphine, an antiparkinsonian drug (Ohta et al., 2000). Moreover, overexpression

of NGF is induced by selegiline, an irreversible monoamine oxidase B inhibitor, which potentiates glial reaction to injury (Niu et al., 1997) or by hydrogen peroxide (Pechan et al., 1992). Unlike in culture, astrocytes do not synthetize NGF *in vivo,* in physiological conditions. However, CNS injuries induce expression of NGF by reactive astrocytes. For example, following traumatic (Goss et al., 1998) or neurotoxic-induced brain injury (Strauss et al., 1994; Fiedorowicz et al., 2001) and after ischemia (Gottlieb and Matute, 1999; Lee et al., 1996,1998), astrocytes are the major source of NGF.

It has been suggested that cultured astrocytes and glioma cells, are able to express p75 (Kahle and Hertel, 1992; Hutton et al., 1992) as well as TrkA (Hutton et al., 1992;Hutton and Perez-Polo, 1995). In culture, under serum-free conditions, exogenous application of NGF causes astrocytes to assume a fibrous morphology (Hutton and Perez-Polo, 1995) and to undergo cell division (Yokoyama, 1993).

In vivo TrkA expression has been detected in selected astrocytes contacting dendritic spines in the rat hippocampal formation, (Barker-Gibb, 2001), and in both white and gray matter of normal spinal cord (Foschini et al., 1994; Oderfeld-Nowak et al., 2001). TrkA expression is modulated during estrus (McCarthy et al., 2002), and by pathological conditions. Strong TrkA upregulation has been observed in reactive astrocytes of spinal cord, in experimental autoimmune encephalomyelitis (Oderfeld-Nowak et al., 2001) or after dorsal rhizotomy (Foschini et al., 1994). High levels of TrkA mRNA and protein has been described in the hippocampus of animal models of Alzheimer's disease (Connor et al., 1996), and in brain lesion induced by colchicines (Aguado et al., 1998) and trimethiltin (Koczyck et al., 1996; Koczyk and Oderfeld-Nowak, 2000).

In the cortex, the main target of NGF produced is the cholinergic neuronal population that resides in the basal forebrain region. These neurons express both NGF receptors TrkA and P75, and are sensitive to NGF. In particular, an exogenous supply of NGF induces an upregulation of choline acetyltransferase (ChAT), the rate limiting enzyme responsible for acetylcholine (ACh) synthesis. Conversely, down regulation of ChAT is obtained by blocking endogenous NGF with antibodies produced by hybridoma cells implanted in the brain (Molnar et al., 1998; Berardi et al., 1994;) or in anti-NGF-transgenic mice (Ruberti et al., 2000). Reactive astrocytes express NGF in response to cholinergic immunolesions of basal forebrain (Rossner et al., 1997) and after a chronic degeneration of cholinergic basal forebrain neurons, due to alcohol-

induced neurtotoxicity (Arendt et al., 1995). These results suggest that astrocytes are sensitive to alteration of cholinergic system.

1.7 THE CHOLINERGIC SYSTEM

Acetylcholine (ACh) is produced and released by cholinergic neurons which are located in the basal forebrain (BF) region and which projecting to several areas of the brain, including the neocortex and the hippocampus. Cholinergic neurons have a large cell body, long axons and dendrites. Based on their projection areas, basal forebrain nuclei give rise to four different pathways: 1) the basal-olfactory bundle, projecting to the olfactory bulb; 2) the basal-hippocampus bundle, formed mainly by the septal nuclei and the vertical limb of diagonal band; this pathway projects to the hippocampus and limbic cortex; 3) the medial cortical pathway, originating mainly in the vertical and horizontal limb of the diagonal band, nucleus basalis. Projections from these nuclei distribute to medial cortical regions including medial occipital cortex; 4) the lateral basal-cortical and basal-amygdala tract, supplying afferents to lateral cortical regions and to the nucleus of amygdala (Fig. 1.7).

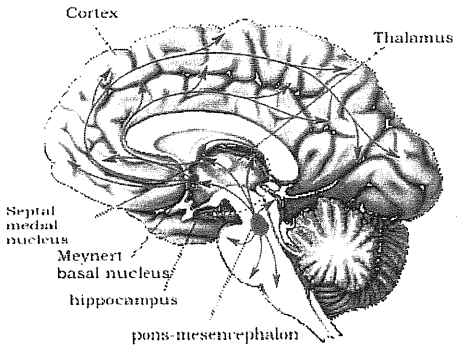


Fig. 1.7 Cholinergic innervation of the brain.

ACh is synthesized from acetyl coenzyme A and choline by the enzyme choline acetyltransferase and is degradated by the enzyme acetylcholinesterase (AchE), which separates choline from its acetyl and therefore terminates the binding of ACh to its receptors. (Fig. 1.8) ACh is stored in a cytoplasmic pool and in synaptic vesicles (Cooper et al., 1991). Upon the arrival of an action potential, ACh from the vescicular fraction, rather than from the cytosolic pool, is released in the synaptic cleft (Carroll, 1983, 1984). A specific low-affinity acetylcholine transporter (VAChT) is responsible for uptake of the transmitters from cytoplasm into vesicles (Schuldiner et al., 1995).

These enzymes are used as markers for the identification of cholinergic neurons. The anatomy of central cholinergic pathways has been well delineated through immunohistochemical studies employing antibodies against ChAT (Eckenstein and Thoenen, 1983; Houser et al., 1983; Levey et al., 1987).

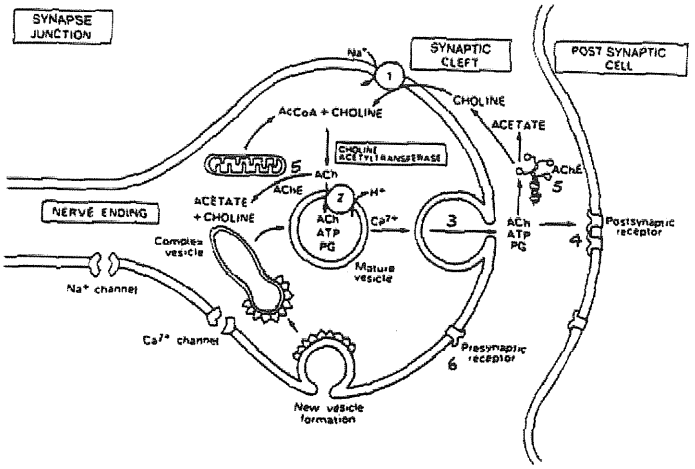


Fig. 1.8 General cholinergic Nerve junction showing ACh methabolism.
(From Katzung, 1992)

In rodent, cholinergic neurogenesis begins at embryonic day 12 and it is completed by embryonic day 17 (Semba and Fibiger, 1988). By using an anterograde tracer, BF it has been shown that axons reach the white matter under the cortical plate around P0. At P3 and successively at P6 the BF axons penetrate into the cortical plate and by the end of the first postnatal week they are found throughout the cortical layers. The BF axons reach an adult-like pattern around P11. Using ChAT as a marker for cholinergic neurons, it has been shown that BF neurons reach the mature pattern of innervation at the end of the second postnatal week. In adult rat visual cortex, cholinergic terminals are distributed throughout the laminae with prominent density in layer I, V and IV.

Cholinergic receptors define the response to Ach released by pre-synaptic terminals. Two main classes of cholinergic receptors are expressed in the nervous system: muscarinic and nicotinic receptors. Muscarinic ACh receptors (mAChRs) comprise 5 different subtypes (M1-M5). Upon binding to ACh the mAChR, which is coupled to G-proteins, activates phospholipase C (PLC) (M_1, M_3, M_5), catalyzes the hydrolysis of PIP2 in diacylgly-cerol (DAG; the physiological activator of PKC) and inositol trisphosphate (IP3, a messenger inducing Ca2+-release from intracellular

stores through binding to IP3 receptors, Berridge and Irvine (1984)). DAG, in concert with Ca2+, activates PKC, which in turn phosphorylates specifc proteins, among others mAChRs (Haga et al., 1990) and in particular the subclasses M1, M3 and M5 (Habecker et al., 1993; Haga et al., 1993). The binding of ACh to the subclasses M2 and M4 induce the G-protein-mediated inhibition of the adenilate cyclase (AC) and the stimulation of guanylyl cyclase, enzymes producing cAMP and gMP respectively. (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 has been found among the immunoreactive patterns of the different receptor subclasses. Highest levels for M_1 are detected in layers II/III, and VI. The M_2 labeling is stronger in layers IVA, IVC and at the border of layers V/VI (Levey et al 1991, Mrzljak et al 1993, Hohmann et al 1995, Tigges et al 1997). M_4 immunoreactivity is lower than 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 have been obtained 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). In mouse forebrain a temporal development of muscarinic receptor subtypes has been described using subtype-specific antibodies. M_2, and M_1, immunoreactivity reach a pattern of distribution characteristic of the adult by P14, while the M_2-IR further increases up to P30 (Hohmann et al 1995).

The neuronal nicotinic receptor class comprises 13 subunit genes, including α2 through α11, which encode the ligand binding subunits, and β2 through β4, which may represent the structural subunits. The receptor appears to be a pentameric ligand-gated ion channel with low selectivity for cations, and high permeability to calcium. Data support the idea that the vast majority (> 90%) of neuronal nicotinic receptors in the rat brain are composed of α4 and β2 subunits (possibly with the combination $(\alpha4)_2(\beta2)_3$) and are α-bungarotoxin insensitive. The second most represented receptors in the cortex contain α-7 subunits (possibly homomeric) and are α -bungarotoxin sensitive. The α-7 receptor exhibits an extraordinary permeability to calcium ions and

is particularly enriched in rat hippocampus (Sargent 1993, Seguela et al 1993, McGehee and Role 1995). Immunohistochemical observations revealed that α4-immunoreactivity is distributed through layers II to VI of the rat cerebral cortex. In a study employing monoclonal antibodies against α 4 and β 2 subunits in the rat brain, immunoreactivity is found in all cortical regions. Immunoreactive cells for muscarinic and nicotinic receptors comprise excitatory pyramidal cells as well as interneurons distributed throughout cortical layers, and in astrocytes.

The Cholinergic system and astrocytes

Several studies, employing different techniques, have demonstrated the presence of functional active acetylcholine receptors in astrocytes. Even thought both muscarinic and nicotinic receptors have been detected in cultured astrocytes (Hosli et al., 1983; 1997; Hosli and Hosli, 1994; Sharma and Vijayaraghavan, 2001), few reports are available for nicotinic receptors, while many authors described the expression of muscarinic receptors by astrocytes. Electrophysiological recording have shown that ACh induces hyperpolarization in cultured astrocytes (Hosli et al., 1988; Hosli et al., 2000). This effect is mediated by nicotinic and muscarinic receptors. However, while stimulation of muscarinic receptors induces hyperpolarization in all astrocytes, nicotinic stimulation induces the same effect only in half of them (Hosly et al., 1997). ACh is also able to stimulate proliferation of astrocytes and this effect is mediated by muscarinic receptors (Guizzetti and Costa, 1996; Ashkenazi et al., 1989). Concerning the different muscarinic subtypes, it was found that cultured rat astrocytes express M3 (Kondou et al., 1994;Guizzetti et al., 1996) and M2 mAChRs (Guizzetti et al., 1996), while cultured mouse astrocytes express M1 and M2 mAChRs (Andre et al., 1994), and human astrocytoma cells express the same subtypes plus M5 mAChRs (Guizzetti et al., 1996). It was suggested that the M3 acetylcholine receptor mediate the proliferation of astrocytes (Guizzetti et al., 1996), as well as the increase of intracellular calcium level in cultured astrocytes (Catlin et al., 2000; Ferroni et al., 2002). The idea that muscarinic receptors mediate the astrocytic response to ACh has been suggested also by experiments conducted in rat hippocampal slices. Combining electrophysiological recording with calcium imaging, a long-lasting inward current (Araque et al., 2002) and an increase of calcium level (Shelton and McCarty, 2000; Araque et al., 2002) have been observed after stimulation of muscarinic receptors. Astrocytic expression of muscarinic receptors has been detected *in situ* in rat brain

with a predominant localization in the most superficial cortical layers and in the white matter of the corpus callosum (van der Zee et al., 1989; 1993). Unluckily, the used antibody (M35) was unable to distinguish among the muscarinic subtypes (Andre et al., 1983) and, thus, at present it is still unknown the muscarinic receptors subtypes expressed by astrocytes *in situ*. Interestingly, the number of astrocytes expressing mAChRs increases in hippocampus after amygdala seizures (Beldhuis et al., 1992) and in gray matter of patients affected by Alzheimer's disease (Messamore et al., 1994).

2. AIM OF THE STUDY

The studies reported in this introduction outline that astrocytes play an important role in nervous system physiology. These studies have prompted neuroscientists to look at the nervous system from a different point of view, considering neuronal functions as the outcome of an active interaction between astrocytes and neurons. To clarify the factors involved in the cross talking between neurons and astrocytes, I studied the effects induced by two molecules, NGF and Ach, on astrocytic phenotype on GFAP expression. NGF and Ach are expressed in the CNS together with their receptors and are known to be important for neuronal survival, differentiation and phenotype maintenance. Experiments were performed in the primary visual cortex of mouse, a well-known area whose function and architecture has been well described. To investigate the effects of NGF deprivation, transgenic mice producing a monoclonal anti NGF antibody, known to block the action of endogenous NGF were utilized. To exclude the possibility that potential effects on astrocytes in this transgenic mice were due to genomic changes, occurring during the manipulation of the genotype, adult wild type mice implanted with hybridoma cells secreting blocking NGF antibodies were also examined. Alterations of astrocytes induced by NGF deprivation were related to neuronal changes such as those occurring in BF cholinergic neurons, the principle target of NGF action in the CNS. Effects induced by deprivation and activation of cholinergic system, including modulation of cholinergic receptors, on astrocytes phenotype were also investigated and related to results obtained in NGF deprived animals.

Since most of the experiments where conducted in young mice, it was mandatory to describe the period of astrocytes maturation and whether visual

experience modulates it. Indeed, sensory experience has been reported to constitute the driving force of visual cortex maturation and so Iinvestigated its role on GFAP maturation.

MATERIALS AND METHODS

Materials and methods

Animals

Experiments were performed in two different strains of mice, C57BL/6J (Jackson Laboratory, Bar Harbor, ME) and C57BL/6 x SJLF2.

C57BL/6J. Experiments were conducted on visual cortex at different postnatal ages: P12, P24, and P60. A different group of animals was reared in darkness from P12 to P24 (dark rearing), five animals for each age and condition were used.

C57BL/6 x SJLF2. Experiments were conducted in transgenic mice adult or wild type of the same littermate.

Experiments were conducted in accordance to the European Community Council Directive for animal treatment. All the animals were housed in groups of 4/5 animals for cage with food and water at libitum. Except the animals used for dark-rearing experiments, the other ones were housed on an artificial 12 hr dark/light cycle. All the animals were housed in the animal house of the University of Trieste. Handling of animals was done in accordance with the Italian law DL 27/1/92 n.116 based on UE rule n.86/609.

Dark Rearing

To evaluate the effect of light deprivation on GFAP expression, mice were kept in complete darkness from the age of P12 to the age of P24. One group was placed in an artificial-light-dark-cycle (12h/12h, control group) whilst the other one remained in the dark for 12 days (dark rearing group). For each litter, pups were randomly assigned to the dark rearing group or the control one. At P24, animals were anesthetized and each experimental group was further divided into two groups: one was perfused and processed for immunohistochemistry while western blot was carried in the other group. After immunohistochemistry sections were couterstained by incubating them in a 0.05% solution of cresyl violet (Sigma, St Louis) and cells were counted.

Flash electroretinogram (F-ERG)

Mice were anaesthetised by an intraperitoneal injection of Urethane (Sigma, St Louis) and loosely mounted in a stereotaxic apparatus with the body temperature maintained at 37.5 °C and the heart rate monitored. F-ERG was evoked by 10 msec flashes of different light intensities generated through a Ganzfeld stimulator (Lace,

Pisa, Italy). The electrophysiological signal was recorded through gold plate electrodes inserted under the lower eyelids, in contact with the cornea previously anaesthetized with ossibuprocaine (Novesine, Novartis Pharma, Switzerland) following methods already described (Domenici et al., 1991b). To minimize the noise ten different responses evoked by light stimulation of both eyes were averaged. The latencies and relative amplitudes of a- and b- were analyzed on-line. After 120 min. of dark adaptation F-ERG was recorded in response to flash of different light intensities ranging from 10^{-4} to 20 cd*m-^{2} sec^{-1}. After completion of responses obtained in dark adaptation conditions the recording session continued with the aim to dissect cone pathway mediating light response. To this aim the f-ERG in response to light of increasing intensity was recorded in the presence of a continuous background light known to adapt rods (background light set at 25 cd*m-^{2}).

Retinal histology.

After F-ERG recordings the eyes were dissected, prepared as eyecups and fixed with 4% buffered paraformaldehyde for 30 minutes at room temperature. Eyecups were washed and crioprotected in Sucrose 30%, embedded and cut on the sagittal plane of cryostat. Retinal sections (16 μm of thickness) were collected on gelatine-subbed slides. Cell nuclei were labeled by incubating the tissue with the nuclear stain 4'6-diamidine-2-phenylindole dihydrochloride, (DAPI, 0.8% in buffer; Sigma, St. Louis) for 20 minutes. Successively, slides containing retinal sections were covered and examined using epifluoscence microscopy (Nikon microscope, model E600). Retinal images were captured by CCD camera (Photometrics, CoolSnap fc).

Transgenic mice

Transgenic mice have been developed by Ruberti et al. (2000).

The plasmids pcDNAI-neo/VKaD11HuCK and pcDNAI-neo/VHaD11HuCg, carrying the light and heavy chain genes, respectively, of the chimeric antibody aD11 (Ruberti et al., 1993) under the transcriptional control of the human cytomegalovirus (CMV) early region promoter [2601 to 216 (Boshart et al., 1985)], were digested with *Kpn*I–*Apa*LI and *Kpn*I–*Xba*I, respectively, to isolate the transcriptional units. The fragments were microinjected in the pronucleus of single-cell fertilized C57BL/6 3 SJLF2 hybrid mouse eggs, either individually or in combination, and the injected eggs were reintroduced into foster pseudopregnant females of an outbred strain different from that of the microinjected eggs. Production of transgenic mice was performed by a custom transgenic service (DNX Corporation, Princeton, NJ). Analysis of transgenic

mice was performed by PCR and dot blot, on genomic DNA from tail biopsies, as described (Piccioli et al., 1995). For the dot blot, the heavy chain probe was a *Bam*HI–*Xba*I fragment from pcDNAI-neo/VHaD11HuCg, encompassing the human heavy chain constant region, whereas the light chain probe was a *Bam*HI–*Apa*LI fragment isolated from plasmid pcDNAI-neo/VKaD11HuCK, encompassing the human light chain constant region. The DNA was quantitated by optical density at 260 nm before loading on the filter. Two founder mice with the light chain transgene, two with the heavy chain transgene, and three double transgenic mice were generated, but despite an intensive breeding program, no offspring were generated. Mice homozygous for the aD11 heavy chain transgene (VH-aD11 mice, lines C and D) and mice homozygous for the aD11 light chain transgene (VK-aD11 mice, lines A and B) were generated from the corresponding founders, by at least two crossings. Homozygosity was verified by genomic PCR analysis on offspring obtained by crossing putative homozygous to negative mice (at least two independent litters for each homozygous line). To obtain mice expressing both chains, and thus reconstituting NGF binding activity, single transgenic mice were intercrossed in different combinations. Two groups of controls were used for the phenotypic analysis of family 1 and family 2 mice: wild-type mice and the corresponding single transgenic controls, expressing only the VH-aD11 heavy chain (C or D, as appropriate). *RNA analysis.* Total RNA was isolated by the guanidineisothiocyanate procedure (Chomcynzski and Sacchi, 1987) and analyzed by RNase protection. The size of the expected protected band was 340 bp for VH and 310 bp for VK. The template for b-actin mRNA transcription was a *Rsa*I, 1700 bp fragment in pGEM4Z. Transcription with T7 polymerase produced a 110 bp probe. VH, VK, and b-actin antisense RNA probes were hybridized to 20 mg of RNA in 80% formamide (46°C for 12–16 hr) and treated with RNase A and T1. Protected fragments were electrophoresed through a 4–6% acrylamide, 8 M urea gel and autoradiographed.

Hybridoma Cells implant

To be sure that the phenotype observed in transgenic mice was due exclusively NGF deprivation, we injected, in the lateral ventricle, hybridoma cell secreting an anti-NGF monoclonal antibody (mAb α-D11) (Cattaneo et al., 1988). Both hybridoma cell line secreting mAb αD11 (αD11-hybridoma cell) and the myeloma cell line P3X63Ag8 (briefly P3U, controls), were grown in Dulbecco's modified Eagle's

medium (DMEM) (Life technologies, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS) (Life technologies). Prior to the injection, cells were washed four times in Hank's balanced salt solution (HBSS) (Life technologies), incubated in DiI for 1 hour at 37 C washed in DMEM supplemented with FBS to absorb the free dye then washed again in HBSS and finally resuspended at $2x10^5$ cell/μL. P3U and αD11-hybridoma cells were injected into the right lateral ventricle of wild type mice at 50 postnatal days (Molnar et al., 1998). For the ventricular injection mice were anesthetized with 2% Tribromoethanol (15 ml/Kg body weight, i.p.) (Sigma-Aldrich ; 400mg/Kg) The injection was assessed using a cannula connected to a Hamilton syringe at the following stereotaxic coordinates: anterioposterior –0.6 mm; mediolateral +1.00 mm relative to the skull surface at bregma; and dorsoventral, -2.2 mm relative to the dura at the injection site. A total of 2 μl was injected. Immediately after injection, animals were treated with cyclosporin A (Sandoz pharma LTD, Basle, Switzerland; 1.5 mg/kg) and treatment was repeated every two days for one week, to prevent implant rejection.

Enzyme linked immunosorbent assay (ELISA)

ELISA was performed on surnatant of both αD11 and P3u cells to test the ability of cells to produce or not the monoclonal antibody and to check the specificity of the cell type (Molnar et al., 1998). Briefly, NUNC Maxi Sorp plates (Applied Scientific; San Francisco, CA, USA) were coated with NGF dissolved in Hydrogen carbonate buffer (5μg/L, overnight at 4 °C) (Alomone Labs, Jerusalem, Israel), to obtain solid phase NGF. Plates were then washed five times in 0.005% Tween 20 in phosphate- buffered saline (PBS) followed by five washes in PBS, and then blocked with 2% defat dry milk in PBS (1 h at 37°C). Surnatant from αD11 or P3U cell culture was collected and diluted in a solution of 2%milk in PBS. Serial dilutions were incubated with solid phase NGF (1 h 37°C). Plates were then washed as above, and incubated, with byotinilated rabbit anti-rat IgG (1:500, 1 h at 37°C) (Dako Laboratories) in PBS plus milk. After washing they were incubated in ABC kit (1:100, 30 min room temperature) (Vector Labs Inc., Burlingame, CA). The peroxidase reaction was detected, after washing, with one-step TMT Turbo ELISA (PIERCE, Rockford, IL, USA) and stopped with 2M sulfuric acid. The reaction was finally analyzed using an ELISA-reader at 450 nm. Only hybridoma cells, actually

expressing mAb-αD11, and P3U, not expressing the antibody, used as control, were implantend.

Drug Treatment

All drug treatments were done in adult C57BL/6 x SJLF2 mice. Five animals per treatment were used. The doses were chosen to be non lethal and care was taken to avoid gross alterations in deambulation and breath rhythm. Control group was injected with vehicle alone (0.9% sodium chloride solution). After the treatment, animals were anesthetized, transcardially perfused with PBS, brain removed and divided into two hemispheres: one was processed for immunohistochemistry and the other one dissected, rapidly frozen and used for western blot.

In the present work, subcutaneous (s.c.) injection of (±)-Vesamicol hydrochloride (RBI, Natick, USA) was administered in a single dose, at two different concentrations: 1.3 mg/Kg or 5 mg/Kg, the effect for each of the two doses was evaluated 5 h later.

Scopolamine

Subcutaneous (s.c.) injection of scopolamine Hydrochloride (Sigma, St Louis, USA) was administered in a single dose, at two different concentrations: 5 mg/Kg or 10 mg/Kg, the effect for each of the two doses was evaluated 5 h later.

Intraperitoneal (i.p.) injection of Mecamylamine hydrochloride (Sigma, St Louis, MO) was administered in a single dose, at the concentrations: of 5 mg/Kg, the effect for each of the two doses was evaluated 5 h later.

Immunohistochemistry

Mice were deeply anesthetized with 20% urethane (Sigma, St. Louis, MO), transcardially perfused with PBS then with 4% paraformaldehyde in 0.1 M Phosphate Buffered Saline (PBS). Brains were removed, post-fixed for 1 hr at 4°C, in the same solution, then cryoprotected in 20% sucrose in PBS. For the experiments on the role of acetylcholine in GFAP expression, after perfusion with PBS, brains were removed and divided into two hemispheres: one was immersed in 4% paraformaldehyde in 0.1 M PBS (ON 4°C) then cryoprotected in 20% sucrose, the other one was processed for western blot. Coronal sections (40 μm thick), containing the primary visual cortex and hippocampus (approximately –1.06 to –5.00 from Bregma, according to the atlas of mouse brain, Franklin and Paxinos, 1997) sections containing basal forebrain and basal nucleus of Meynert (approximately 1.42 to –1.34 from Bregma) were obtained

using a freezing sliding microtome and serial sections were collected. Sections were washed three times in PBS plus 0.05% Triton X-100 then in 3% hydrogen peroxide and washed again in PBS/Triton. After washing, sections containing visual cortex and hippocampus were preincubated with M.O.M mouse Ig Blocking reagent (Vector Labs Inc., Burlingame, CA), diluted in PBS/Triton, for 1 h at room temperature to block non-specific binding sites. Sections were incubated overnight, at 4°C, with a mouse anti-GFAP monoclonal antibody (Sigma-Aldrich, clone G-A-5; 1:100) or with a mouse anti-S-100 monoclonal antibody (Neo Markers, Fremont, Ca, USA; 1:100), recognizing both the α- and the β-form, diluted in PBS/Triton, plus M.O.M diluent. Sections containing basal forebrain and basal nucleus of Meynert, were washed and treated with hydrogen peroxidase, then preincubated in 10% fetal calf serum and 5% bovine serum albumin diluted in PBS/Triton for 1h at room temperature to block non-specific binding sites. After that, sections were incubated overnight at 4°C with a goat anti-ChAT polyclonal antibody (Chemicon international, Temecula, Ca, USA; 1:500), to stain cholinergic neurons. After the incubation with the primary antibodies, for detection of both GFAP and S100, sections were incubated with a biotinylated anti-mouse IgG (Vector Labs Inc., Burlingame, CA; 1:200), diluted in PBS plus M.O.M. diluent (3 hr at room temperature). For the detection of ChAT-IR neurons, after the incubation with the primary antibody, sections were incubated with a biotinylated anti-goat IgG (Vector Labs Inc; 1:200), diluiuted in PBS plus 5% fetal calf serum (3h at room temperature). For all the antibodies used, the horseradish peroxidase conjugated avidin-biotin complex (Elite Standard kit; Vector Labs Inc; 1:200) was used for 1 hr at room temperature. The peroxidase complex was visualized using 3,3-diamminobenzidine tetrachloride (Sigma-Aldrich) dissolved in Tris/HCl, pH 7.5. Cells positive for GFAP, S100 and ChAT, were counted using a grid and a 40x objective. To distinguish different cortical layers, sections coming from dark reared animals and its control group, were counterstained with cresyl-violet acetate (0.05%, Sigma).

Cell Counts

Cells counts were performed for GFAP, S100 and ChAT immunoreactive cells, in all the treated and controls animals and in all the transgenic and wild type for each age studied as previously described.

Sections containing visual cortex and hippocampus were divided in 10 consecutive series (four-five slices/series) and sections containing basal forebrain in 6 consecutive series (Howard & Reed, 1998). In this way, respectively 10, or 6, series of 4-5 sections each were sampled random uniformly. A single random series (1/12 or 1/6) of coronal sections/animal was analyzed for each antibody, thereby allowing all visual cortical sections to be sampled with the same probability. After processing for immunohistochemistry, GFAP and S100-IR cells in the visual cortex and hippocampus or ChAT-IR neurons in basal forebrain and basal nucleus of Meynert, were counted using a 40x objective. A 300 μm wide grid was superimposed on the visual cortex to delimit the area of sampling. Cells within each frame were manually counted, in different focal planes, using the "optical dissector" method (Gundersen, 1986).

Western Blot analysis

To evaluate the amount of GFAP in cortical areas, proteins were extracted from visual cortex or hippocampus, as described later, and the amount of GFAP was analyzed. Mice were deeply anesthetized with 20% urethane, the brain was removed and the visual cortex and hippocampus was separated from the rest of the brain, utilizing the same stereotaxic coordinates as for the immunohistochemistry. Cortex was homogenized in an extracting buffer (1 g tissue/ 5 ml buffer) containing 50 mM Tris HCl pH 8, 150 mM EDTA, 0.1% deoxycholic Acid, 1 mM aprotinin, 5 mg/ml phenilmethylsulfonilfluoride, 1mM iodoacetamide, 5 mg/ml leupeptin, 4 mg/ml soy bean trypsin inhibitor, 10 mg/ml turkey egg white inhibitor. Protein amount was determined by using the bicinchoninic acid protein assay kit (Sigma) following the manufacturer instruction.

For each lane 7.5 μl of the entire extract was loaded with 2.5 μl to SDS gel-loading buffer (exept for the analysis of vesamicol's effect, in which 100μl of total protein were loaded in eache lane), and resolved in a 12% SDS polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane. Membrane was blocked for one hour at 37° C with 5% non fat dry milk in phosphate-buffered saline (PBS) plus 0.05% of Tween 20 (PBST) then incubated overnight at 4° C with the antibody dissolved in PBST. We used a goat anti-GFAP polyclonal antibody (1:300) (Santa Cruz Biotechnology and Laboratories) and a rat anti-tubuline monoclonal antibody (mAb YOL-1, Kindly provided by Cesar Milstein, MRC, Cambridge UK) (1:250).

After washing in PBST the membranes were incubated for 2 hours at 37° C with a biotinylated anti-goat IgG (1:1000) (Vector Laboratories) or biotinylated anti-rat IgG (1:500) (Dako Laboratories), washed and incubated for 30 min at room temperature with alkaline-phosphatase conjugated ABC kit (1:1000) (Vector Laboratories). Reaction was revealed using p-Nitro Blue Tetrazolium chloride (NBT) (Sigma, 0.5 mg/ml) and 5-Bromo-4-Chloro-3-Indoyl Phosphate p-toluidine salt (BCIP) (Sigma, 0.25 mg/ml) in developing buffer (0.1 M Tris, 0.5 mM MgCl2, pH 9.5). Each western blot was run in duplicate. For a semiquantitative analysis, after western blot, membranes were scanned and the density of the single bands was determined using dedicated software (NIH-SCION IMAGE, Scion Corporation, MD). For each band, optical density (OD) was calculated multiplying the mean optical density by the area of the band and values were given as relative units (RU) according to the follow formula: (OD of GFAP/ OD of Tubulin)*100.In the experiment with dark reared animals, to assess the effectivness of dark rearing protocol, the expression of NR2A subunit of NMDAr was evaluated. For detection of this protein, an 8% SDS polyacrylamide gel was used and western blot analysis was performed as described above. The membrane was incubated overnight with a rabbit anti- NMDAR2A policlonal antibody (Chemicon International, Temecula CA) (1:300). After washing membrane was incubated, for 2 h at 37 °C, with a biotinylated anti-rabbit IgG (1:500) (Vector Laboratories) and then processed as described above, for the other antibodies.

TrkA reverse trascription-polimerase chain reaction (RT-PCR)

Presence of TrkA mRNA was checked in visual cortex by using RT-PCR. To detect protein-specific mRNA, total RNA was extracted from visual cortex using TRIZOL Reagent (GIBCO BRL). A 1-μg aliquot of total RNA was reverse transcribed (RT) into cDNA using Moloney-Murine leukaemia virus (M-MLV) reverse transcriptase (GIBCO BRL) with random priming. After RT, 1 μl of cDNA was used as template in 25 μl of PCR mixture containing 0.5 mM final concentration of protein-specific primers, 0.2 mM deoxinucleotides, GeneAmp 10X buffer and 0.5 U of Platinum Taq DNA Polymerase (GIBCO BRL). Primers for *TrkA* and for β-actin were designed from published sequence data as follows: (1) *TrkA*, accession #M85214 (*Rattus rattus*), *TrkA* (2253) = 5'-ggtaccagctctccaacactgagg-3', *TrkA* (2456c) = 5'-ccagaacgtccaggtaactcggtg-3', 204 bp amplified product ; (2) β- actin, accession #X03672 (*Mus musculus*), actin (182) = 5'-cgtgggccgccctaggcacca-3', actin

(424c) = 5'-ttggccttagggttcaggggggg-3',243 bp amplified product. Number in brackets refer to the position of he primer in the nucleotide sequence, "c" denotes the complementary strand. PCR steps were as follow: after a denaturation step of 5 min 95°C, amplification was carried out for 35, in the case of *TrkA*, or 25 cycles, for β-actin, of 94°C/30 sec, 60°/30 sec, and 72°/1 min. As positive control, mRNA extracted from Basal forebrain, was used. Final product was analyzed on a 1.5 agarose/TBE gel.

RESULTS

4.1 GFAP Development in mouse visual cortex

Distribution of GFAP-IR astrocytes in postnatal development

The first aim of my work was to describe the distribution of GFAP immunoreactive (IR) astrocytes in mouse visual cortex during postnatal development; moreover the amount of GFAP at different ages was evaluated. The meaning of this study, inside my thesis, resides to the fact that all the subsequent experiments I conducted, aimed to interfere with adult- like, regular GFAP organization in this animal model. Therefore, it was important to understand the period in which the shift from a developing stage, to a stable, adult stage occurs. To evaluate the cellular distribution of GFAP in developing mouse visual cortex we used a monoclonal antibody against GFAP. GFAP expression was evaluated at three different postnatal ages: P12, before eye opening, P24 which corresponds to the middle stage of postnatal development when the cortex is sensitive to MD, and P60 when development and the critical period are by and large over (Gordon and Stryker, 1996).

Twelve days of age. At this age eyes are still closed and no interference of light input can act on GFAP-IR cell organization. Immunohistochemical analysis revealed cells heavily stained in different cortical layers and white matter. In gray matter (Fig.4.1 a, d, g) the highest cellular density was observed in layers I and VI (Fig. 4.1 a and g). Heavy stained cells are also abundant on the pial surface (Fig.4.1a). These cells are absentin layer II-III and layer IV/V (Fig. 4.1a and d), where it is possible to observe thick filaments, reminiscent of radial glia (Fig. 4.1 a and d, arrowheads). A dense plexus of GFAP-IR cells is visible in layer VI (Fig. 4.1 g). At higher magnification, cells in layer I show bushy perykaria with short multipolar processes, characteristic of cells moving to become mature protoplasmic astrocytes (Fig. 4.2a).

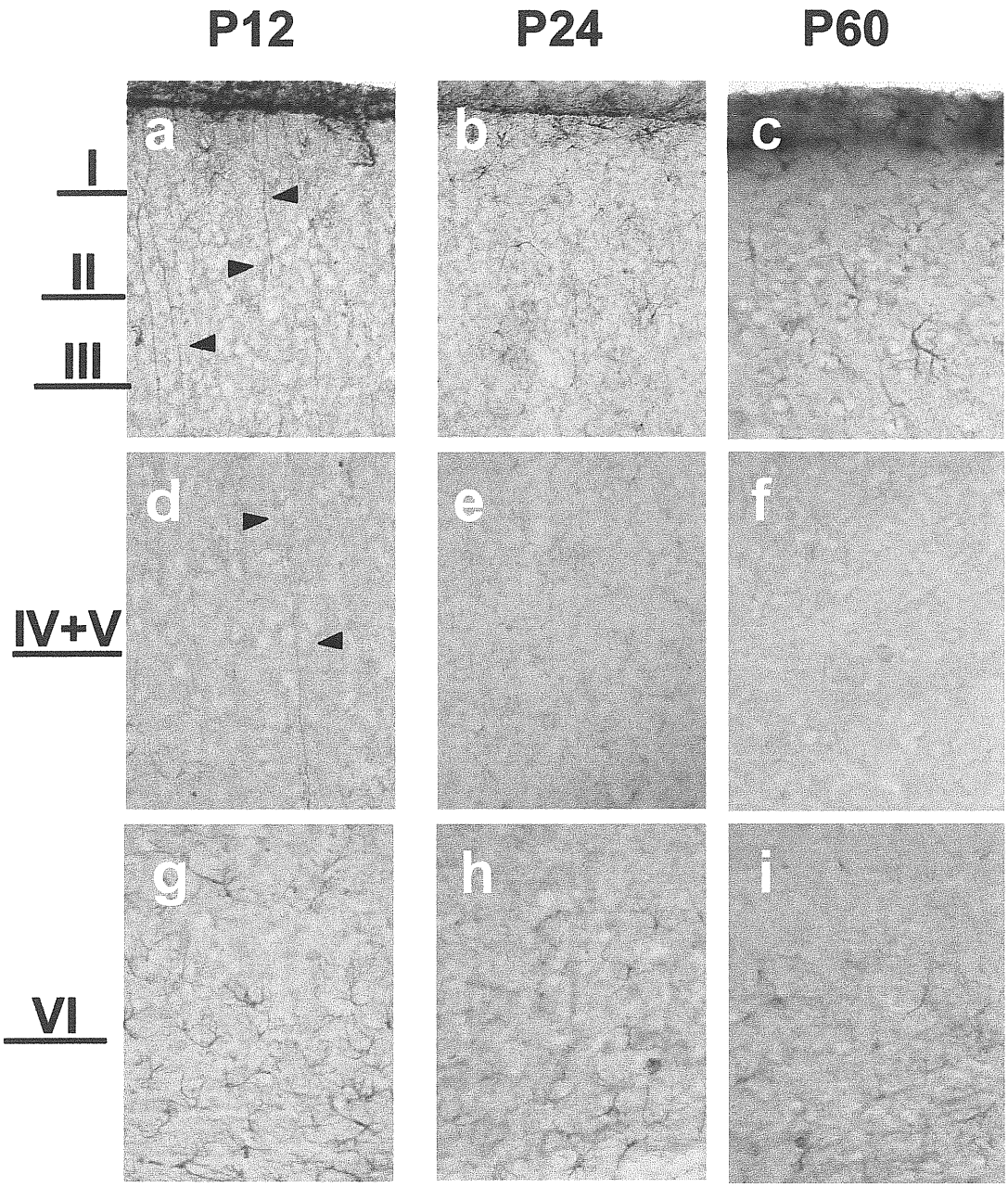


Fig. 4.1: GFAP immunoreactivity in developing mouse visual cortex.

In P12 mice (A, D, G), labeled radial glia spans the entire cortical depth (arrowheads). At P24 (B, E, G) and in adult mice (C, F, H), labelled radial glia disappears and GFAP-stained astrocytes are localized in the infragranular and supragranular layers.

Scale bar: 50 μm

Fibers reminiscent of radial glia, extending radially into the gray matter, without evident staining of cell bodies, are visible in layer II-III and layer IV/V (Fig. 4.2b). Cells present in layer VI show a small soma with few short processes located among fibers of radial glia (Fig. 4.2c). Heavily stained cells were also located in the white matter (Fig. 4.2f) where they form a dense plexus in which cells show a fibrous aspect with few long processes which sometimes span the entire cortex.

Twenty-four days of age. At this age, radial glia is no longer present in any of the cortical layers. Bushy, star shaped cells are wydely distributed in layer I and in a dense plexus confined on the pial surface (Fig. 4.1b). Fewer astrocytes, with the same phenotype as in layer I, are also present in layer II. Different from P12, no GFAP immunoreactivity was found in layers IV and V (Fig. 4.1e). Bushy cells were also observed in layer VI (Fig. 4.1h) and on the contrary to that observed at P12, they are less diffuse, moving to form a thin layer on the border with the white matter. A few cells similar to those observed at P12 were also present at P24. At higher magnification (Fig. 4.2d), astrocytes display a heavily stained soma, bigger than the one present at P12, from which multipolar processes branch off and can be followed for short distance. The highest staining level for GFAP was observed in white matter (Fig. 4.2g). Numerous fibrous astrocytes are densely packed and highly immunoreactive, radial glia is absent and processes spanning the entire cortical layer are no longer present. Also in adult mice, we did not observe radial processes running towards the pial surface or those reminiscent of radial glia.

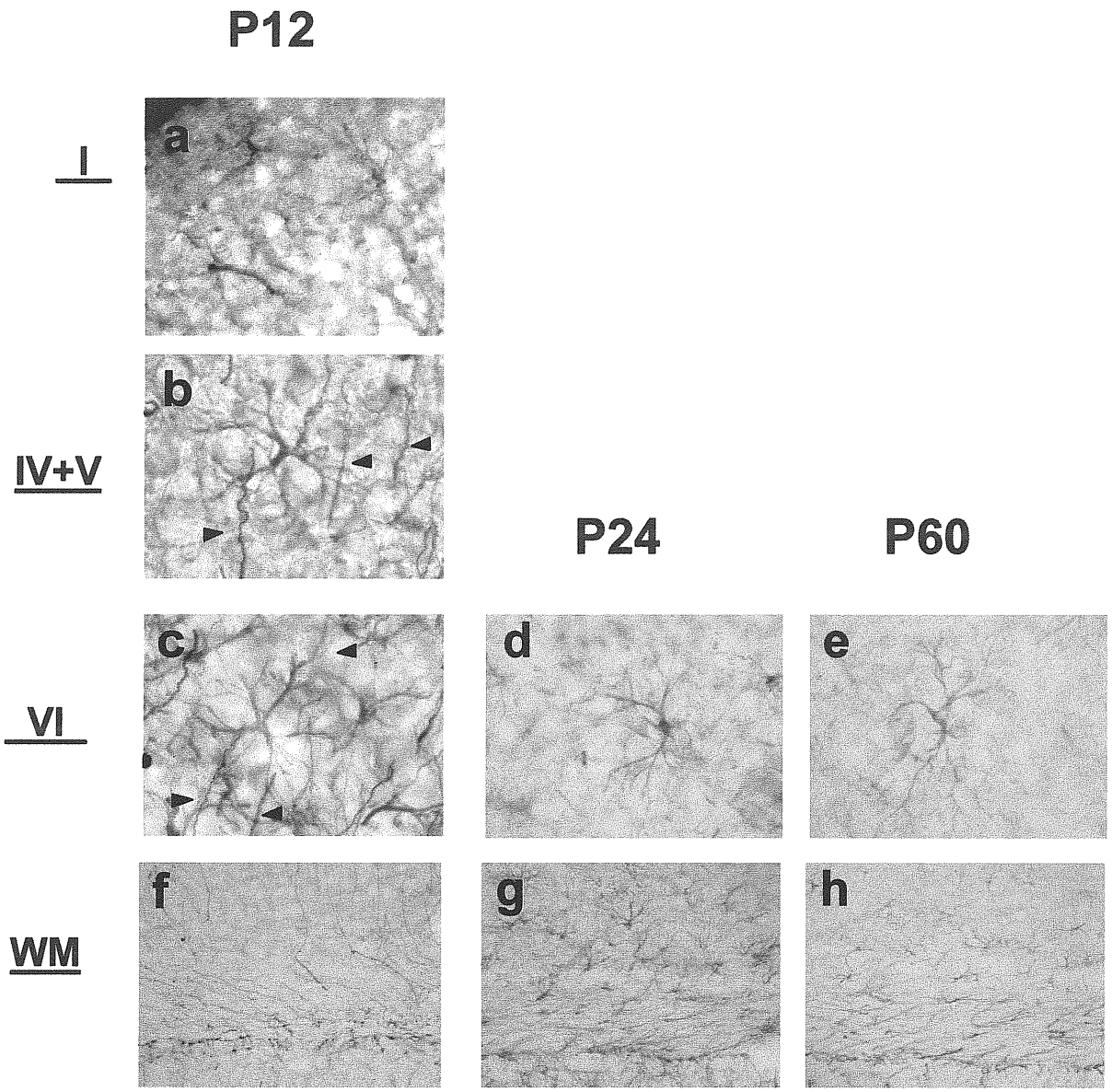


Fig. 4.2: GFAP immunoreactivity in developing mouse visual cortex.
In P12 mice (A, D, G), labeled radial glia spans the entire cortical depth (arrowheads). At P24 (B, E, G) and in adult mice (C, F, H), labeled radial glia disappears and GFAP-stained astrocytes are localized in the infragranular and supragranular layers.
Scale bar: 50 μm

Two months of age. At P60, the cortical distribution of GFAP was similar to that observed at P24. GFAP immunopositive cells showed the same preference for pial surface, layer I (Fig.4.1c), layer VI (Fig. 4.1i) and white matter (Fig. 4.2h). No GFAP-IR cells were observed in layer IV and V (Fig. 4.1f). However, different with respect to the previous age, the number of GFAP-IR cells was reduced and astrocytes in layer I (Fig. 4.1c) were less densely packed at the pial surface, and were widespread in the entire layer I. Astrocytes in layer II/III showed, more or less, the same pattern as layer I. Also in layer VI (Fig. 4.1 i) the number is reduced with respect to P24 and astrocytes are confined in a layer close to white matter. At high magnification (Fig. 4.2e) immunostained cells revealed a large perykaria whose shape was polygonal with the center devoid of staining (probably mirroring the package of GFAP inside the cell); processes were highly branched, long and tapered. GFAP-IR astrocytes in white matter (Fig.4.2h) were sparse resulting in a lower intensity of the immunostaining.

Cellular amount of GFAP in the developing Visual Cortex

To establish if the change of distribution of GFAP immunostained cells in the visual cortex during postnatal development corresponds to different levels of proteins, tissue from the primary visual area was extracted, homogenized and a western blot was performed. Samples were taken from the visual cortex, using mice of the same age and strain of those used for immunohistochemistry. Membranes were probed with a monoclonal antibody recognizing GFAP and a densitometric analysis was carried on the membranes. Density of GFAP positive bands (Fig. 4 lower panel), were normalized, for total protein content, with anti-tubulin (Fig. 4.3a, upper panel). The analysis showed that the protein expression was high at P12 ($P<0.05$) and decreased, significantly, at later postnatal ages (Fig. 4.3a, lower panel; b). Indeed, at P24 the mean relative level of GFAP was significantly lower than that found at P12. No significant difference was found between P24 and P60 (Fig. 4.3 a and b; $P>0.05$). Our findings indicate that GFAP cellular expression is developmentally regulated in the mouse visual cortex with a progressive restriction of immunostained cells to the most superficial and deep cortical layers. This progressive segregation of labeled cells corresponds to a reduction in the amount of GFAP.

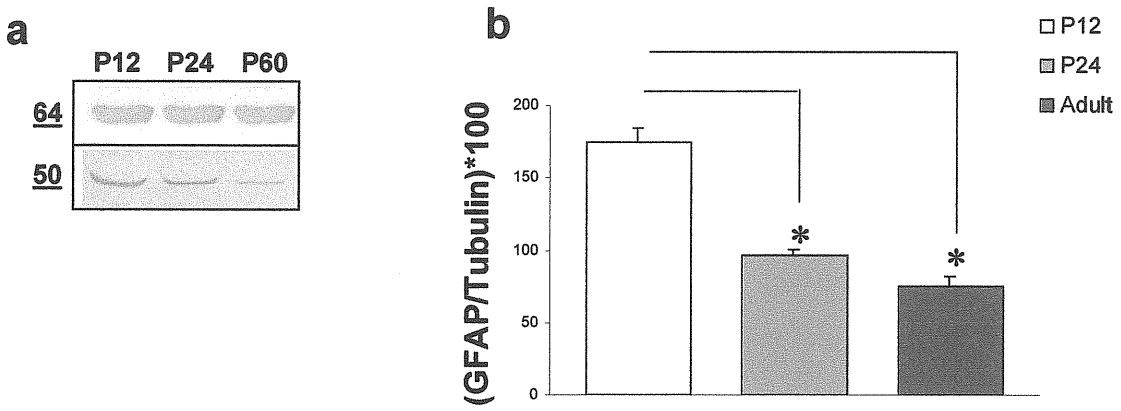


Fig. 4.3: Time course of GFAP expression in mouse visual cortex.
(a) Western blot of visual cortex taken at different stages of postnatal development, probed with tubulin (upper panel) or GFAP (lower panel) antibody. One representative western blot is reported for each age. (b) The plot reports the percentage ratio between the expression of GFAP and tubulin- optical density values (mean±SEM). The GFAP level was significantly different between P12 and P24 and between P12 and adult mice (ANOVA and post hoc Tukey's test, *$P<0.05$,$N=5$). No significant difference was found between P24 and adult mice.

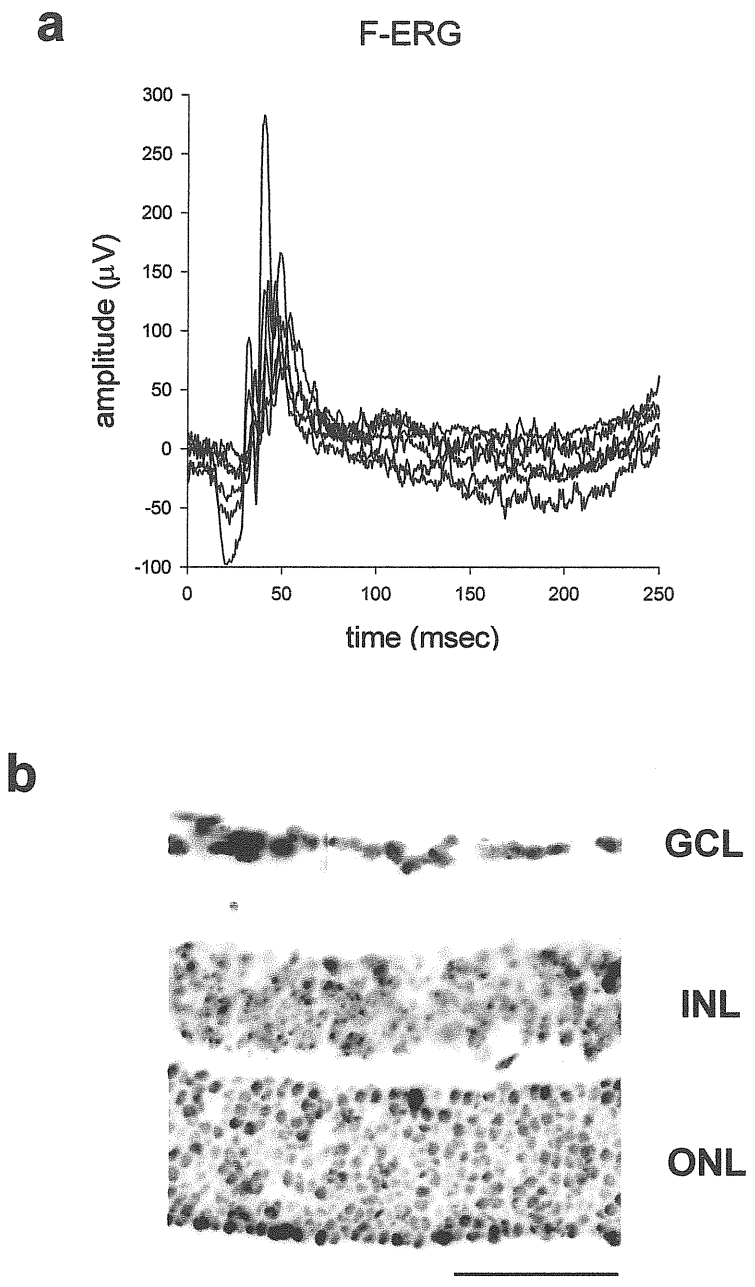


Fig. 4.4: F-ERG and histology of retina are not altered in C57BL mice. In a is shown the F-ERG obtained in dark adapted conditions to different intensities of light (0.001, 0.01, 0.1, 1, 10, 20 $cd^{*m-2.}$ Each trace corresponds to a different light intensity. In b is shown retinal image of typical DAPI stained retinal section. Outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer appear normal. Scale bar = 50 μm.

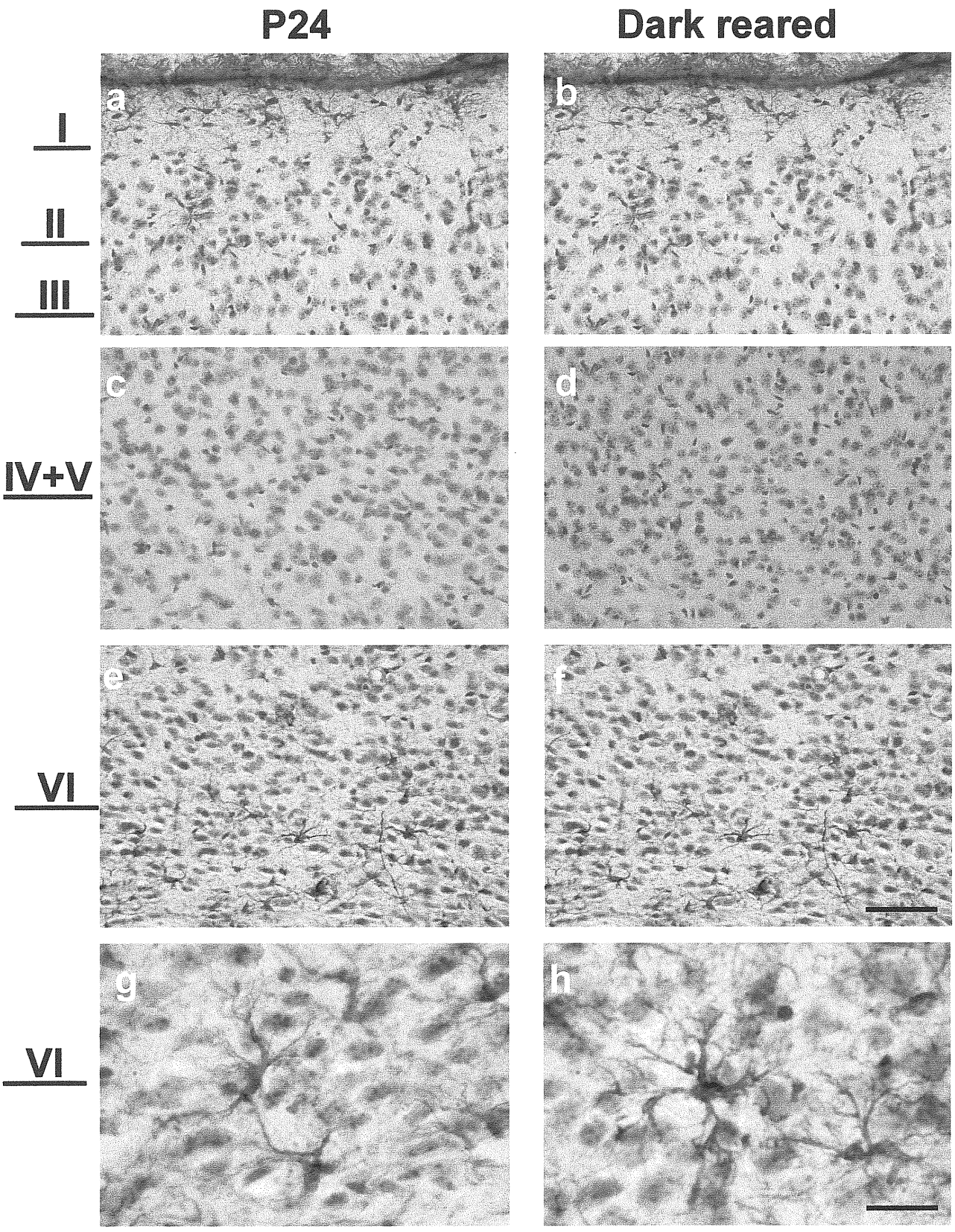
4.2 Effect of sensory deprivation on GFAP-positive distribution

We have shown that after eight days of light from the time of eye opening the GFAP expression in the visual cortex reaches an adult pattern of expression. To investigate the influence of light on GFAP expression, mice were kept in darkness for 12 days, from P12, i.e. before the eye opening until P24, when both the cellular expression and level of GFAP can be considered adult-like. Since recent data have shown that naturally occurring mutations should lead to retinal degeneration (rd) it was essential to eliminate this possibility in our colony of C57BL mice (Chang et al., 2002). In three mice randomly chosen we have recorded the electroretinogram evoked by flash (F-ERG) in dark and light adapted conditions. After recording the F-ERG the mice were sacrificed and the retinae dissected out for histology. The results showed that the F-ERG was normal both in dark (Fig. 4.4a) and adapted conditions (data not shown). Histology of retina excluded the presence of rd (Fig. 4.4b).

After 12 days of dark rearing, GFAP in visual cortex did not change with respect to normal-reared mice of the same age. GFAP cellular expression in P24 dark reared animals (Fig.4.5b, d and f), was similar to that described in P24 normally reared mice (Fig.4.5a, c and e) with cells confined at the pial surface, at layer II and layersV/VI. Astrocytes showed a bushy morphology without gross differences between control (Fig. 4.5g) and dark reared mice (Fig.4.5h). We did not observe labeled astrocytes reminiscent of radial glia. To obtain a quantitative evaluation of GFAP cellular expression, four to five serial sections for each animal, within the region of visual cortex, were processed for immunohistochemistry and counterstained with cresyl-violet. To sample cells in different cortical layers we chose a grid 300 μm large, spanning all cortical layers in height. We have used this method to evaluate the number of astrocytes expressing GFAP in different cortical layers. Cell counts of GFAP stained cells showed no statistically significant differences between dark reared and control mice in all cortical layers (Fig. 4.7a) ($P>0.05$). Finally, we measured the level of GFAP with western blot in dark reared and age matched control mice (Fig. 4.6b). From the histogram in Fig. 4.6c, it is clear that the mean relative value of GFAP was not statistically different between dark reared and control mice ($P>0.05$). In order to exclude the possibility that our dark rearing protocol was too short and too mild to produce visible changes in visual cortex, we measured the endogenous level

of a protein, which is known to be sensitive to visual deprivation. From previous studies conducted in rats, it is known that the subunit NR2A of NMDA-receptor is expressed in visual cortical neurons (Tongiorgi et al., 1999) and is down regulated in visual cortex of dark reared animals of about 50% (Quinlan EM et al, 1999). In contrast to what was shown for GFAP, we found that NR2A amount was less than 50% in dark reared mice compared to age-matched controls (Fig. 4.7b).

Thus, under our experimental conditions, light deprivation does not affect GFAP maturation.


P24
Dark reared
I
II
III
IV+V
VI
VI
a
b
c
d
e
f
g
h

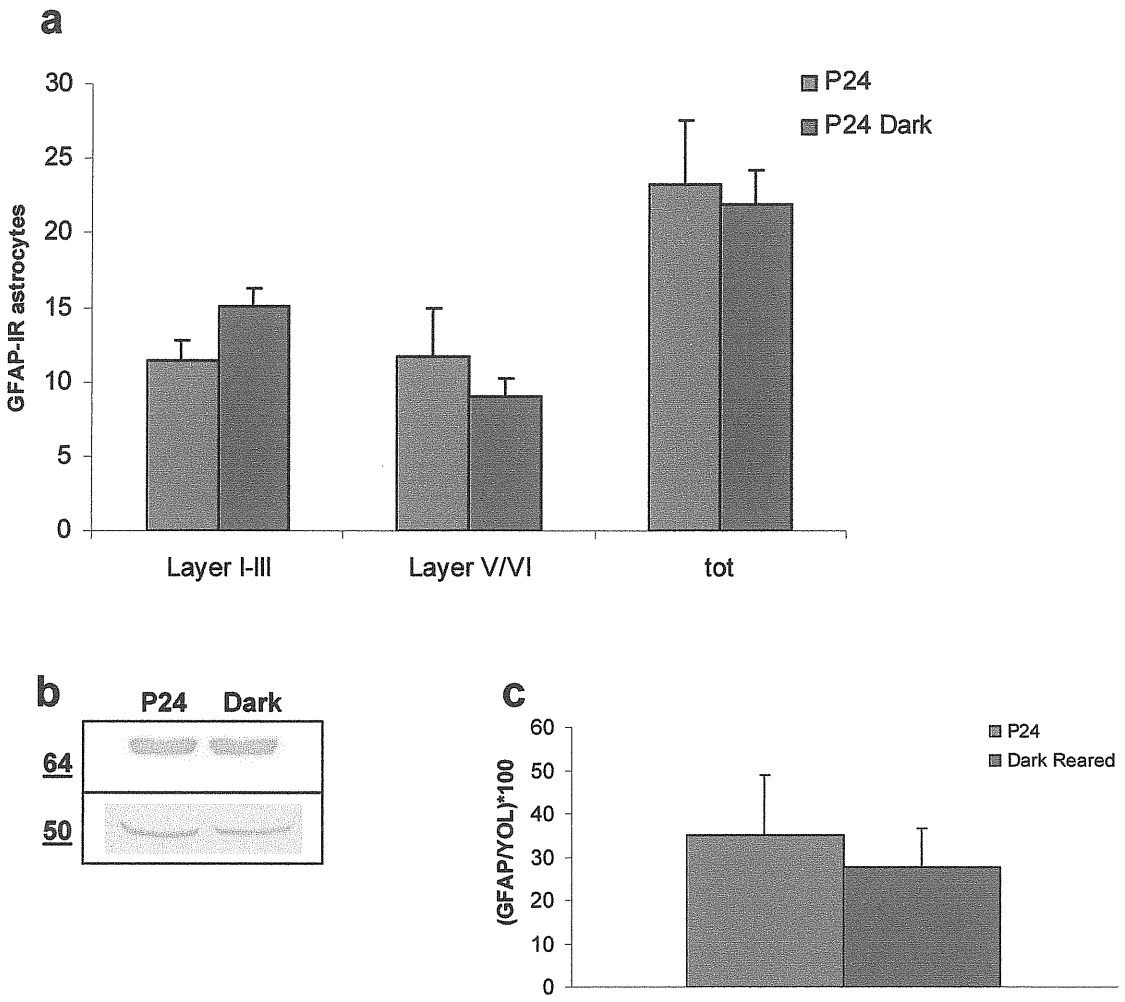


Fig. 4.6: GFAP expression in dark reared and control mice.
GFAP positive cells were counted in different layers of visual cortex. a. Cell counts of GFAP stained cells. The number of GFAP stained cells in different cortical layers and in the whole visual cortex (total) is plotted for dark reared (black columns) and control animals (white columns). No significant difference was found between the two groups of. (t test, P>0.05,N=5). **b. GFAP level in dark reared and control mice.** Immunoblots were probed with anti-tubulin (b, upper panel) and anti-GFAP (b, lower panel). Dark reared animals were compared with P24 normal reared mice. The plot (c) reports the percentage ratio between the expression of GFAP and Tubulin- optical density values, in the two groups. Data are reported as mean±SEM (t test, P>0.05,N=5).

Fig. 4.5: GFAP-IR astrocytes in dark reared and control mice.
GFAP positive cells in different layers of visual cortex of normal reared (a,c,e) and dark reared mice (b,d,f). Cell were counterstained with cresyl violet to identify different layers. It is possible to note that there are no difference between the two groups. The shape of astrocytes does not change, as shown at higher magnification (g,h).

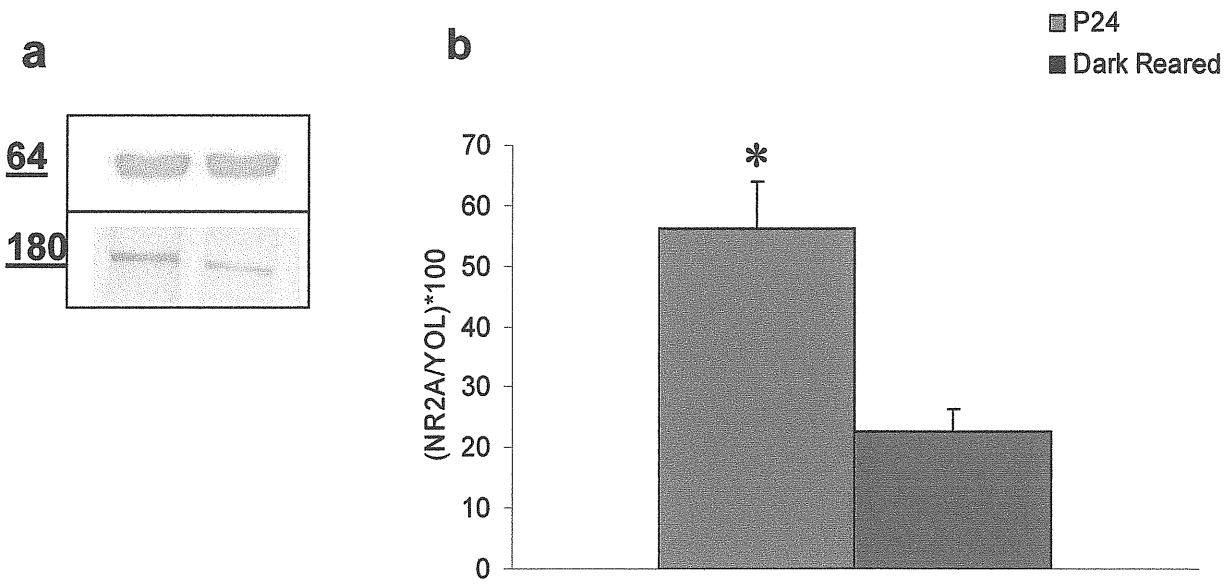


Fig. 4.7: Expression of the NMDAr subunit NR2A in dark reared and control mice.
a) A representative example of western blot. b) The plot reports the percentage ratio between the expression of GFAP and tubulin- optical density values (mean±SEM). The expression of NR2A in dark reared group was decreased with respect to the control one. (t test, $P<0.05$,$N=5$).

4.3 NGF effect on GFAP expression

Since we have characterized the postnatal development of GFAP we then investigated the factors controlling GFAP expression at an age when the maturation period was completely over. As mentioned before, in the introduction, GFAP expression can be modulated by several factors, included neurotrophins. The aim of the present study was to investigate the effect of NGF deprivation on GFAP immunoreactive astrocytes in adult mice. Since classical NGF-knock out mice die in the first weeks of postnatal life (Crowley *et al.*, 1994), we used transgenic mice expressing the neutralizing anti-NGF immunoglobulin (anti NGF transgenic mice) during postnatal life, which are also living in adulthood. The expressio of antibody was under the transcriptional control of the human cytomegalovirus (CMV) early region promoter [2601 to 216 (Boshart et al., 1985)]. Anti-NGF transgenic mice express low amounts of anti NGF antibodies in the first month of the postnatal age. The level of anti-NGF antibodies increases three fold during the second postnatal month (Ruberti et al., 2000). All the experiments reported in the present paper, were conducted on two month old NGF-transgenic mice and on age matched wild type mice (control mice). It has been shown that anti-NGF transgenic mice are charachterized by a cholinergic impairment (Ruberti et al., 2000). To check the effectivness of NGF blockade, basal forebrain neurons (BF), were investigated. Cholinergic neurons were counted in the medial septum and in the diagonal band (MS+DB) (Fig. 4.8, a and b), and the basal nucleus of Meynert (BNM) (Fig. 4.8, d and e). A strong reduction of BF cholinergic neurons was found in both MS+DB (Fig. 4.8c) and in BNM (Fig. 4.8f) of anti-NGF transgenic mice with respect to control group. In addition, the staining of remaining cholinergic cells was lower with respect to that found in wild type mice. Thus, the BF cholinergic system was profoundly impaired in anti NGF-transgenic mice.

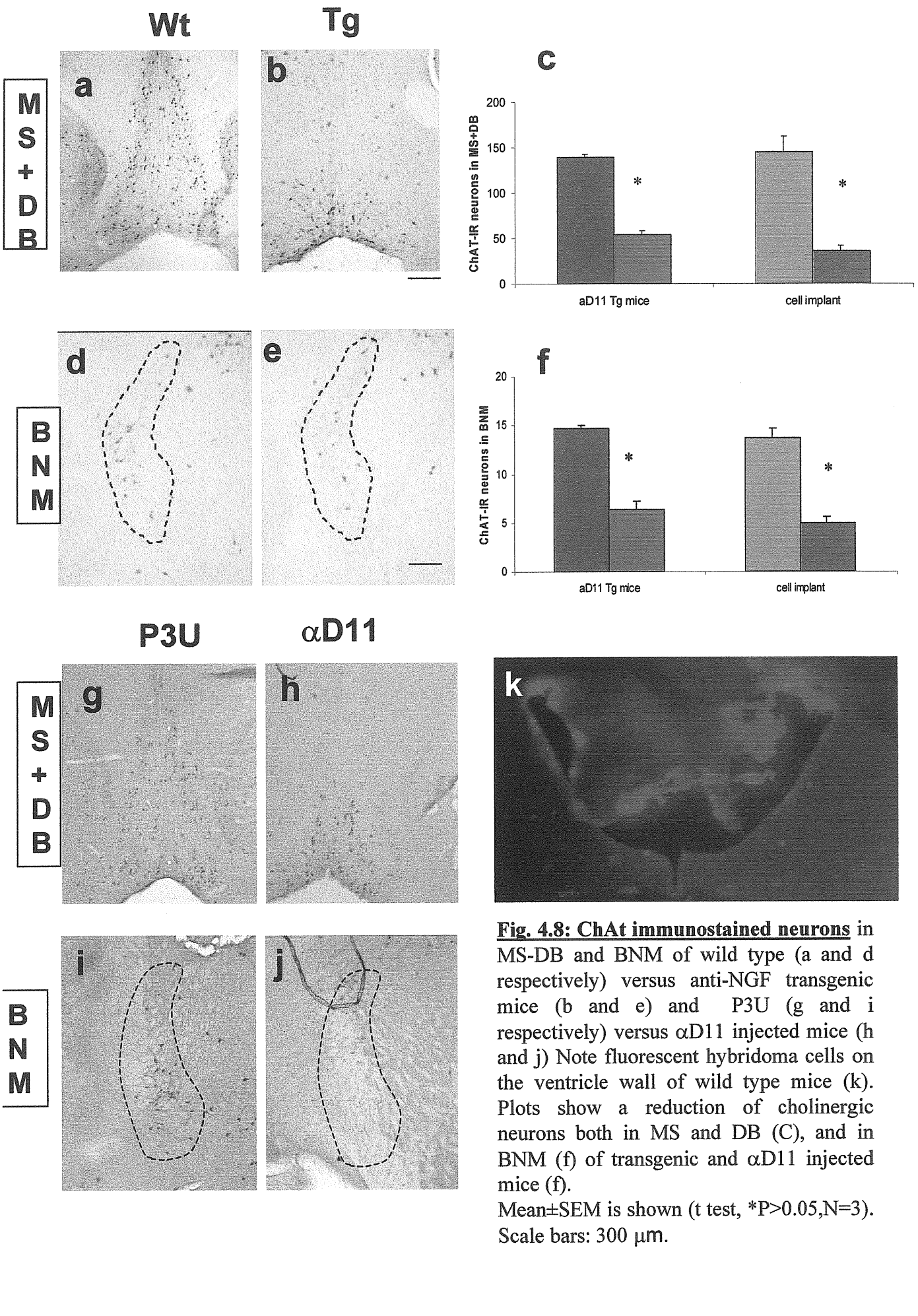


Fig. 4.8: ChAt immunostained neurons in MS-DB and BNM of wild type (a and d respectively) versus anti-NGF transgenic mice (b and e) and P3U (g and i respectively) versus αD11 injected mice (h and j) Note fluorescent hybridoma cells on the ventricle wall of wild type mice (k). Plots show a reduction of cholinergic neurons both in MS and DB (C), and in BNM (f) of transgenic and αD11 injected mice (f).
Mean±SEM is shown (t test, *P>0.05,N=3).
Scale bars: 300 μm.

GFAP-IR astrocytes in visual cortex of NGF-transgenic mouse

As shown before in the visual cortex of wild type mice, GFAP-IR astrocytes were preferentially localized in layers I-II and VI, with layer IV almost devoid of immunostained cells (Fig. 4.9a, c and e). In anti-NGF transgenic mice (Fig. 4.9b,d and f), GFAP immunoreactive cells were reduced in all cortical layers. At higher magnification (Fig.4.9g and h) it can be observed that the morphology of GFAP immunopositive cells is different between transgenic and control mice. Wild type astrocytes (Fig. 4.9g) showed the classic astrocytic phenotype, with a star shaped soma with branch cytoplasmic processes. The rare GFAP-IR astrocytes still present in the visual cortex of transgenic mice (Fig. 4.9h) were less stained with respect to the control mice and characterized by small soma and short and thin processes. To obtain a quantitative evaluation of GFAP labeled cells, astrocytes were counted using a grid of 300 μm large over-imposed on primary visual cortex. Four to five serial sections for each animal, within the region of visual cortex, were evaluated. Results (Fig.4.10b) clearly showed that GFAP-IR astrocytes were reduced by more than 50 % in the visual cortex of anti NGF-transgenic mice, with respect to the control group (t-Test $P<<0.05$).

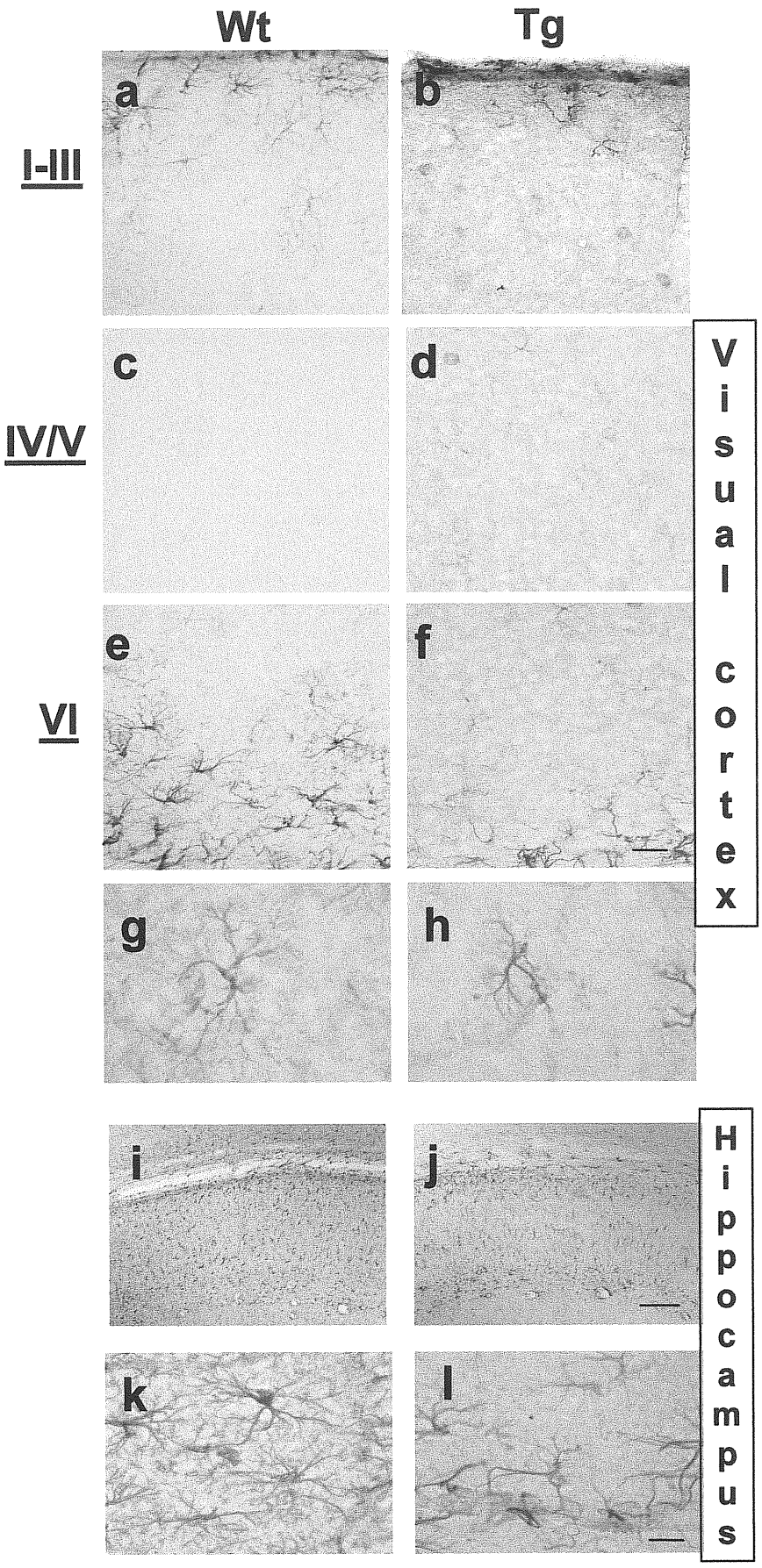
Cellular amount of GFAP in visual cortex of anti-NGF transgenic mice

The endogenous amount of GFAP was measured by western blot. Samples were taken from visual cortex of transgenic and wild type mice. Membranes were probed with a monoclonal antibody recognizing GFAP and a densitometric analysis was carried out on the membranes. Density of GFAP positive bands (Fig. 4.11a, upper panel) was normalized for total protein content, with anti-tubulin (Fig. 4.11a, lower panel) and values are given as relative units. The analysis of GFAP bands (Fig.4.11b) showed that the protein expression was significantly lower (about 30%) in NGF-transgenic mice compared to wild type mice (t-Test $P<0.05$). These results indicate that anti-NGF transgenic mice are characterized by the reduction of GFAP-IR cells, which corresponds to a lower level of the protein as assessed by western blot.

GFAP-IR cells in hippocampus

To check whether alteration of GFAP expression in anti-NGF transgenic mice was confined to visual cortex the hippocampus area CA-1 was analyzed. Clearly GFAP immunoreactivity in area CA-1 of transgenic mice (Fig. 4.9j) was lower with respect to control mice (Fig.4.9i). In addition, the morphology of immunopositive

astrocytes was altered in transgenic mice (Fig. 4.9l). Cell counts (Fig. 4.11d) revealed a reduction of about 30% in the anti-NGF transgenic mice hippocampus with respect to the wild type mice (t-Test, $P<<0.05$). These results suggest that NGF deprivation effects on the phenotype of astrocytes were not restricted to the visual cortex.


Wt
Tg
a
b
I-III
c
d
IV/V
e
f
VI
Visual cortex
g
h
i
j
k
l
Hippocampus

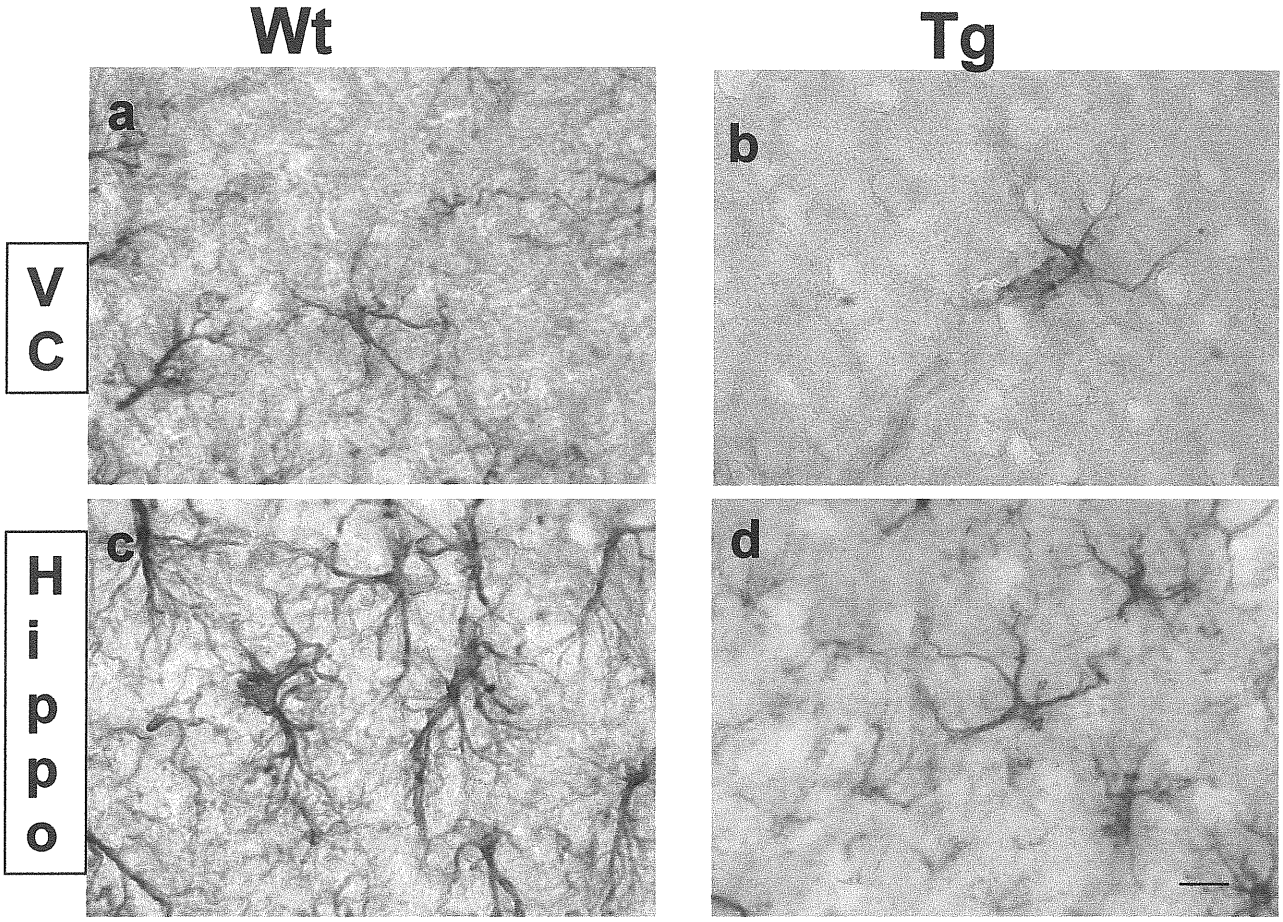


Fig. 4.10 Higher magnification shows the morphological alteration, due to NGF deprivation, in GFAP-IR astrocytes of (b) visual cortex and (d) hippocampus. Scale bar: 20 μm.

Fig. 4.9. GFAP immunoreactivity in (a-f) different layer of visual cortex and in (I and j) hippocampus of anti-NGF transgenic mice. In anti NGF-transgenic mice, it is possible to note the reduction of GFAP-IR astrocytes in (b, d and f) visual cortex and (j) hippocampus . Higher magnification shows the morphological alteration of GFAP-IR in (h) visual cortex and (l) hippocampus. Scale bar: a-f, i and j, 100 μm; g, h, k and l, 20 μm.

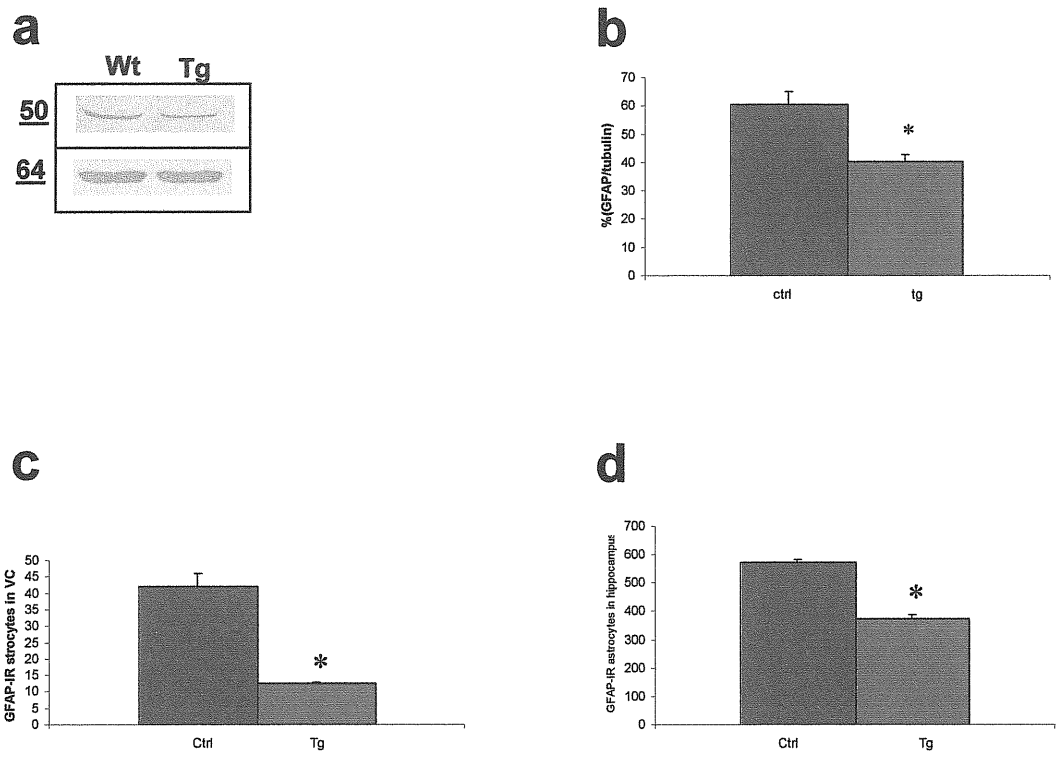


Fig.4.11: (a) Western blot of visual cortex in wild type and anti-NGF transgenic mice probed with or GFAP (upper panel) or Tubulin (lower panel) antibody, one representative sample for each group. (b) The plot reports the percentage ratio between the expression of GFAP and Tubulin-optical density values (mean±SEM), in wild type and transgenic animals. (c) GFAP-IR cells in visual cortex and (c) in hippocampus of NGF-transgenic mice versus the control group NGF deprivation provoked a reduction of GFAP-IR astrocytes. (T-test, *P<0.05,N=3).

Evaluation of S100-IR astrocytes

Cellular distribution of another astrocytic marker, the protein S-100 was investigated in both wild type and transgenic mice. In wild type mice, S-100 was widely distributed throughout all the visual cortical layers (Fig. 4.12a). In anti-NGF transgenic mice (Fig. 4.12b), the distribution of S-100-IR astrocytes was similar to that observed in wild type mice. The same situation was observed when the hippocampus of control mice (Fig. 4.12c) was compared to that of NGF-transgenic mice (Fig. 4.12d). Astrocytes were counted, as previously described. No differences were found between control and transgenic mice, neither in visual cortex (Fig. 4.12e), nor in the hippocampus (Fig.4.12f) (t-Test, $P>0.05$). Thus, NGF deprivation induces a down regulation of GFAP expression but not a reduction of the astrocytic number.

Implant of hybridoma cells releasing αD11 antibody

To check whether alteration of GFAP immunopositive astrocytes was because of NGF deprivation and not because of insertion of a transgene, endogenous NGF was blocked by intraventricular implants of hybridoma cells producing the neutralizing NGF antibody αD11. Hybridoma cells injected into the lateral ventricle can adhere to the wall of the ventricle itself (note the Fig. 4.8k, in which hybridoma cells were stained with the vital dye DiI) and release the antibody both into the brain parenchyma and in the CSF (Domenici et al., 1994; Berardi et al., 1994; Molnar et al., 1998). In previous papers, it was shown that hybridoma cells release αD11 in the ventricle for at least one month and that the level of antibodies in hippocampus and visual cortex remained high and stable for three to four weeks after the implant (Berardi et al., 1994; Molnar et al., 1998). Sections containing basal forebrain and sections containing visual cortex were processed for detection of ChAT positive neurons and GFAP-IR astrocytes, respectively. The effectiveness of the implant was checked by the analysis of ChAT positive neurons both in MS+DB and in BNM. Two weeks after implantation, reduction of cholinergic neurons was evident both in MS+DB (Fig.4.8h) and in BNM (Fig.4.8j). ChAT-IR neurons were significantly reduced (t-Test, $P<<0.05$) both in MS+DB (Fig. 4.8c) and in basal nucleus of Meynert (Fig. 4.8f) with the reduction in both areas comparable to that obtained in the transgenic mouse. In control mice implanted with P3U cells, cholinergic neurons were not affected thus excluding a pathological effect due to the implant itself. GFAP-IR

astrocytes were counted in visual cortex and hippocampus of hybridoma and P3U implanted mice.

GFAP distribution in hybridoma cells implanted mice

Analysis of GFAP-IR cells in both visual cortex (Fig. 4.13a and b) and hippocampus (Fig. 4.12c and d), showed that they are reduced in αD11 hybridoma injected mice (Fig. 4.13a and c) with respect to control mice (Fig. 4.13b and d). The shape of astrocytes in αD11 injected mice was also altered both in visual cortex (Fig. 4.13b) and hippocampus (Fig. 4.13d). Plots show that reduction induced by hybridoma implant was statistically significant respect to control group both in visual cortex (Fig. 4.13e) and hippocampus (Fig. 4.13f) (*$P<0.05$). Furthermore, the difference between hybridoma cell- and transgenic mice was not statistically significant (t-Test, $P>0.05$, Fig. 4.18c and d). We conclude that GFAP down regulation was induced by NGF deprivation.

Expression of TrkA mRNA in mouse visual cortex

The expression of TrkA mRNA is very low in rat visual cortex and has been detected using PCR (Cellerino et al., 1996). TrkA mRNA was checked in the mouse visual cortex by PCR. Little TrkA was detected in wild type mouse visual cortex. In Fig. 4.14, side a of the panel reports the bands corresponding to β-actin fragment, a house keeping gene used as an internal control, and side b shows the RT-PCR product relative to TrkA fragment, both in the visual cortex and basal forebrain. The intensity of the TrkA mRNA-band is very weak in visual cortex while it is high in basal forebrain, suggesting that TrkA produced, locally, in the mouse visual cortex is low.

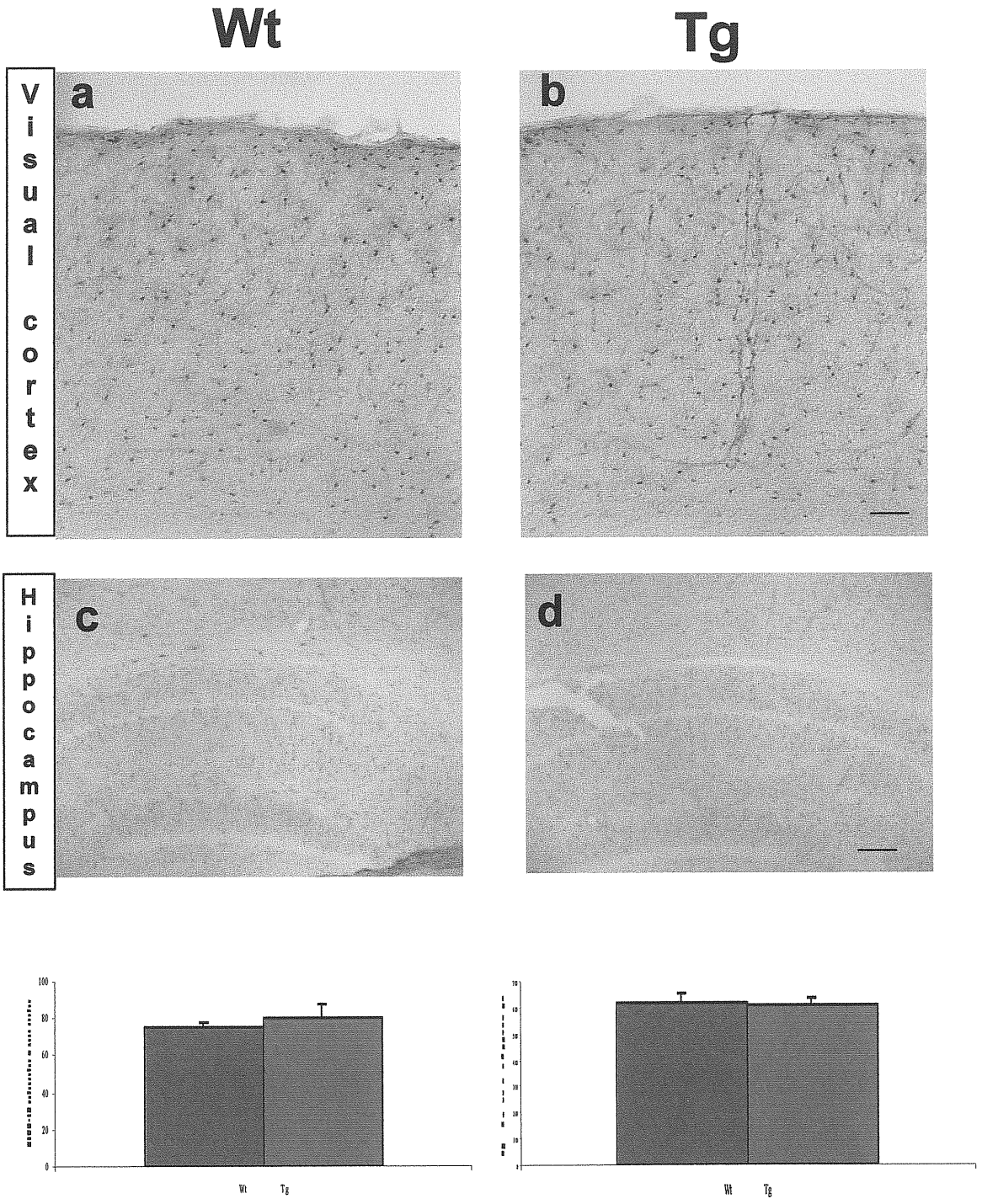


Fig. 4.12. S-100 immunostaining in (b) visual cortex and (d) hippocampus of anti-NGF transgenic mice. As shown, no difference was found both in (e) visual cortex and in (f) hippocampus. Scale bar: 100 μm.

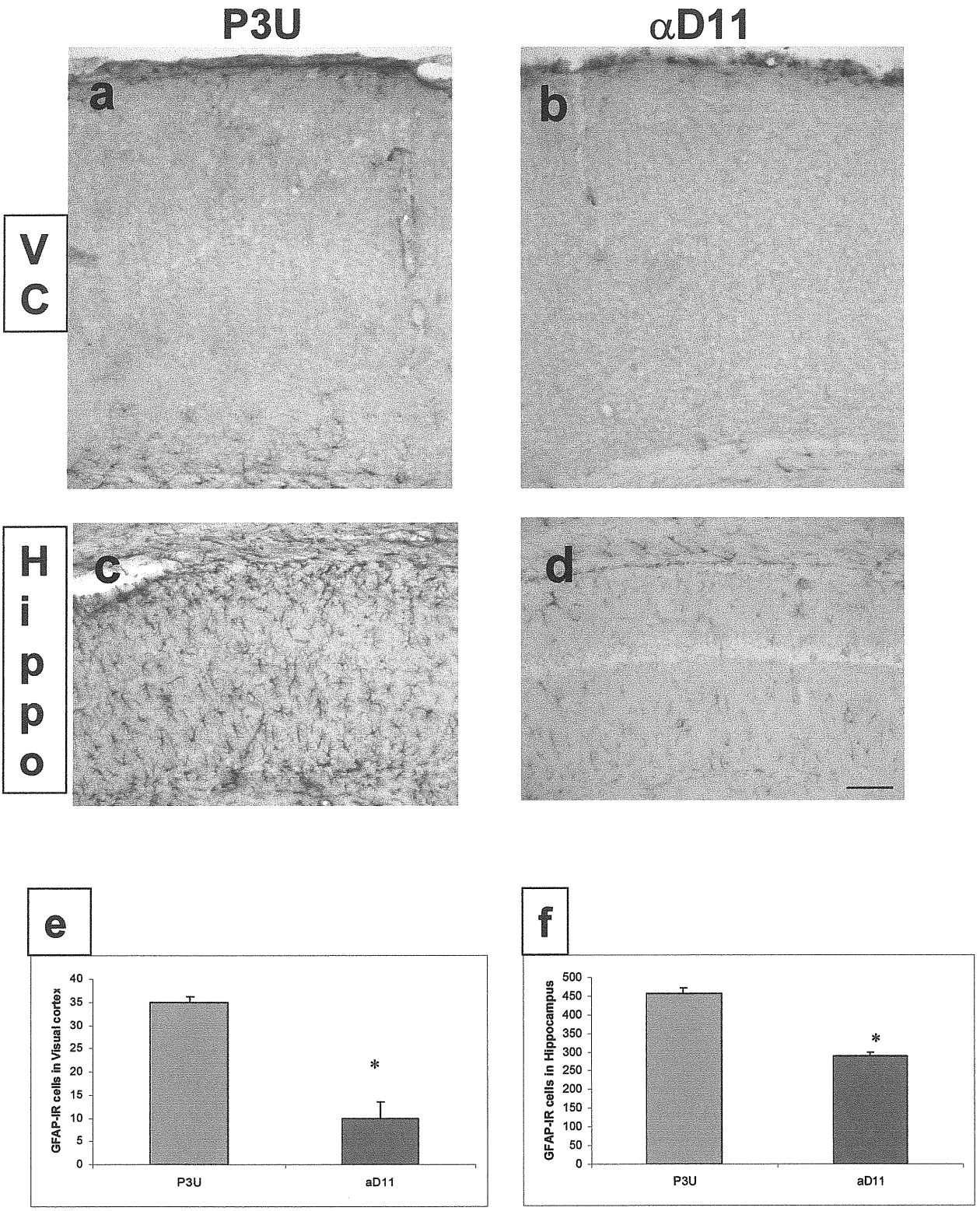


Fig.4.13: GFAP-IR astrocytes are reduced in αD11 injected mice. (a-b) GFAP-IR cells in visual cortex and (c-d) in hippocampus of NGF-deprived mice versus mieloma injected mice. (T-test, *P<0.05,N=5).

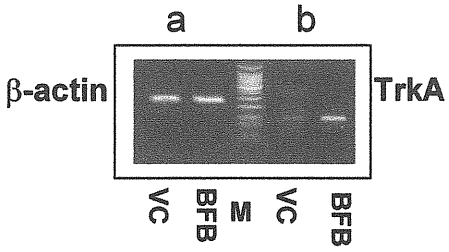


Fig. 4.14. RT-PCR on mouse visual cortex. β-actin, side a and TrkA, side b. RNA from BFB was used as positive control. It is possible to note the light intensity of the band relative to TrkA in visual cortex. M=molecular weight marker.

NGF Indirect modulation of GFAP

By using RT-PCR only a small amount of TrkA was found and, *in situ*, I did not detect neither TrkA mRNA nor the protein (data not shown). These results prompted me to test the hypothesis that GFAP down regulation was secondary to other systems and/or factors sensitive to NGF deprivation. Since the cholinergic system was highly impaired in NGF deprived mice and astrocytes express muscarinic receptors (Van der Zee et al., 1989; 1993). Therefore NGF could affect astrocytic phenotype in an indirect way through the cholinergic system. To test this hypothesis it was essential to know whether a reduction of BF cholinergic neurons, in absence of NGF deprivation could mimic the results on GFAP expression obtained in transgenic mice. To test this, it was therefore important to use an animal model deprived of cholinergic input.

To block Ach release we used Vesamicol. Vesamicol is known to inhibit transport of ACh into synaptic vesicles both *in vitro* and *in vivo* (Kobayashi et al., 1997). ACh is stored in cytoplasmic and synaptic vesicles (Cooper et al., 1991). Release of ACh in the synaptic cleft involves release of ACh from the vesicular fraction rather than from the cytosolic pool (Carroll, 1983, 1984). A specific low-affinity acetylcholine transporter is responsible for uptake of the transmitters from cytoplasm into vesicles (Prior et al., 1992; Shuldiner et al., 1995). This transporter is selectively blocked by 2-(4-phenylpiperidino)-cyclohexanol (Fig. 4.15), also known as vesamicol (Marshall and Parson, 1987). This compound inhibits the transport of ACh into purified synaptic vesicles from the electric organ of the Torpedo (Rogers and Parsons, 1993; Rogers et al., 1989) and it prevents cholinergic transmission in several *in vitro* models (Searl et al., 1990;Collier et al., 1986; Jope and Johnson, 1986). Furthermore it was found to inhibit the release of ACh *in vivo* (Boccafusco et al., 1991; Marien et al., 1991) and to cross the BBB if administered peripherally (Kobayashi et al., 1997).

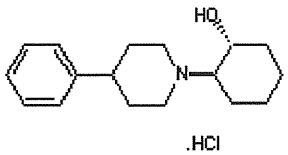


Fig. 4.15 (±)-Vesamicol hydrochloride- Molecular structure

Vesamicol was injected subcutaneously, as previously described in the material and methods section, in adult wild type mouse, and the effect on GFAP immunopostive astrocytes was evaluated five hours later. Similar to what was shown for anti-NGF transgenic mice and for hybridoma-implanted mice, this treatment caused a reduction of GFAP immunoreactivity in both visual cortex (Fig. 4.16b) and hippocampus (Fig. 4.16d). Also in this case morphology of astrocytes was altered in both the areas (Fig. 4.17b-visual cortex-and e- hippocampus). Plots show a reduction of GFAP immunoreactivity in both the areas investigated (Fig. 4.16g-visual cortex-and h-hippocampus). Western blot analysis was performed to evaluate the amount of GFAP after the treatment. We found that the amount of GFAP was not different between saline and control (Fig. 4.18c). Thus, in contrast with results obtained in NGF deprived mice, vesamicol treated mice were characterized by a reduction of GFAP immunoreactivity without concomitant reduction of GFAP total amount (summary plot, Fig. 4.19b,c and d) ($P>0.05$).

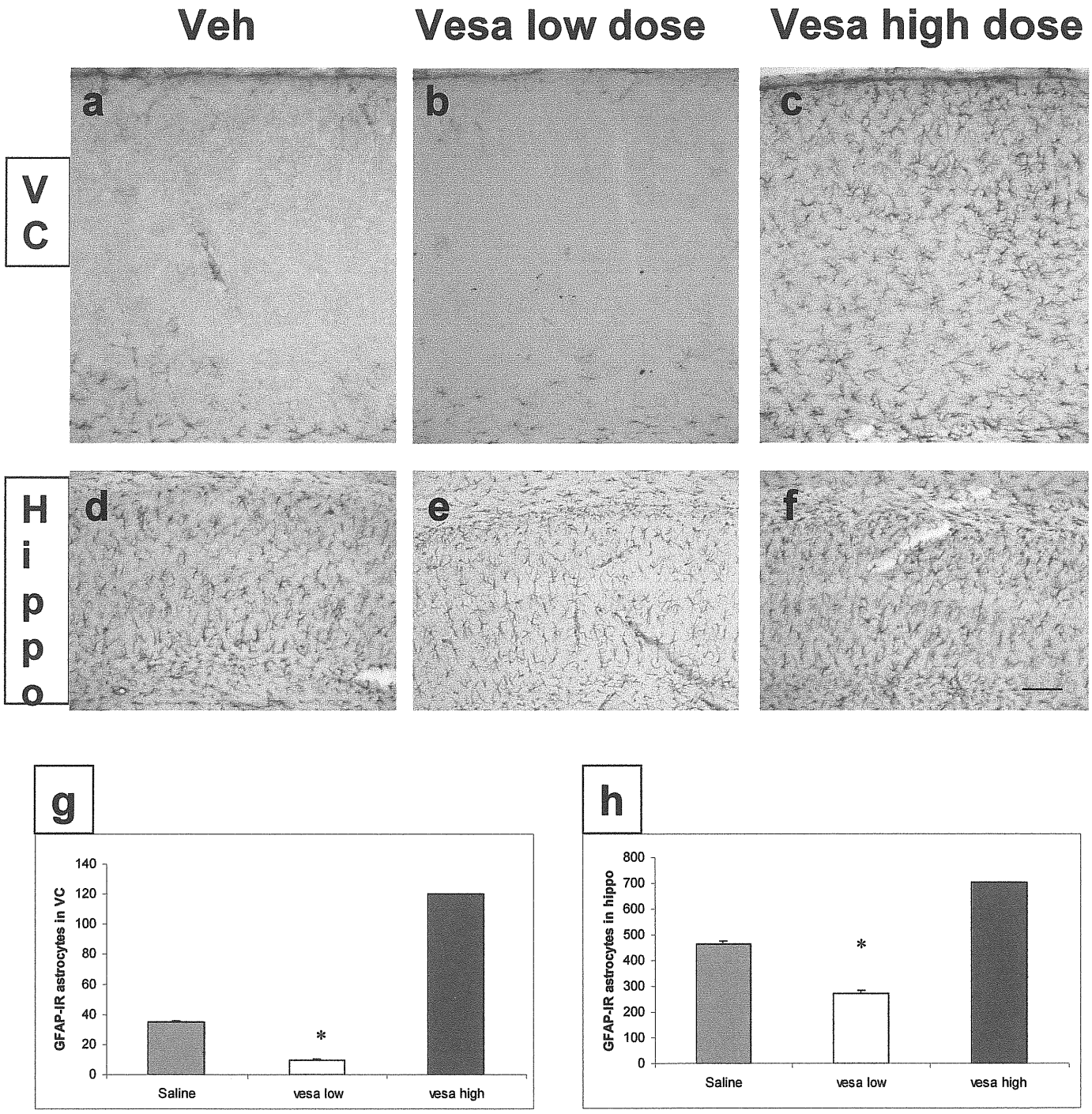


Fig. 4.16. GFAP immunoreactivity in vesamicol injected mice is dose dependent.GFAP-IR astrocytes are reduced after a mild Ach reduction in (b) visual cortex and in (e) hippocampus whilst are increased after a strong Ach reduction in both (c) visual cortex and in (f) hippocampus.The plot reports GFAP-IR astrocytes in (g) visual cortex and (h) hippocampus in vehicle injected, vesamicol low dose and vesamicol high dose. (*P<0.05, t-Test, N=5). Scale bar: 100 μm.

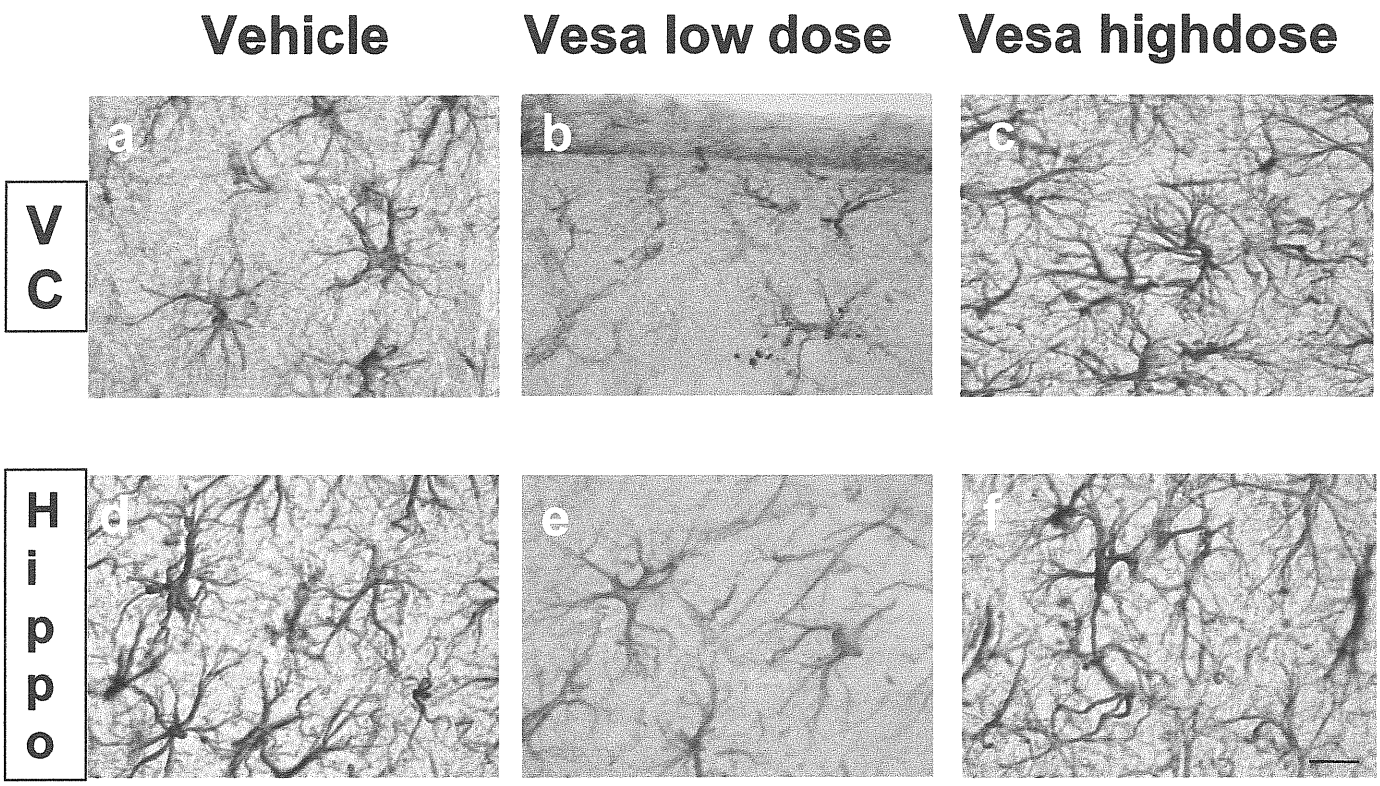


Fig. 4.17 Ach reduction provoked alteration in the shape of GFAP-IR astrocytes when comparable to vehicle injected animals. Higher magnification shows the morphological alteration, comparable to that induced by NGF deprivation. Mild Ach reduction, induced an alteration of astrocytes comparable to that provoked by NGF deprivation in (b) visual cortex and (d) hippocampus. After strong Ach reduction (c-f) GFAP-IR are also altered but display a gliotic phenotype in both the areas investigated. Scale bar: 20 μm.

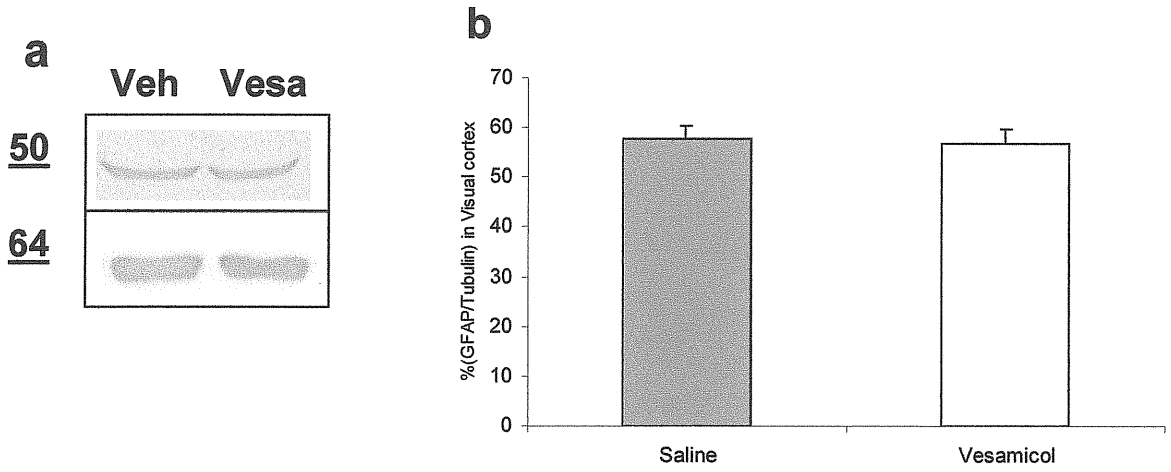
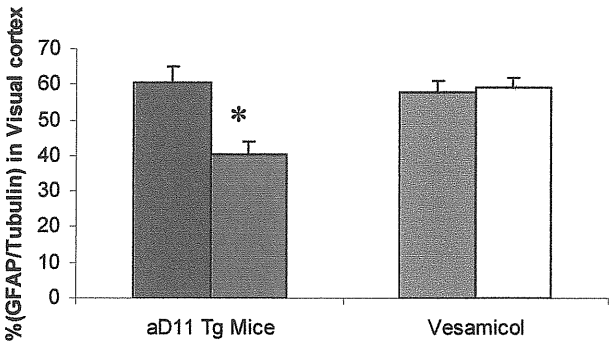


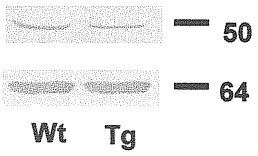
Fig. 4.18: Low dose of vesamicol did not reduce the amount of GFAP.
a) A representative example of western blot. b) The plot reports the percentage ratio between the expression of GFAP and tubulin- optical density values (mean±SEM). The expression of GFAP after injection of low dose of vesamicol was not reduced with respect to the vehicle injected group. (t test, P>0.05,N=5).

Fig.4.19 : Summary plots of GFAP expression and distribution after NGF or Ach deprivation.
(a) Western blot of visual cortex in anti-NGF transgenic mice probed with or GFAP (upper panel) or Tubulin (lower panel) antibody, one representative sample for each group. NGF deprivation provoked GFAP downregulation whilst no difference was found after treatment with low dose of vesamicol (b) The plot reports the percentage ratio between the expression of GFAP and Tubulin-optical density values (mean±SEM), in transgenic versus wild type and in vesamicol-treated animals versus the control group.. (c) GFAP-IR cells in visual cortex and (d) in hippocampus of NGF-transgenic mice, hybridoma implanted and vesamicol treated animals, versus the respective control group. All the treatment induced a similar reduction of GFAP-IR astrocytes (T-test, *P<0.05,N=3 for transgenic and relative control, and 5 for vesamicol- treated and hybridoma implanted, and relative control groups).

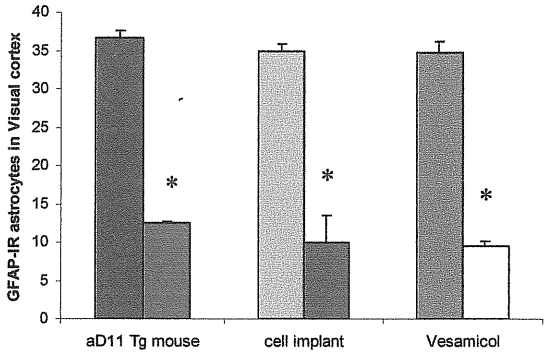
a

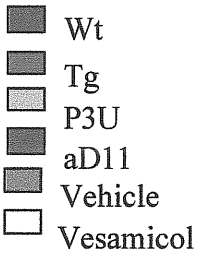

%(GFAP/Tubulin) in Visual cortex
70
60
50
40
30
20
10
0
*
aD11 Tg Mice
Vesamicol

b

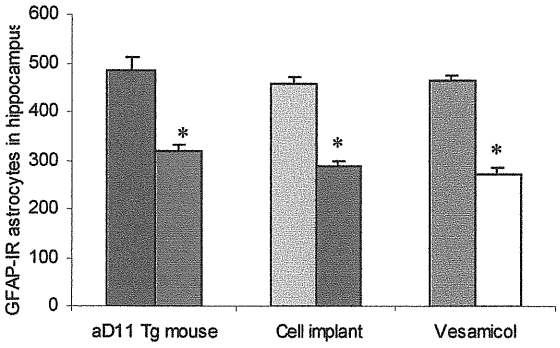

50
64
Wt
Tg

c


GFAP-IR astrocytes in Visual cortex
40
35
30
25
20
15
10
5
0
*
*
*
aD11 Tg mouse
cell implant
Vesamicol


Wt
Tg
P3U
aD11
Vehicle
Vesamicol

d


GFAP-IR astrocytes in hippocampus
600
500
400
300
200
100
0
*
*
*
aD11 Tg mouse
Cell implant
Vesamicol

GFAP expression is modulated by acetylcholine in a dose dependent manner

Cholinergic impairment has been classically described to induce GFAP upregulation and gliosis instead of GFAP down regulation. Pharmacological lesion of cholinergic nuclei caused gliosis in cholinergic target areas (Iacson et al., 1987; Anezaki et al., 1992; Monzon-Mayor et al., 2000). However, cholinergic lesions were induced in a very invasive way and with a pharmacological approach which, rather than being selective for cholinergic neurons, caused more generalized neuronal death. Vesamicol allowed us to selectively reduce acetylcholine release, without affecting the release of other molecules. It could be possible that acetylcholine reduction *per se* does not induce any gliotic response. However, we cannot exclude the alternative possibility that the dose of vesamicol we used was too mild to induce GFAP upregulation and gliosis. Following this hypothesis mild reduction of ACh release leads to GFAP down regulation while strong reduction of ACh induces GFAP upregulation. Thus, reaction of astrocytes to acetylcholine reduction could be dose-dependent. To test this hypothesis, a high dose of vesamicol was used to induce a strong reduction of ACh release. The high dose of vesamicol caused an increase in GFAP immunoreactivity in both visual cortex and hippocampus. Saline injected animals (control) show the classic distribution observed in normal conditions both in visual cortex (Fig. 4.16a) and hippocampus (Fig. 4.16d). Vesamicol treatment induced a strong immunoreactivity in all cortical layers of visual cortex (Fig. 4.16c) and an alteration of the morphology, with astrocytes showing hypertrophic soma and thick protoplasmic processes (Fig. 4.17c). Furthermore, strong GFAP immunoreactivity was also observed in cortical layers such as layer III and IV where GFAP-IR astrocytes are normally absent. Intense staining (Fig. 4.16f) and altered cell morphology (Fig. 4.17f) was also observed in hippocampus. Cell counts revealed a huge increase of astrocytic number in visual cortex (almost 80% more astrocytes were found after the Vesamicol treatment). Thus, reaction of astrocytes to ACh deprivation is directly related to the degree of reduction of cholinergic input.

DISCUSSION

DISCUSSION

Evidences collected from the last decade have let investigators to think the central nervous system as a network composed by different cell types cooperating with each other to create a unitary response to physiological and pathological changes of extracellular environment. On this view, astrocytes actively participate with neurons in the process of neuronal information. The ratio of glia to neurons is low in inferior species and increases during phylogenesis. In *Caenorhabditis elegans* there are 302 neurons and only 56 glial and associated support cells (Sulston et al., 1983). The human brain contains the greatest numbers of glia and the highest ratio of glia to neurons (at least 10:1). This may suggest that glial cells, and particularly astrocytes, play a role in brain processes normally associated with higher species, such as cognitive functions. There is little information about functional interactions between neurons and astrocytes in cognitive processes. Recent studies have shown that astrocytic stimulation leads to a potentiation of the inhibitory synaptic transmission in hippocampal slices (Kang et al., 1998) and, *viceversa,* neuronal activity can induce an activation of an astrocytic response, as measured by the increase of calcium waves (Dani and Smith, 1995; Porter and McCarthy 1996). GFAP-deficient mice exhibit an enhancement of LTP (McCall et al., 1996), and impaired LTD (Shibuki et al., 1996) suggesting that astrocytes are required for the normal expression of synaptic plasticity. It must be mentioned that synaptic plasticity represents the functional substrate of cognitive functions such as learning and memory. One of the areas in which neuronal plasticity has been widely investigated is the primary visual cortex. This area is sensitive to manipulation of visual input, not only during restricted temporal windows of postnatal development (critical period) but also in adulthood. During the critical period regressive processes such as neuronal death are already over (for review see Zilles, 1985) and LGN fibers have already reached the visual cortex, making synapses with the specific targets (Lund and Mustari, 1977); at this time the visual cortex is highly plastic and sensitive to visual manipulations such as monocular deprivation. For example, monocular deprivation (MD) of the type introduced by Wiesel and Hubel (1965) produces irreversible alterations in the visual cortex, with minor differences between rat and mouse. Visual cortical neurons cease responding to the deprived eye and, as a consequence, the number of binocular units is reduced in

rodent visual cortex (Domenici et al., 1991; Maffei *et al.*, 1992; Fagiolini *et al.*, 1994; Hensch *et al.*, 1998; Hanover *et al.*, 1999). Geniculate neurons receiving input from the deprived eye and sending projections to primary visual cortex shrink (Domenici *et al.*, 1993). However, neuronal plasticity is not limited to the critical period. Indeed, there are other forms of neuronal plasticity associated with visual learning and changes of the topographic sensory map induced by a peripheral lesion (for example in the retina, Gilbert and Wiesel, 1992; Obata *et al.*, 1999). Deprivation of visual input (dark rearing) results in two types of effects: 1) it increases the length of the critical period for monocular deprivation and 2) it delays the maturation of the functional properties of visual cortical neurons. All these results indicate that mammalian visual cortex has well-defined periods for cortical circuit refinement and represents, therefore, a good model for studying activity-dependent neuronal plasticity and maturation. However, a holistic view of CNS prompted us to extend these studies and look at the interaction between neurons and astrocytes both during postnatal development and in adulthood.

First of all we clarified the time course of astrocyte maturation in mouse visual cortex. In particular, we were interested in the time employed by astrocytes to acquire an adult phenotype. To this aim we followed the maturation of GFAP expression during postnatal development. We performed this study in the mouse since this animal species assumed a strategic importance for the availability of transgenic mice.

5.1 Postnatal development of GFAP in mouse visual cortex

As a marker of glia maturation I used GFAP since previous studies have shown that this cytoskeletal protein is regulated during postnatal development (Stichel et al., 1990; Muller, 1992) and that it is sensitive to functional changes of the environment (Jones et al., 1996) and injuries of the CNS (for a review see Eng et al., 2000). Other protein of astrocytes intermediate filaments, such as nestin and vimentin, are also useful markers to study the development of the cortex but their expression, in physiological conditions, is limited to early stage of postnatal development, before eye opening and, therefore, they were excluded (Kalman and Ajtai, 2001). Three different postnatal ages have been studied: P12, i.e. before eye opening, P24, when visual cortex is highly sensitive to MD (Gordon and Stryker, 1996) and P60 when the critical period and the maturation of the visual cortex are by and large over. I have found that GFAP is regulated during postnatal development: at P12 its endogenous

level is high and GFAP labeled astrocytes are distributed throughout all cortical layers. At this postnatal age, GFAP positive radial glia was easily identified whilst at later postnatal ages this type of glia was no longer present. The fact that radial glia disappeared from visual cortex of P24 mice is in agreement with previous results obtained in kittens and rat pups (Stichel et al., 1990) and supports the possibility that also in the mouse visual cortex radial glia plays a role during an early postnatal stage, before eye opening. In fact, different studies demonstrated that, in the developing cerebral cortex, radial glial cells guide radially migrating neurons and are the precursor of astrocytes (Hatten, 1990; Mission et al., 1991). More recently, a few studies (Malatesta, 2000; Noctor, 2001) highlighted the importance of radial glia also in neurogenesis suggesting that radial glia includes multipotent cells able to differentiate in two lines of progenitors cells dividing in neurons or in astrocytes.

Concerning the distribution pattern before eye opening, GFAP immunopositive cells were distributed throughout all the cortical layers and in white matter. At P24, the maturation of GFAP immunopositive astrocytes can be considered over; indeed, both the intensity of staining and the cellular distribution did not change between P24 to P60. Reduction of GFAP-IR cells is accompanied by a down regulation of GFAP content, as measured by western blot. In particular, GFAP level is high at P12 and is reduced after eye opening (P24) and remains stable thereafter. Thus, reduction of GFAP cellular expression in different cortical layers corresponds to a reduced level of GFAP and occurs early in postnatal development. Previous studies on GFAP maturation have been performed in the cat and in the rat. In kittens, GFAP immunoreactivity increases during postnatal development and the period of GFAP maturation is much longer with respect to the mouse (Muller et al., 1992). In the rat, GFAP decreases during postnatal development, as well as we have shown in the mouse, but the period of GFAP maturation is longer with respect to the mouse and cannot be considered over before P50 (Stichel et al., 1991). Interestingly, the period of maturation of GFAP largely overlaps with the critical period for monocular deprivation both in the cat and in the rat. Experiments designed to describe the mouse critical period for MD established that MD effects start to be detected at P19 with P29 corresponding to the peak of the critical period (Gordon and Stryker, 1996). The fact that GFAP maturation, in visual cortex, can be considered over by P24 when the visual cortex is highly sensitive to MD indicates that the time windows for both critical period and GFAP maturation do not overlap. Thus, it appears that there are

species differences in GFAP maturation and also in relation to visual cortical plasticity.

5.2 Influence of visual activity on GFAP development

Changes of GFAP expression occurring soon after eye opening raises the interesting question of whether neuronal activity, triggered by light, may represent a key factor in regulation of maturation of astrocytes. It is known that visual experience constitutes the driving force for the maturation of neurons and refinement of neuronal connections in the visual cortex (for a review see Berardi et al., 2000). Sensory dependent modifications of neurons can be considered a component of visual cortical plasticity. Indeed, deprivation of visual input slows down the functional and structural maturation of visual cortical neurons and increases the length of the critical period for MD (Fagiolini et al., 1994). Concerning astrocytes, it has been reported that environmental stimuli are able to interfere with GFAP expression. Indeed, surface density of astrocytes within layer II/III increases in the rat cortex, after a short period of complex housing (Jones et al, 1996). In addition, it has been shown that prolonged periods of light deprivation from birth provoke a reduction of GFAP immunostaining in kittens (Muller, 1990) and GFAP level in rats (Stewart et al, 1986). These results helped to raise the idea that the maturation of GFAP is retarded in dark reared animals. To establish if GFAP development in the mouse visual cortex is modulated by light, an experiment of light deprivation was conducted. Animals were kept in complete darkness from P12, before eye opening, until P24 when the period of GFAP maturation is over, as established in the study on GFAP development. To eliminate the possibility that retinal degeneration could affect the transmission of visual input from eyes (Chang et al., 2002), flash electroretinogram and retinal histology were performed in a few mice of our colony. Functional and histological results excluded the presence of retinal degenerations.

Results obtained in dark reared mice showed that the maturation of GFAP is not affected by dark rearing. To discard the possibility that the dark rearing protocol was too short or mild to influence cell phenotype a control experiment was carried out using a neuronal marker known to be sensitive to visual deprivation and involved in cortical plasticity, namely the subunit of NMDAr called NR2A. NR2A is expressed in visual cortical neurons (Tongiorgi et al., 1999) and its maturation is altered by visual deprivation in cats (Chen et al., 2000) and rats (Nase et al., 1999; Quinlan et al.,

1999). I found that NR2A was reduced in the visual cortex of dark reared mice suggesting that the adopted dark rearing protocol was strong enough to produce changes of cell phenotype. However, to be conclusive on this issue we must exclude that GFAP might be regulated with a very slow kinetic so that GFAP changes are not detectable using short dark rearing protocols. It is know that thee are two components of GFAP turnover: a fast-decaying pool (turnover comprised in a range hours) and a more stable pool (turnover comprised in a range of several weeks) (DeArmond et al., 1983; 1986;Goldman and Chiu 1984). In the present work, mice were kept in darkness for 2 weeks, between P10 to P24, in a period in which, in normal conditions of light, GFAP expression is known to decrease (Corvetti et al., 2003 and as shown above). Indeed, after light deprivation it is possible to note a GFAP expression comparable to P24 animals reared in normal condition of light exposure. Other authors observed, in rat, a GFAP reduction after a long protocol of light deprivation (Stewart et al., 1986). Thus it would be possible that the reduction observed by these authors was due to a component with fast turnover. However, one cannot exclude that the ligt deprivation-induced reduction of was due to phenomena of protein disassembly rather than GFAP turnover.

Possible discrepancies between dark reared results reported in the present paper and those shown in previous papers (Stewart et al., 1987; Muller et al., 1990) may be because I studied mouse cortex, while other authors investigated on cat and rat cortex. An alternative hypothesis takes into account the different dark rearing protocol used. Indeed, a long protocol of dark rearing, starting at birth and spanning all the critical period, was utilized in kittens and rats to induce modification of both neuronal and astrocytic phenotype, while to study mouse visual cortex, dark rearing lasted only 12 days. I consider unlikely the possibility that the short dark rearing protocol adopted is not adequate to investigate light deprivation effects on GFAP maturation since it covers the period of GFAP maturation and starts before eye opening. On the other hand, in the visual cortex there are processes and factors in which maturation is light independent. For example, refinement of functional properties such as development of orientation selectivity of visual cortical cells and of ocular dominance columns do not need visual experience for early development (Chapman et al., 1999).

A corollary and intriguing hypothesis assumes that the maturation of GFAP is independent of light but the maintenance of GFAP phenotype can be affected by a long period of visual deprivation. In agreement with this hypothesis, it has been

reported that very long periods of light deprivation affects GFAP expression (Stewart et al., 1987; Muller et al., 1990).

5.3 Role of NGF on GFAP expression in adulthood

As mentioned before, visual cortex is highly plastic not only during postnatal development, but also in adulthood. A bulk of evidence reports that neurotrophins and in particular NGF are factors influencing synaptic plasticity (Domenici et al., 1991; Maffei et al., 1992; Domenici et al., 1993; Berardi et al., 1994; Domenici et al., 1994; Pesavento et al., 2000). However, little information is available on the role of this neurotrophin in astrocytes. We approached this issue by investigating effects induced by NGF deprivation on GFAP expression. At first we used an anti-NGF transgenic mouse, producing intracellular antibodies that are released in the extracellular space to block NGF. Anti-NGF transgenic mice represent one of the most comprehensive models for Alzheimer's disease (AD). Aged (15 month old) anti-NGF mice display neurodegeneration characterized by cholinergic loss, insolubility and hyperphosphorylation of tau protein in cortical and hippocampal neurons (Capsoni et al., 2000), tangle-like accumulation (Capsoni et al., 2001) and β-amyloid plaques (Capsoni et al., 2002). The neurodegeneration is associated with behavioural deficits (Ruberti et al., 2000; Capsoni et al., 2000) and decreased cortical synaptic plasticity (Pesavento et al., 2002). In anti-NGF mice the neurodegeneration is characterized by a rather slow progression. At 2 months of age, the neurodegeneration is not overt and anti-NGF mice display just cholinergic loss and tau hyperphosphorylation in the entorhinal cortex (Capsoni et al., 2002). Thus, young anti-NGF mice allow the study of effects produced by NGF deprivation on CNS. I found that anti-NGF transgenic mice are characterized by a reduction of the number of astrocytes expressing GFAP. This was associated to a low amount of endogenous GFAP level in visual cortex and hippocampus. Reduction of GFAP expression was not due to cell loss as assessed by another marker of mature astrocytes, the calcium binding protein S-100, which was not affected by NGF deprivation, suggesting that NGF modulates GFAP.

To exclude the possibility that GFAP down regulation was due to unspecific results related to transgene insertion, NGF was chronically deprived by using intraventricular implant of hybridoma cells delivering the same anti-NGF antibody, αD11, expressed by anti-NGF transgenic mice. The hybridoma cell technique allows

us to obtain a high level of NGF antibody in the CSF and in the brain parenchyma for at least 2 weeks (Molnar *et al*, 1998). Results showed that GFAP was reduced in hybridoma cell implanted mice, thus indicating that this effect was because of NGF deprivation.

We have shown that the reduction of GFAP cellular expression is paralleled by the concomitant reduction of endogenous GFAP level in the visual cortex, as assessed by western blot. This suggests that GFAP is down regulated in astrocytes of anti-NGF mice.

In vitro reconstitution studies revealed that assembly and morphology of GFAP filaments are affected by various physical factors such as GFAP concentration, pH, divalent cations, ionic strength, phosphorylation of aminoacids residues, and temperature (Inagaki et al., 1990; Herrmann et al., 1993; Nakamura et al., 1991). Phosphorylation and dephosphorylation of the GFAP head region (Inagaki et al., 1990; Inagaki et al., 1987) and change of GFAP turnover, including synthesis and degradation of GFAP, have a critical role for the assembly and maintenance of the correct structure of GFAP. Since NGF deprived mice are characterized by a reduction of cellular expression, which corresponds to a reduced amount of GFAP, I suggest that the change of protein turnover, including synthesis and degradation, may account for GFAP down regulation. However, I cannot exclude the fact that modification of the phosphorylation state, in addition to turnover change, is to some extent responsible for the GFAP immunoreactivity reduction in NGF deprived mice. To discriminate whether the reduction of GFAP is due to the turnover of the protein it might be useful to check the status of GFAP mRNA. In fact the reduction of the protein could reflect a reduced expression of the mRNA. In this context *in situ* hybridization could be useful to check the GFAP mRNA status in the single cells.

Concerning the mechanisms underlying NGF regulation of GFAP expression two possibilities can be taken into account: i) NGF acts directly on astrocytes through NGF receptors and ii) GFAP down regulation is indirect and mediated by other systems sensitive to NGF deprivation. The first hypothesis assumes that NGF receptors are expressed in cortical and hippocampal astrocytes. NGF effects are mediated by its binding to two separate receptor classes, the Trk family of tyrosine kinase receptors and the p75 neurotrophin receptor, a member of the tumor necrosis factor receptor superfamily (Chao and Hempstead, 1995). The use of immunohistochemical and in situ hybridization techniques has given conflicting

results on this issue. In a recent study Trk-A immunoreactive astrocytes were observed in rat hippocampus (McCarty *et al*, 2002). However other authors failed to detect TrkA immunoreactivity in the human brain (Aguado *et al*, 1998) and in rat hippocampus (Koczyk and Oderfeld-Nowak, 2000). I failed to detect TrkA mRNA and protein (data not shown) both in the hippocampus and in the visual cortex of mice by using non-isotopic *in situ* hybridization and polyclonal antibodies. Using RT-PCR I detected a small amount of TrkA mRNA, in visual cortex in contrast with the BF region where I found high level of TrkA mRNA. Even though RT-PCR does not account for cell type expressing trkA mRNA, one cannot completely exclude the possibility that astrocytes express low amounts of TrkA, under the detection threshold of *in situ* hybridization and immunological techniques, which might, to some extent, account for NGF deprivation effects on GFAP expression.

Another hypothesis is that NGF deprivation affects astrocytes in an indirect way, through factors and/or systems sensitive to NGF and able to directly interact with astrocytes. Anti-NGF transgenic mice are characterized by loss of cholinergic neurons of basal forebrain. As a consequence, in the visual cortex of anti-NGF transgenic mice, the cholinergic input is reduced and this in turn might regulate, in an acute/subacute way, the expression of GFAP in astrocytes. Furthermore, reduction of GFAP immunoreactivity was generalized and not localized to visual cortex. In agreement with this idea, astrocytes express muscarinic receptors (Ashkenazi 1989; Hosli et al., 1988; Hosli and Hosli, 1988; van der Zee et al., 1989; 1993) and are susceptible to acetylcholine (Araque et al., 2002).

Cortical areas and hippocampus receive strong cholinergic input from basal forebrain neurons, which are sensitive to NGF deprivation. It would be important to investigate GFAP in areas that do not receive cholinergic input. Cholinergic system, as well as the other neuromodulatory systems, sends diffuse projections to almost all areas in the CNS. Cerebellum can be considered the region receiving less cholinergic input respect to other nuclei such as cortical areas, hippocampus and the other limbic areas, amygdala and olfactory bulb. This diffuse projection prevented a systematic and comparative analysis of areas target of cholinergic neurons versus areas that do not receive cholinergic input. To afford this important question we looked for nuclei of CNS that receive input from cholinergic neurons are not sensitive to NGF. For example, the visual thalamic nuclei (dorsal lateral geniculate- LG, lateral posterior- LP, and perigeniculate- PG) receive projections from cholinergic neurons in the

brainstem (Heckers et al., 1992; Smith et al., 1988). It has been shown that brainstem cholinergic neurons do not express NGF receptors (Hecker et al., 1992; Sobreviela et al., 1994; Holtzman et al., 1995). Preliminary data obtained in 2 mo. anti-NGF transgenic mice showed that GFAP-IR astrocytes of the LG are slightly reduced in NGF transgenic mice compared to control. However, quantitative data, including analysis of brainstem cholinergic neurons are needed before giving the definitive conclusion.

To mimic cholinergic impairment without affecting NGF, 2-(4-phenylpiperidino)-cyclohexanol, vesamicol (Prior et al., 1992) was used. This compound inhibits the transport of ACh into synaptic vesicles of the electric organ of Torpedo (Rogers and Parsons, 1993; Rogers et al., 1989) and inhibits the release of ACh *in vivo* (Buccafusco et al., 1991; Marien et al., 1991). Vesamicol crosses the BBB and therefore was administered peripherally, following methods previously shown (Kobayashi et al., 1997). I found that the cholinergic impairment, provoked by vesamicol, in normal mice, leads to a reduction of GFAP cellular expression comparable to that obtained in anti-NGF and hybridoma implanted mice.

In contrast with results obtained in NGF deprived mice, reduction of GFAP cellular expression is not associated with a concomitant reduction of protein, as assessed by western blot. To explain the intracellular machinery responsible for these results I advance the idea that ACh depletion may induce a change of the GFAP phosphorylation. As reported above, phosphorylation of amino acid residues in the head domain inhibits polymerization and depolimeryzes filaments (Inagaki et al., 1990). Phosphorylation dependent disassembly of GFAP can result in a decrease of GFAP immunoreactivity. Phosphorylation could mask the epitope recognized by the antibody when used *in situ*, but not when protein is in a soluble pool and this could explain the discrepancies between data obtained using immunohistochemistry and western blot. In agreement with the idea that ACh deprivation leads to modification of GFAP phosphorylation is the result that GFAP reduction is very rapid, occurring within 5 hours after the injection of Vesamicol.

To test whether the observed reduced GFAP within astrocytes is really due to a process of phosphorylation induced-disassembly of intermediate filaments we will use an antibody specific for the phosphorylated form of the protein that has been, only recently, rendered available for research purpose.

Results on GFAP immunoreactivity obtained in NGF deprived and ACh depleted mice point to similar conclusions, i.e. that GFAP is regulated by NGF and ACh. However, one should consider differences of effects found with NGF blocking and ACh release blocking such as the differential effect on the amount of GFAP amount arising from (1) acute ACh depletion versus chronic NGF deprivation and (2) different mechanisms and/or pathways activated by ACh versus NGF.

A hypothesis is that GFAP down-regulation in anti-NGF trasngenic mice is independent of ACh. Anti-NGF transgenic mice represent a good models for neurodegenerative disease, in particular Alzheimer's disease (AD). Aged (15 month old) anti-NGF mice display neurodegeneration characterized by cholinergic loss, insolubility and hyperphosphorylation of tau protein in cortical and hippocampal neurons (Capsoni et al., 2000), tangle-like accumulation (Capsoni et al., 2002b) and β-amyloid plaques (Capsoni et al., 2002b). The neurodegeneration is associated with behavioural deficits (Ruberti et al., 2000; Capsoni et al., 2000) and decreased cortical synaptic plasticity (Pesavento et al., 2002). In anti-NGF mice the neurodegeneration is characterized by a rather slow progression. At 2 months of age, anti-NGF transgenic mice are characterized by a cholinergic alteration in basal forebrain, abnormal phosphorylation of the microtubule associated protein tau and increased APP staining in the wall of some cerebral vessels (Capsoni et al., 2002b). Thus, in 2 months old anti-NGF mice, two mechanisms may regulate GFAP, one encompassing cholinergic system and the other one involving tau protein. Interestingly, phosphorylation of neurofilaments and tau protein in neurons is associated with changes of astrocytes shape (Arendt et al., 1994). Thus, we cannot discard the possibility that phosphorylation of tau may contribute to GFAP regulation.

5.4 Role of acetylcholine on GFAP expression

These results are difficult to reconcile with previous data obtained in animal models of neurodegeneration. Indeed, cholinergic impairment has been classically described to induce gliosis instead of GFAP down regulation. Pharmacological lesioning of cholinergic nuclei caused gliosis in cortex (Iacson et al., 1987; Anezaki et al., 1992; Monzon-Mayor et al., 2000). Gliosis was observed also in animal models of Alzheimer's disease (Beach et al., 1989; Delacourte, 1990). In contrast, data obtained in both transgenic and vesamicol treated mice indicate the absence of gliosis.

This was also confirmed by investigating the immunoreactivity of S-100. It is known that the expression of the β-form is upregulated after traumatic brain injury (Cerutti and Chadi, 200; Hinkle et al., 1997) and after neurodegenerative diseases (Mossakowski and Weinrauder, 1986; Sheng et al., 2000; Griffin et al., 1998; Sheng et al., 1996). The pattern of distribution of this protein was not altered in anti-NGF transgenic mice, confirming the absence of gliosis.

However, cholinergic lesions (Iacson et al., 1987; Anezaki et al., 1992; Monzon-Mayor et al., 2000) were induced in an invasive way and with a pharmacological approach which rather than being selective for cholinergic neurons, caused more generalized neuronal death. Furthermore, Alzheimer's disease, beyond inducing cholinergic impairment, is a neurodegenerative condition, which involve several factors and affect many cell types, thus inducing an astrocytic reaction with the final aim of promoting neuronal restoration. Vesamicol, allowed selectively reduction in acetylcholine release, without interfering with the release of other molecules. It could be possible that acetylcholine reduction *per se* does not induce any gliotic response. However, I cannot exclude the alternative possibility that the dose of vesamicol I used was able to cause a mild reduction of ACh release, thus resulting in GFAP down regulation. Higher dose/and or concentration of vesamicol should theoretically lead to the opposite effect, i.e. GFAP upregulation and gliosis.

To test this hypothesis a higher dose of vesamicol was injected to induce a strong reduction of acetylcholine release. As expected, after five hours from the injection, the early response to cholinergic reduction was an increase in GFAP immunoreactivity with astrocytes displaying the classic gliotic morphology. This result suggests that acetylcholine affects astrocytic phenotype in a dose dependent manner. The present result is also important to understand the temporal changes occurring in GFAP immunopositive astrocytes in animal models of Alzheimer disease. I have shown that at an early stage NGF deprivation (anti-NGF mice) induces an impairment of basal forebrain cholinergic neurons associated with GFAP down regulation. At later stages when cortex is fully degenerated with tangles, β-amyloid plaques and the impairment of cholinergic neurons is higher than in two months, the GFAP expression in anti-NGF mice is also increased and astrocytes appear hypertrophic, thus showing the classical pattern of gliotic response (Capsoni et al., submitted).

Thus, the temporal changes of GFAP expression in astrocytes can be considered a hallmark of Alzheimer disease progression.

Altogether, data obtained studying NGF and acetylcholine suggest that acetylcholine and NGF are molecules that are reciprocally modulated and active on both neurons and astrocytes. The cartoon (Fig. 5.1) summarizes the synergistic effect of NGF and acetylcholine in modulating GFAP expression. Briefly, NGF sustains viability of basal forebrain cholinergic neurons and acetylcholine released by projecting fibers maintains the regular astrocytic phenotype. NGF subtraction causes a reduction of cholinergic neurons and finally of acetylcholine released with a consequent alteration of glial fibrillary acidic protein.

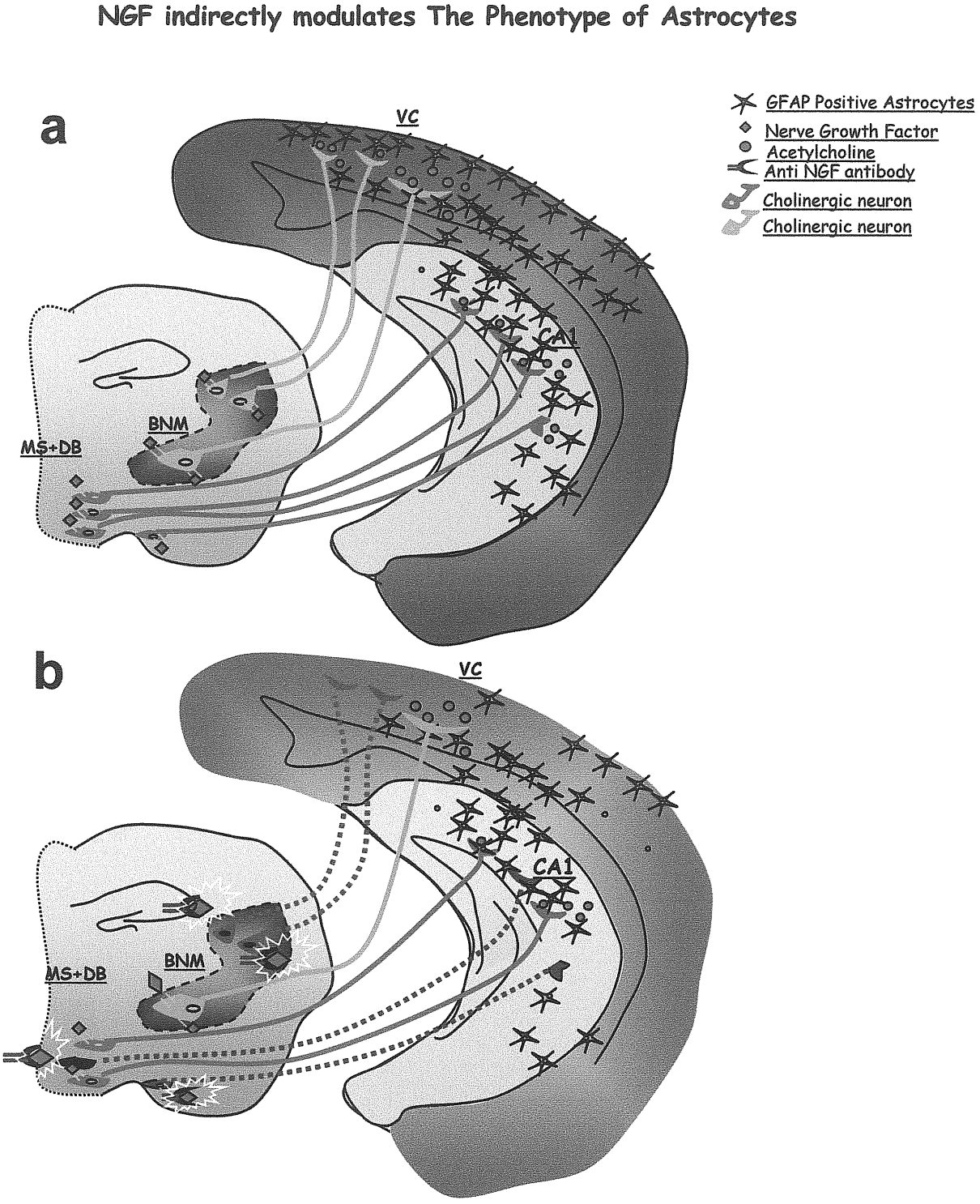


Fig. 51 NGF and acetylcholine synergistically modulate GFAP expression.
(a) NGF sustains the viability of basal forebrain cholinergic neurons. ACh released by fibers projecting to visual cortex or hippocampus maintains the regular astrocytic phenotype. (b) The reduction of NGF provokes a reduction of cholinergic neurons and finally of ACh released with a consequent alteration of GFAP expression.

5.5 Concluding remark and future strategies.

During the course of this thesis I had the opportunity to study factors and mechanisms which modulate astrocytic phenotype. My work has focused on GFAP, a member of intermediate filaments family. This protein is involved in astrocytic motility, astrocytic surface density and it has been shown to be sensitive to changes in the extracellular environment. Since it has been reported that GFAP-IR astrocytes have a role in neuronal maturation and plasticity, the first step of my study was to describe the period of maturation of GFAP in mouse visual cortex. Following experiments were focused on the role of visual experience in GFAP maturation, since previous studies showed that this represents an important factor for maturation of visual cortical neurons. In this experiment the protocol of sensory deprivation was restricted to the developmental time window of GFAP maturation. Light deprivation during this period did not affect GFAP expression, suggesting that light does not regulate neurons and astrocytes in the same way. It will be important to test if light is involved mainly in the maintenance of GFAP phenotype instead of GFAP maturation. For this purpose long period of visual deprivation will be utilized. Furtehrmore, it will be interesting to check wheter vimentin and nestin, two markers of immature astrocytes, are expressed in dark reared animals. This will clarify wheter light deprivation retard the maturation of astrocytes or it is important for the maintainance of the correct phenotype. Another interesting issue to be investigated is whether light deprivation affects the expression of other proteins, intracellular calcium level or electrophysiological properties in astrocytes. Beyond sensorial experience I investigated the role of NGF and acetylcholine on GFAP expression at an age well beyond the end of the GFAP maturation period. I found that NGF can modulate the regular astrocytes phenotype and, in particular, GFAP expression. The action of NGF deprivation was studied both in the transgenic mouse producing antibodies blocking free NGF action and in mice implanted with hybridoma cells releasing anti-NGF antibodies into the lateral ventricle. The results were similar in the two experimental groups and demonstrate that NGF deprivation induces a down regulation of GFAP in visual cortex. This effect was not confined to visual cortex since it was present also in hippocampus.

What might be the functional implication of the present finding? We have shown that at an early stage chronic NGF deprivation does not induce an astrogliosis. It is known that the first hallmark of reactive astrocytes is pathological accumulation

of cytoskeletal proteins such as GFAP (Brock and O'Callaghan 1987; Cheung et al., 1999; Latov et al., 1979; Mathewson and berry, 1985; Smith et al., 1983) and an increase of S-100-IR (Cerutti and Chadi, 200; Hinkle et al., 1997). In contrast, using two independent markers, namely GFAP and S100, both upregulated during gliosis, I found an absence of reactive process in two month old anti NGF-transgenic mice. As the neurodegeneration proceeds astrocytes start to accumulate GFAP and in aged anti-NGF mice gliosis is apparent (Capsoni, Giannotta and Cattaneo, unpublished data). In agreement with this observation, gliosis has been described in patients affected by Alzheimer disease when different systems and factors are altered by the neurodegenerative process (Beach et al., 1989; Delacourte, 1990).

Data I found lead to the idea that young anti-NGF mice reflects conditions occurring at very early stage of AD. Furthermore I speculate that the reduction of GFAP and the retraction of astrocytic processes observed in young anti-NGF mice may contribute to the disruption of the BBB. Indeed, it is known that astrocytes actively contribute to the function of the BBB (Abbott 2002) and that the impairment of BBB and extracellular deposition of β-amyloid (Kalaria 1997) are associated with the onset of inflammatory processes in the brain (Tomimoto et al., 1996). Interestingly, recent data obtained in our lab (Capsoni, Giannotta and Cattaneo, unpublished data) show that the BBB is already impaired in anti-NGF transgenic mice at an early stage of the neurodegenerative process.

It is known that vimentin is a protein transiently expressed in astrocytes, during early postnatal development (Stichel et al., 1991; Sancho-Tello et al., 1995). Vimentin is re-expressed in adult animals after traumatic injury (Poltorak et al., 1993; Hill et al., 1996) or neurodegenerative disease (Cammer et al., 1990; Smith et al., 1990), and it is a good marker for gliosis. Thus I expect that vimentin would reappere in the aged but not in two-month-old anti-NGF transgenic-mice. However, this does not exclude the fact that vimentin expression could be checked in a future to further confirm the absence of gliosis at an early stage of neurodegeneration.

As discussed above, a two-month-old anti-NGF transgenic mouse might represent a model two study very early stage of neurodegeneration. In this model, at a mild impairment of the cholinergic system correspond an absence of gliosis a down regulation of GFAP. However this does not mean that NGF deprivation induce always a GFAP down regulation. In fact, aged anti-NGF mice, which are characterized by a

strong neurodegeneration, show a GFAP upregulation (Capsoni et al., unpublished data). Thus GFAP might be regulated in function of the grade of neurodegeneration. It is known that lesions induce a GFAP upregulation around the lesioned area (Hill et al., 1996). Lesion is a good method to induce a localized strong neurodegeneration. In light of the events occurring in aged transgenic animals, in lesioned two month-old anti NGF transgenic mice, I would aspect a GFAP upregulation similar to that described in wild type mice.

We mimicked the results obtained in NGF deprived mice by reducing the acetylcholine release in wild type mice, thus suggesting that NGF deprivation may act on astrocytes through the cholinergic system. Interestingly, in ACh depleted mice, reduction of GFAP immunoreactivity is not accompanied by a decrease in GFAP as assessed by western blot. This represents also a difference compared to NGF deprived mice, which are characterized by a reduction of both GFAP immunoreactivity and amount of protein. As discussed, this discrepancy could be because of a phosphorylation-induced disassembly of GFAP in ACh depleted mice versus turnover changes in NGF deprived mice. Future experiments will be addressed to clarify the grade of GFAP phosphorylation in ACh depleted animals. Unluckily, antibodies recognizing phosphorylated amino acid residues of GFAP protein are not presently available.

Results on GFAP immunoreactivity obtained in NGF deprived and ACh depleted mice point to similar conclusions, i.e. that GFAP is regulated by NGF and ACh. However, one should consider differences of effects found with NGF blocking and ACh release blocking such as the differential effect on the amount of GFAP amount arising from (1) acute ACh depletion versus chronic NGF deprivation and (2) different mechanisms and/or pathways activated by ACh versus NGF.

A hypothesis is that GFAP down-regulation in anti-NGF trasngenic mice is ACh independent. As described before, anti-NGF transgenic mice represent one of the best models to study AD. Indeed, aged (15 month old) anti-NGF mice display sign of neurodegeneration characterstic of AD, included insolubility and hyperphosphorylation of tau protein in cortical and hippocampal neurons (Capsoni et al., 2000), tangle-like accumulation (Capsoni et al., 2002b) and β-amyloid plaques (Capsoni et al., 2002b). The neurodegeneration is associated with behavioural deficits (Ruberti et al., 2000; Capsoni et al., 2000) and decreased cortical synaptic plasticity

(Pesavento et al., 2002). At 2 months of age, anti-NGF transgenic mice are characterized by a cholinergic alteration in basal forebrain, abnormal phosphorylation of the microtubule associated protein tau and increased APP staining in the wall of some cerebral vessels (Capsoni et al., 2002b). Thus, in 2 months old anti-NGF mice, two streams may control GFAP expression, one encompassing cholinergic neurons and the other involving microtubule associated tau protein. Interestingly, phosphorylation of neurofilaments and tau in neurons is associated with changes of astrocytes shape (Arendt et al., 1994). Thus, I cannot discard the possibility that phosphorylation of tau may contribute to GFAP regulation.

With respect to ACh we showed that effects on GFAP phenotype are dose-dependent: mild reduction of ACh release induces reduction of GFAP immunoreactivity while strong reduction induced the opposite result, i.e. increase of GFAP expression. These data show for the first time the ACh effect on GFAP astrocytes. However, the intracellular pathway mediating such an effect is still unknown. Thus, it will be important to know which type of acetylcholine receptors mediate ACh receptors by using pharmacological tools. In this thesis I reported experiments showing that astrocytes express muscarinic receptors and therefore it is conceivable that this subclass of receptors mediate the cholinergic effect on GFAP. In addition, it will be important to clarify the intracellular pathway of signal transduction.

Preliminary data showed that blockade of muscarinic receptors by scopolamine increased GFAP-IR astrocytes in both visual cortex and hippocampus (Fig. 5.2) with astrocytes becoming hypertrophic (Fig. 5.3). Blockade of nicotinic receptors by mecamylamine leaft GFAP astrocytes completely unaffected, thus indicating that muscarinic but not nicotinic receptors are involved in GFAP regulation (Fig.5.4, 5.5). Finally, it will be important to clarify whether chronic depletion of ACh affects GFAP expression similarly to acute blockade of ACh release. Indeed, we can consider discrepancies with respect to the amount GFAP between NGF and ACh deprived mice arising from (1) acute ACh depletion versus chronic NGF deprivation and (2) different mechanisms and/or pathways activated by ACh versus NGF.

The final remark is dedicated to the functional meaning of the present results. It is generally assumed that kinetics of intermediate filaments (IF) such as GFAP is important for intracellular IF network regulating cell shape and plasticity especially in the perisynaptic region. We formulate the hypothesis that neuronal activity through

secretion of neurotransmitters such as Ach may influence IF network this reflecting on the feedback communication between astrocytes and neurons and potentially on other target cells such as endothelial cells of blood vessels. Preliminary data obtained in our laboratory showed that specific patterns of afferent electrical activity to the primary visual cortex regulate GFAP expression in astrocytes, possibly through release of neurotransmitters. If we couple this result to previous data showing that GFAP is involved in regulation of synaptic efficacy, we thus depict a neural network controlling the flow of information in the brain both in normal and pathological conditions.

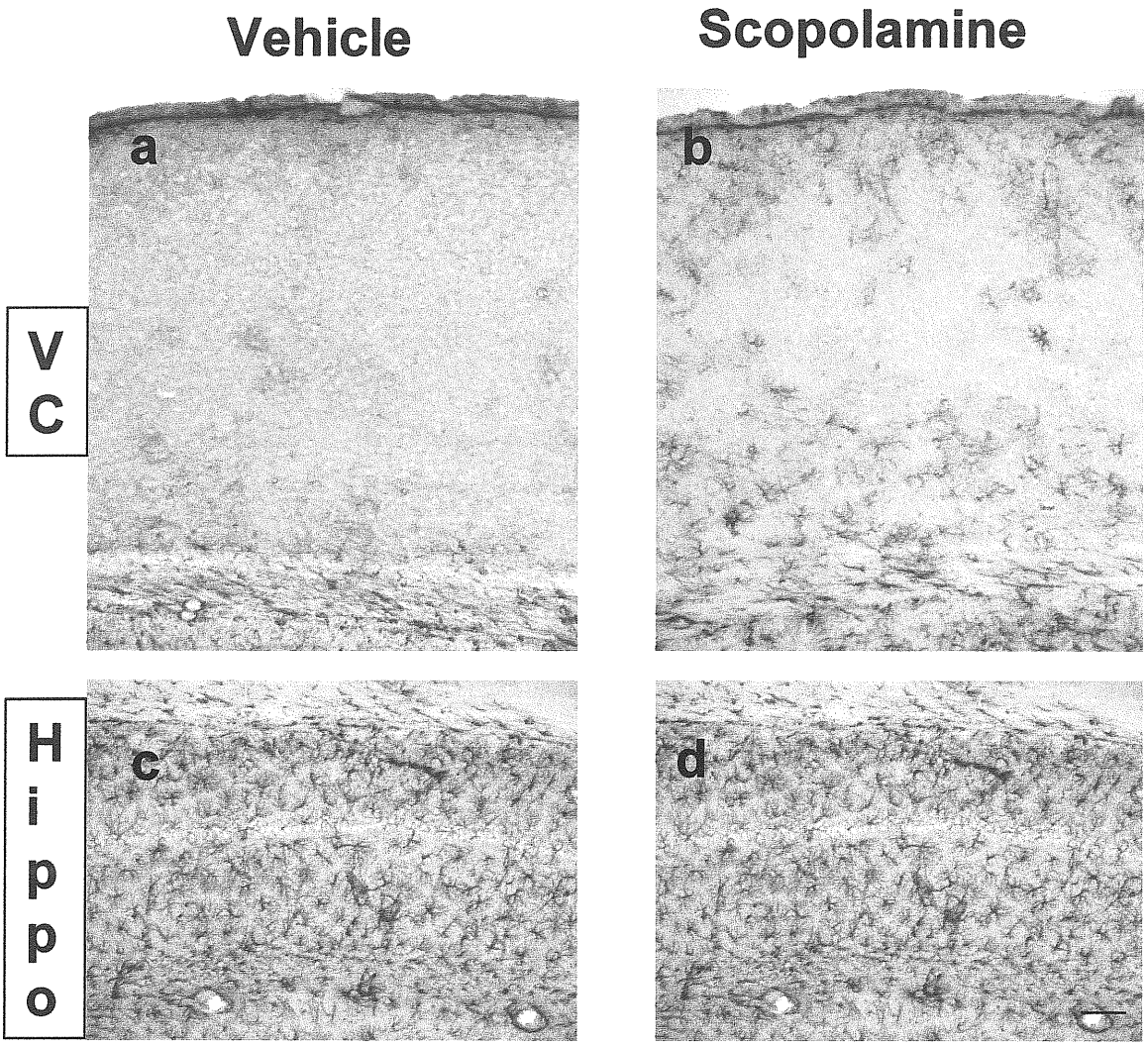


Fig. 5.2. GFAP-IR in scopolamine injected mice.GFAP-IR astrocytes are increased after blockade of muscarinic receptors reduction in both (b) visual cortex and in (d) hippocampus. Scale bar: 100 μm.

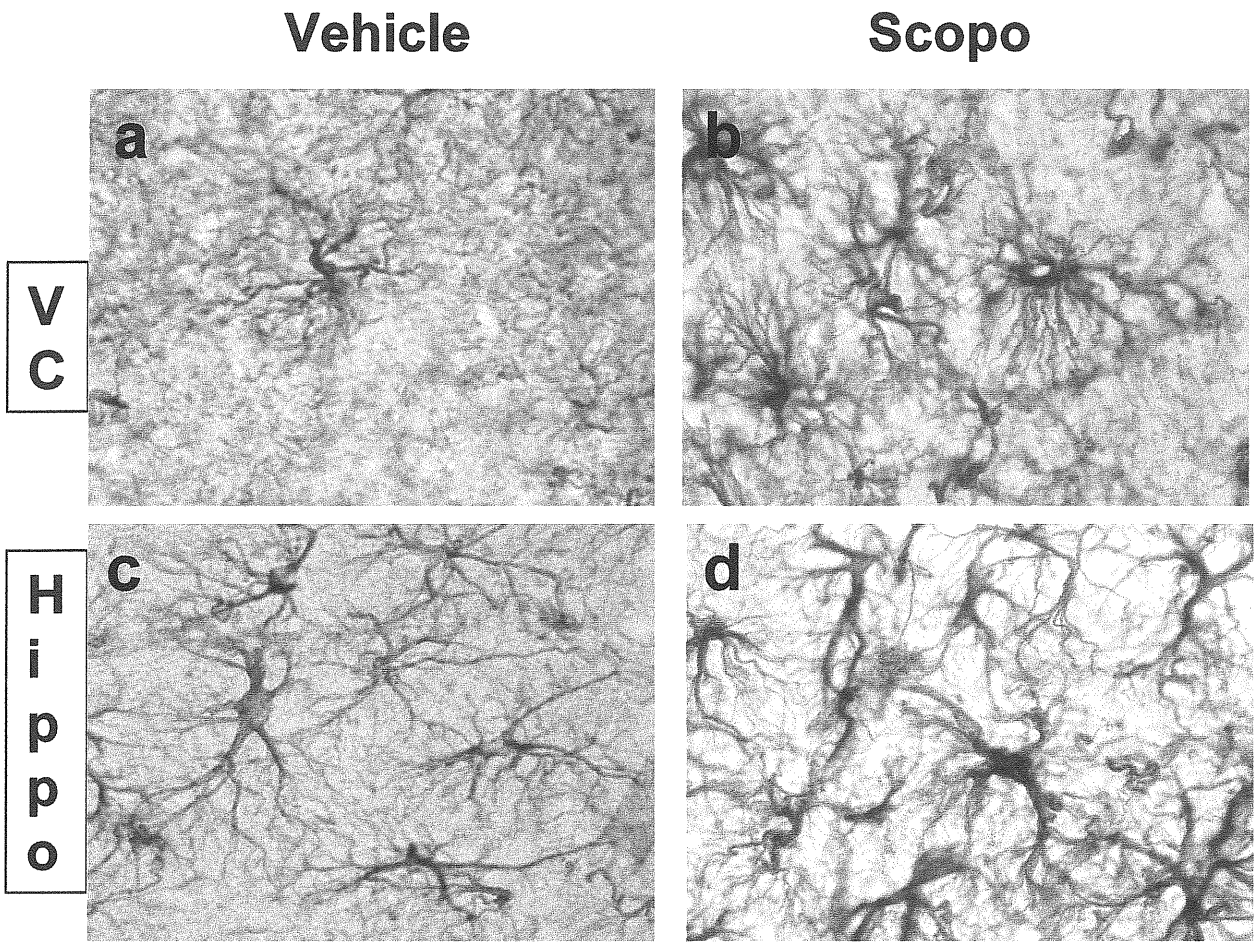


Fig. 5.3 Blockade of muscarinic receptors induce alteration in the shape of GFAP-IR astrocytes. Higher magnification shows the morphological alteration, comparable to that induced by NGF deprivation. After injection of scopolamine, astrocytes in both (b) visual cortex and (d) hippocampus. Scale bar: 20 μm.

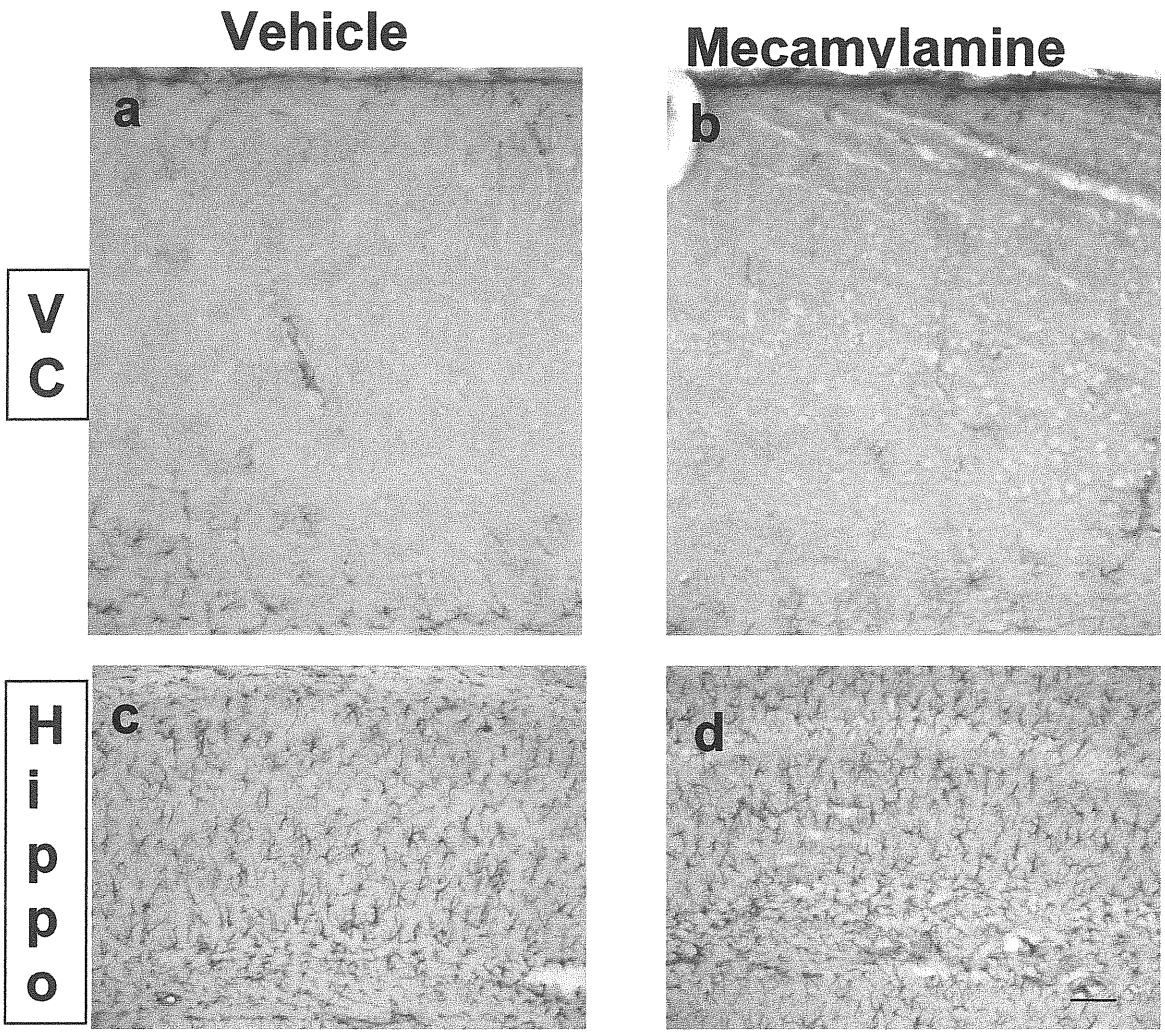


Fig. 5.4. GFAP-IR in mecamylamine injected mice.GFAP immunoreactivity is not affected by blockade of nicotinic receptors reduction in both (b) visual cortex and in (d) hippocampus. Scale bar: 100 μm.

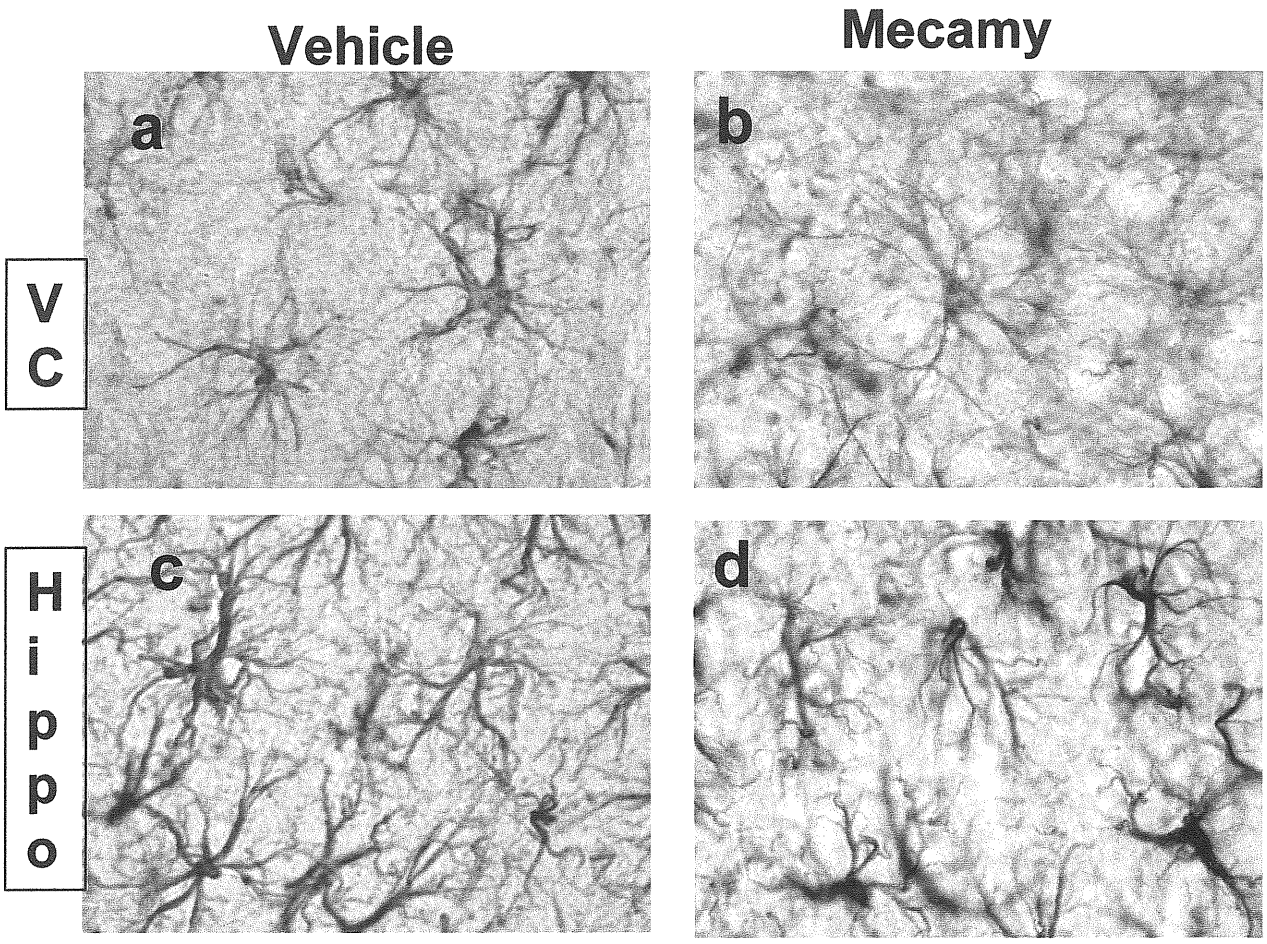


Fig. 5.5 Blockade of nicotinic receptors does not alter the shape of GFAP-IR astrocytes. Higher magnification shows that astrocytic shape in mice injected with (a-c) saline alone is comparable to that in (b-d) mecamylamine injected animals. Scale bar: 20 μm.

REFERENCES:

1. Aguado F, Ballabriga J, Pozas E, Ferrer I. (1998) TrkA immunoreactivity in reactive astrocytes in human neurodegenerative diseases and colchicine-treated rats. Acta Neuropathol (Berl) 96:495-501.
2. Ahmed S, Reynolds BA, Weiss S (1995) BDNF enhances the differentiation but not the survival of CNS stem cell-derived neuronal precursors. J Neurosci 15:5765-78.
3. Alvarez-Buylla A, Garcia-Verdugo JM, Tramontin AD. (2001) A unified hypothesis on the lineage of neural stem cells. Nat Rev Neurosci 2: 287-93.
4. Amaducci L, Forno KI, Eng LF (1981) Glial fibrillary acidic protein in cryogenic lesions of the rat brain. Neurosci Lett 21:27-32.
5. Andre C, Dos Santos G, Koulakoff A (1994) Muscarinic receptor profiles of mouse brain astrocytes in culture vary with their tissue of origin but differ from those of neurons. Eur J Neurosci 6:1702-9.
6. Anezaki T, Yanagisawa K, Takahashi H, Nakajima T, Miyashita K, Ishikawa A, Ikuta F, Miyatake T (1992) Remote astrocytic response of prefrontal cortex is caused by the lesions in the nucleus basalis of Meynert, but not in the ventral tegmental area. Brain Res 574:63-9.
7. Aquino DA, Chiu FC, Brosnan CF, Norton WT (1988) Glial fibrillary acidic protein increases in the spinal cord of Lewis rats with acute experimental autoimmune encephalomyelitis. J Neurochem 51:1085-96.
8. Araque A, Carmignoto G, Haydon PG. (2001) Dynamic signaling between astrocytes and neurons. Annu Rev Physiol 63:795-813
9. Araque A, Martin ED, Perea G, Arellano JI, Buno W (2002) Synaptically released acetylcholine evokes Ca2+ elevations in astrocytes in hippocampal slices. J Neurosci 22:2443-50.
10. Arendt T, Bruckner MK, Krell T, Pagliusi S, Kruska L, Heumann R. (1995) Degeneration of rat cholinergic basal forebrain neurons and reactive changes in nerve growth factor expression after chronic neurotoxic injury--II. Reactive expression of the nerve growth factor gene in astrocytes. Neuroscience 65:647-59.

11. Ashkenazi A, Peralta EG, Winslow JW, Ramachandran J, Capon DJ. (1989) Functional diversity of muscarinic receptor subtypes in cellular signal transduction and growth. Trends Pharmacol Sci Suppl:16-22.
12. Ashkenazi A, Ramachandran J, Capon DJ. (1989) Acetylcholine analogue stimulates DNA synthesis in brain-derived cells via specific muscarinic receptor subtypes. Nature 340:146-50.
13. Banner DW, D'Arcy A, Janes W, Gentz R, Schoenfeld HJ, Broger C, Loetscher H, Lesslauer W. (1993) Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. Cell 73:431-45.
14. Barbacid M. The Trk family of neurotrophin receptors. (1994) J Neurobiol Nov 25:1386-403.
15. Barde YA, Edgar D, Thoenen H. (1982) Purification of a new neurotrophic factor from mammalian brain. EMBO J 1:549-53.
16. Barde YA. The nerve growth factor family. (1990) Prog Growth Factor Res 2:237-48.
17. Barker-Gibb AL, Dougherty KD, Einheber S, Drake CT, Milner TA. (2001) Hippocampal tyrosine kinase A receptors are restricted primarily to presynaptic vesicle clusters. J Comp Neurol 430:182-99.
18. Barrett GL. The p75 neurotrophin receptor and neuronal apoptosis. (2000) Prog Neurobiol 61: 205-29.
19. Beach TG, Walker R, McGeer EG. (1989) Patterns of gliosis in Alzheimer's disease and aging cerebrum. Glia 2:420-36.
20. Beldhuis HJ, Everts HG, Van der Zee EA, Luiten PG, Bohus B. (1992) Amygdala kindling-induced seizures selectively impair spatial memory. 2. Effects on hippocampal neuronal and glial muscarinic acetylcholine receptor. Hippocampus 2:411-9.
21. Benedetti M, Levi A, Chao MV. (1993) Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. Proc Natl Acad Sci U S A 90:7859-63.
22. Bentivoglio M, Mazzarello P. (1999) The history of radial glia. Brain Res Bull 49:305-15.

23. Berardi N, Cellerino A, Domenici L, Fagiolini M, Pizzorusso T, Cattaneo A, Maffei L (1994) Monoclonal antibodies to nerve growth factor affect the postnatal development of the visual system. Proc Natl Acad Sci U S A 91:684-8.
24. Berardi N, Pizzorusso T, Maffei L. (2000) Critical periods during sensory development. Curr Opin Neurobiol 10:138-45.
25. Berkemeier LR, Winslow JW, Kaplan DR, Nikolics K, Goeddel DV, Rosenthal A. (1991) Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. Neuron 7:857-66.
26. Bernier L, Colman DR, D'Eustachio P. (1988) Chromosomal locations of genes encoding 2',3' cyclic nucleotide 3'-phosphodiesterase and glial fibrillary acidic protein in the mouse. J Neurosci Res 20:497-504.
27. Berridge MJ, Irvine RF. (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 312:315-21
28. Biffo S, Offenhauser N, Carter BD, Barde YA. (1995) Selective binding and internalisation by truncated receptors restrict the availability of BDNF during development. Development 121:2461-70.
29. Bongcam-Rudloff E, Nister M, Betsholtz C, Wang JL, Stenman G, Huebner K, Croce CM, Westermark B. (1991) Human glial fibrillary acidic protein: complementary DNA cloning, chromosome localization, and messenger RNA expression in human glioma cell lines of various phenotypes. Cancer Res 51:1553-60.
30. Boothe RG, Dobson V, Teller DY. (1985) Postnatal development of vision in human and nonhuman primates. Annu Rev Neurosci 8:495-545.
31. Boshart M, Weber F, Jahn G, Dorsch-Hasler K, Fleckenstein B, Schaffner W. (1985) A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell 41:521-30.
32. Bovolenta P, Liem RK, Mason CA. (1984) Development of cerebellar astroglia: transitions in form and cytoskeletal content. Dev Biol 102:248-59.
33. Boyer S, Montagutelli X, Gomes D, Simon-Chazottes D, Guenet JL, Dupouey P. (1991) Recent evolutionary origin of the expression of the glial fibrillary acidic protein (GFAP) in lens epithelial cells. A molecular and genetic analysis of various mouse species. Brain Res Mol Brain Res 10:159-66.
34. Brock TO, O'Callaghan JP. (1987) Quantitative changes in the synaptic vesicle proteins synapsin I and p38 and the astrocyte-specific protein glial fibrillary

acidic protein are associated with chemical-induced injury to the rat central nervous system. J Neurosci 7:931-42.

35. Brodie C, Goldreich N, Haiman T, Kazimirsky G. (1998) Functional IL-4 receptors on mouse astrocytes: IL-4 inhibits astrocyte activation and induces NGF secretion. J Neuroimmunol 81:20-30.
36. Buccafusco JJ, Wei J, Kraft KL. (1991) The effect of the acetylcholine transport blocker vesamicol on central cholinergic pressor neurons. Synapse 8:301-6.
37. Cajal SR. (1909) Histologie du Systeme Nercveux de l'Homme et des Vertebres. A Maloine, Paris.
38. Cameron RS, Rakic P. (1991) Glial cell lineage in the cerebral cortex: a review and synthesis. Glia 4:124-37.
39. Cammer W, Tansey FA, Brosnan CF. (1990) Reactive gliosis in the brains of Lewis rats with experimental allergic encephalomyelitis. J Neuroimmunol 27:111-20.
40. Canady KS, Olavarria JF, Rubel EW. (1994) Reduced retinal activity increases GFAP immunoreactivity in rat lateral geniculate nucleus. Brain Res 663:206-14.
41. Capsoni S, Giannotta S, Cattaneo A (2002) beta-Amyloid Plaques in a Model for Sporadic Alzheimer's Disease Based on Transgenic Anti-Nerve Growth Factor Antibodies. Mol Cell Neurosci 21:15.
42. Capsoni S, Ugolini G, Comparini A, Ruberti F, Berardi N, Cattaneo A (2000) Alzheimer-like neurodegeneration in aged antinerve growth factor transgenic mice. Proc Natl Acad Sci U S A 97:6826-31.
43. Carroll PT. (1983 Spontaneous release of acetylcholine and acetylhomocholine from mouse forebrain minces: cytoplasmic or vesicular origin. Neurochem Res 8:1271-83.
44. Carroll PT. (1984) Veratridine-induced release of acetylcholine from mouse forebrain minces: dependence on the hydrolysis of cytoplasmic acetylcholine for a source of choline. Brain Res 321:55-62.
45. Catlin MC, Kavanagh TJ, Costa LG. (2000) Muscarinic receptor-induced calcium responses in astroglia. Cytometry 41:123-32.
46. Caulfield MP, Birdsall NJ. (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 50:279-90.

47. Cellerino A (1996) Expression of messenger RNA coding for the nerve growth factor receptor trkA in the hippocampus of the adult rat. Neuroscience 70:613-16

48. Cerutti SM, Chadi G (2000) S100 immunoreactivity is increased in reactive astrocytes of the visual pathways following a mechanical lesion of the rat occipital cortex. Cell Biol Int 24:35-49.

49. Chanas-Sacre G, Thiry M, Pirard S, Rogister B, Moonen G, Mbebi C, Verdiere-Sahuque M, Leprince P. (2000) A 295-kDA intermediate filament-associated protein in radial glia and developing muscle cells in vivo and in vitro. Dev Dyn. 219:514-25.

50. Chang B., Hawes NL, Hurd RE, Davisson MT, Nusinowitz S, Heckenlively JR (2002) Retinal degeneration mutants in the mouse. Vision Res. 42, 517-525.

51. Chao MV, Bothwell MA, Ross AH, Koprowski H, Lanahan AA, Buck CR, Sehgal A. (1986) Gene transfer and molecular cloning of the human NGF receptor. Science 232:518-21.

52. Chao MV, Hempstead BL. (1995) p75 and Trk: a two-receptor system. Trends Neurosci 18:321-6.

53. Chen L, Cooper NG, Mower GD. (2000) Developmental changes in the expression of NMDA receptor subunits (NR1, NR2A, NR2B) in the cat visual cortex and the effects of dark rearing. Brain Res Mol Brain Res 78:196-200.

54. Cheung WM, Wang CK, Kuo JS, Lin TN. (1999) Changes in the level of glial fibrillary acidic protein (GFAP) after mild and severe focal cerebral ischemia. Chin J Physiol 42:227-35.

55. Chiu FC, Goldman JE. (1984) Synthesis and turnover of cytoskeletal proteins in cultured astrocytes. J Neurochem 42:166-74.

56. Choi BH, Lapham LW. (1978) Radial glia in the human fetal cerebrum: a combined Golgi, immunofluorescent and electron microscopic study. Brain Res 148:295-311.

57. Chomczynski P, Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156-9.

58. Chung IY, Benveniste EN. (1990) Tumor necrosis factor-alpha production by astrocytes. Induction by lipopolysaccharide, IFN-gamma, and IL-1 beta. J Immunol 144:2999-3007.

59. Clary DO, Reichardt LF. (1994) An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin 3. Proc Natl Acad Sci U S A 91:11133-7.

60. Cohen S. (1960). Purification of a nerve-growth promoting protein from the mouse salivary gland and its neurocytotoxic antiserum. Proc Natl Acc Sci USA 46:302-311.

61. Collier B, Welner SA, Ricny J, Araujo DM. (1986) Acetylcholine synthesis and release by a sympathetic ganglion in the presence of 2-(4-phenylpiperidino) cyclohexanol (AH5183). J Neurochem 46:822-30.

62. Condorelli DF, Dell'Albani P, Kaczmarek L, Messina L, Spampinato G, Avola R, Messina A, Giuffrida Stella AM. (1990) Glial fibrillary acidic protein messenger RNA and glutamine synthetase activity after nervous system injury. J Neurosci Res 26:251-7.

63. Condorelli DF, Salin T, Dell' Albani P, Mudo G, Corsaro M, Timmusk T, Metsis M, Belluardo N. (1995) Neurotrophins and their trk receptors in cultured cells of the glial lineage and in white matter of the central nervous system. J Mol Neurosci 6:237-48.

64. Connor B, Young D, Lawlor P, Gai W, Waldvogel H, Faull RL, Dragunow M. (1996) Trk receptor alterations in Alzheimer's disease. Brain Res Mol Brain Res Nov 42:1-17.

65. Cooper JR, Bloom FE, Roth RH. (1991) The biochemical basis of neuropharmacology, 6th ed. Neww York & Oxford: Oxford University Press.

66. Cordon-Cardo C, Tapley P, Jing SQ, Nanduri V, O'Rourke E, Lamballe F, Kovary K, Klein R, Jones KR, Reichardt LF, et al. (1991) The trk tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. Cell 66:173-83.

67. Corvetti L, Capsoni S, Cattaneo A, Domenici L. (2003) Postnatal development of GFAP in mouse visual cortex is not affected by light deprivation. Glia 41:404-414.

68. Crockard HA, Gadian DG, Frackowiak RS, Proctor E, Allen K, Williams SR, Russell RW. (1987) Acute cerebral ischaemia: concurrent changes in cerebral

blood flow, energy metabolites, pH, and lactate measured with hydrogen clearance and 31P and 1H nuclear magnetic resonance spectroscopy. II. Changes during ischaemia. J Cereb Blood Flow Metab 7:394-402

69. Crowley C, Spencer SD, Nishimura MC, Chen KS, Pitts-Meek S, Armanini MP, Ling LH, MacMahon SB, Shelton DL, Levinson AD, et al (1994) Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76:1001-11.
70. Culican SM, Baumrind NL, Yamamoto M, Pearlman AL. (1990) Cortical radial glia: identification in tissue culture and evidence for their transformation to astrocytes. J Neurosci 10:684-92.
71. Cynader M. (1983) Prolonged sensitivity to monocular deprivation in dark-reared cats: effects of age and visual exposure. Brain Res 284:155-64.
72. da Cunha A, Jefferson JJ, Tyor WR, Glass JD, Jannotta FS, Vitkovic L. (1993) Control of astrocytosis by interleukin-1 and transforming growth factor-beta 1 in human brain. Brain Res 631:39-45.
73. Dani JW, Smith SJ. (1995). The triggering of astrocytic calcium waves by NMDAinduced neuronal activation. Ciba. Found. Symp. 188:195–205
74. DeArmond SJ, Fajardo M, Naughton SA, Eng LF. (1983) Degradation of glial fibrillary acidic protein by a calcium dependent proteinase: an electroblot study. Brain Res 262:275-82.
75. DeArmond SJ, Lee YL, Kretzschmar HA, Eng LF. (1986) Turnover of glial filaments in mouse spinal cord. J Neurochem 47:1749-53.
76. Delacourte A. (1990) General and dramatic glial reaction in Alzheimer brains. Neurology 40:33-7.
77. Di Loreto S, Corvetti L, Maccarone R, Piancatelli D, Adorno D. 2000 Interleukin 1-beta modulates the effects of hypoxia in neuronal culture. J Neuroimmunol 106:32-42.
78. DiStefano PS, Friedman B, Radziejewski C, Alexander C, Boland P, Schick CM, Lindsay RM, Wiegand SJ. (1992) The neurotrophins BDNF, NT-3, and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. Neuron 8:983-93.
79. Domenici L, Cellerino A, Berardi N, Cattaneo A, Maffei L. (1994) Antibodies to nerve growth factor (NGF) prolong the sensitive period for monocular deprivation in the rat. Neuroreport. 5:2041-4.

80. Domenici L, Cellerino A, Maffei L. (1993) Monocular deprivation effects in the rat visual cortex and lateral geniculate nucleus are prevented by nerve growth factor (NGF). II. Lateral geniculate nucleus. Proc R Soc Lond B Biol Sci 251:25-31.

81. Domenici L, Gravina A, Berardi N, Maffei L. (1991) Different effects of intracranial and intraorbital section of the optic nerve on the functional responses of rat retinal ganglion cells. Exp Brain Res 86:579-84.

82. Drager UC, Hubel DH. (1978) Studies of visual function and its decay in mice with hereditary retinal degeneration. J Comp Neurol 180:85-114.

83. Du S, Rubin A, Klepper S, Barrett C, Kim YC, Rhim HW, Lee EB, Park CW, Markelonis GJ, Oh TH. (1999) Calcium influx and activation of calpain I mediate acute reactive gliosis in injured spinal cord. Exp Neurol 157:96-105.

84. Eckenstein F, Thoenen H. (1983) Cholinergic neurons in the rat cerebral cortex demonstrated by immunohistochemical localization of choline acetyltransferase. Neurosci Lett 36:211-5.

85. Edmondson JC, Hatten ME. (1987) Glial-guided granule neuron migration in vitro: a high-resolution time-lapse video microscopic study. J Neurosci 7:1928-34.

86. Eng LF, Vanderhaghen JJ, Bignami A, Gerstl B. (1971). An acidic protein isolated from fibrous astrocytes. Brain Res, 28: 351-354.

87. Eng LF, Yu AC, Lee YL. (1992). Astrocytic response to injury. Prog Brain Res; 94:353-65.

88. Eng LF. (1980). The glial fibrillary acidic (GFA) protein. In: Proteins of the Nervous System, 2nd Ed. R.A. BRADSHAW AND D.M. SCHNEIDER, eds. Raven Press, New York, pp.85-117.

89. Ernfors $P_{(a)}$, Ibanez CF, Ebendal T, Olson L, Persson H. (1990) Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. Proc Natl Acad Sci U S A 87:5454-8.

90. Ernfors $P_{(b)}$, Wetmore C, Olson L, Persson H. (1990) Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. Neuron 5:511-26.

91. Fagiolini M, Pizzorusso T, Berardi N, Domenici L, Maffei L. (1994) Functional postnatal development of the rat primary visual cortex and the role of visual experience: dark rearing and monocular deprivation. Vision Res, 34:709-20
92. Fagiolini M, Pizzorusso T, Berardi N, Domenici L, Maffei L. (1994) Functional postnatal development of the rat primary visual cortex and the role of visual experience: dark rearing and monocular deprivation. Vision Res 34:709-20.
93. Feinstein DL, Weinmaster GA, Milner RJ. (1992) Isolation of cDNA clones encoding rat glial fibrillary acidic protein: expression in astrocytes and in Schwann cells. J neurosci Res 32: 1-14.
94. Feng L, Hatten ME, Heintz N. (1994) Brain lipid-binding protein (BLBP): a novel signaling system in the developing mammalian CNS. Neuron 12:895-908.
95. Ferroni S, Marchini C, Ogata T, Schubert P. (2002) Recovery of deficient cholinergic calcium signaling by adenosine in cultured rat cortical astrocytes. J Neurosci Res 68:615-21.
96. Fiedorowicz A, Figiel I, Kaminska B, Zaremba M, Wilk S, Oderfeld-Nowak B. (2001) Dentate granule neuron apoptosis and glia activation in murine hippocampus induced by trimethyltin exposure. Brain Res 912:116-27.
97. Foschini DR, Kestler AM, Egger MD, Crockett DP. (1994) The up-regulation of trkA and trkB in dorsal column astrocytes following dorsal rhizotomy. Neurosci Lett169:21-4.
98. Fox K, Zahs K. (1994) Critical period control in sensory cortex. Curr Opin Neurobiol 4:112-9
99. Fox K. (1995) The critical period for long-term potentiation in primary sensory cortex. Neuron 15:485-8.
100. Furukawa S, Furukawa Y, Satoyoshi E, Hayashi K. (1987) Regulation of nerve growth factor synthesis/secretion by catecholamine in cultured mouse astroglial cells. Biochem Biophys Res Commun 147:1048-54.
101. Furukawa S, Furukawa Y, Satoyoshi E, Hayashi K. (1986) Synthesis and secretion of nerve growth factor by mouse astroglial cells in culture. Biochem Biophys Res Commun 136:57-63.
102. Gadisseux JF, Evrard P, Mission JP, Caviness VS Jr. (1992) Dynamic changes in the density of radial glial fibers of the developing murine cerebral wall: a quantitative immunohistological analysis. J Comp Neurol 322:246-54.

103. Gasser UE, Hatten ME. (1990) Neuron-glia interactions of rat hippocampal cells in vitro: glial-guided neuronal migration and neuronal regulation of glial differentiation. J Neurosci 10:1276-85.
104. Geisler N, Weber K. (1983) Amino acid sequence data on glial fibrillary acid protein (GFA); implication for subdivision of intermediate filaments into epithelial and non epithelial members, EMBO J 2:2059-2063.
105. Gilbert CD, Wiesel TN. (1992) Receptive field dynamics in adult primary visual cortex. Nature 356:150-2.
106. Giulian D, Lachman LB. (1985) Interleukin-1 stimulation of astroglial proliferation after brain injury. Science 228:497-9.
107. Giulian D, Woodward J, Young DG, Krebs JF, Lachman LB. (1988) Interleukin-1 injected into mammalian brain stimulates astrogliosis and neovascularization. J Neurosci 8:2485-90.
108. Golgi C. 1885. Sulla fina anatomia degli organi centrali del sistema nervoso. Riv Sper Fremiat Med Legal Alienazioni Ment. 11:72-123.
109. Goodman CS, Shatz CJ. (1993) Developmental mechanisms that generate precise patterns of neuronal connectivity. Cell 72 Suppl: 77-98.
110. Gordon JA, Stryker MP. (1996) Experience-dependent plasticity of binocular responses in the primary visual cortex of the mouse. J Neurosci16: 3274-86.
111. Goss JR, Finch CE, Morgan DG. (1991) Age-related changes in glial fibrillary acidic protein mRNA in the mouse brain. Neurobiol Aging 12: 165-70.
112. Goss JR, O'Malley ME, Zou L, Styren SD, Kochanek PM, DeKosky ST. (1998) Astrocytes are the major source of nerve growth factor upregulation following traumatic brain injury in the rat. Exp Neurol 149:301-9.
113. Gottlieb M, Matute C. (1999) Expression of nerve growth factor in astrocytes of the hippocampal CA1 area following transient forebrain ischemia. Neuroscience 91:1027-34.
114. Gotz M, Hartfuss E, Malatesta P. (2002) Radial glial cells as neuronal precursors: a new perspective on the correlation of morphology and lineage restriction in the developing cerebral cortex of mice. Brain Res Bull 57:777-88.
115. Griffin WS, Sheng JG, Mrak RE (1986) Senescence-accelerated overexpression of S100beta in brain of SAMP6 mice. Neurobiol Aging 19:71-6.
116. Guizzetti M, Costa LG. (1996) Inhibition of muscarinic receptor-stimulated glial cell proliferation by ethanol. J Neurochem 67:2236-45.

117. Gundersen HJ (1986) Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. J Microsc 143:3-45.
118. Habecker BA, Tietje KM, van Koppen CJ, Creason SA, Goldman PS, Migeon JC, Parenteau LA, Nathanson NM. (1993) Regulation of expression and function of muscarinic receptors. Life Sci 52:429-32.
119. Haga K, Haga T, Ichiyama A. (1990) Phosphorylation by protein kinase C of the muscarinic acetylcholine receptor. J Neurochem 54:1639-44
120. Haga T, Haga K, Kameyama K, Nakata H. (1993) Phosphorylation of muscarinic receptors: regulation by G proteins. Life Sci 52:421-8
121. Hallbook F, Ibanez CF, Persson H. (1991) Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron 6:845-58.
122. Hanover JL, Huang ZJ, Tonegawa S, Stryker MP. (1999) Brain-derived neurotrophic factor overexpression induces precocious critical period in mouse visual cortex. J Neurosci19: RC40.
123. Hatten ME. (1990) Riding the glial monorail: a common mechanism for glial-guided neuronal migration in different regions of the developing mammalian brain. Trends Neurosci13: 179-84.
124. Hatten ME. (2000) Central nervous system neuronal migration. Annu Rev Neurosci 22:511-39.
125. Hawrylak N, Greenough WT. (1995) Monocular deprivation alters the morphology of glial fibrillary acidic protein-immunoreactive astrocytes in the rat visual cortex. Brain Res 683:187-99.
126. Herrmann H, Eckelt A, Brettel M, Grund C, Franke WW. (1993) Temperature-sensitive intermediate filament assembly. Alternative structures of Xenopus laevis vimentin in vitro and in vivo. J Mol Biol 234:99-113.
127. Heumann R. Neurotrophin signalling. (1994) Curr Opin Neurobiol 4:668-79.
128. Hill SJ, Barbarese E, McIntosh TK. (1996) Regional heterogeneity in the response of astrocytes following traumatic brain injury in the adult rat. J Neuropathol Exp Neurol 55:1221-9.
129. Hinkle DA, Baldwin SA, Scheff SW, Wise PM. (1997) GFAP and S100beta expression in the cortex and hippocampus in response to mild cortical contusion. J Neurotrauma 14:729-38.

130. His W. (1889) Die Neuroblasten und deren Entstehung im embryonalen Mark. Abhandl Marh Phys Kl Akad Wis.15: 311-373.
131. Hohmann CF, Potter ED, Levey AI. (1995) Development of muscarinic receptor subtypes in the forebrain of the mouse. J Comp Neurol 358:88-101.
132. Hosli E, Hosli L. (1994) Binding of cholecystokinin, bombesin and muscarine to neurons and astrocytes in explant cultures of rat central nervous system: autoradiographic and immunohistochemical studies. Neuroscience 61:63-72.
133. Hosli E, Ledergerber M, Kofler A, Hosli L. (1997) Evidence for the existence of galanin receptors on cultured astrocytes of rat CNS: colocalization with cholinergic receptors. J Chem Neuroanat 13:95-103.
134. Hosli E, Ruhl W, Hosli L. (2000) Histochemical and electrophysiological evidence for estrogen receptors on cultured astrocytes: colocalization with cholinergic receptors. Int J Dev Neurosci 18:101-11.
135. Hosli L, Hosli E, Della Briotta G, Quadri L, Heuss L. (1988) Action of acetylcholine, muscarine, nicotine and antagonists on the membrane potential of astrocytes in cultured rat brainstem and spinal cord. Neurosci Lett 92:165-70.
136. Hosli L, Hosli E, Kaser H. (1993) Colocalization of cholinergic, adrenergic and peptidergic receptors on astrocytes. Neuroreport Jun 4:679-82.
137. Houser CR, Crawford GD, Barber RP, Salvaterra PM, Vaughn JE. (1983) Organization and morphological characteristics of cholinergic neurons: an immunocytochemical study with a monoclonal antibody to choline acetyltransferase. Brain Res 266:97-119.
138. Howard C.V. & Reed M.G (1998) Unbiased stereology. Springer-Verlag, New York NY
139. Hubel DH, Wiesel TN. (1973) A re-examination of stereoscopic mechanisms in area 17 of the cat. *J Physiol*, 232:29P-30P.
140. Hutton LA, deVellis J, Perez-Polo JR. (1992) Expression of p75NGFR TrkA, and TrkB mRNA in rat C6 glioma and type I astrocyte cultures. J Neurosci Res 32:375-83.
141. Hutton LA, Perez-Polo JR. (1995) In vitro glial responses to nerve growth factor. J Neurosci Res 41:185-96.
142. Inagaki M, Gonda Y, Nishizawa K, Kitamura S, Sato C, Ando S, Tanabe K, Kikuchi K, Tsuiki S, Nishi Y. (1990) Phosphorylation sites linked to glial

filament disassembly in vitro locate in a non-alpha-helical head domain. J Biol Chem 265:4722-9.

143. Inagaki M, Nishi Y, Nishizawa K, Matsuyama M, Sato C. (1987) Site-specific phosphorylation induces disassembly of vimentin filaments in vitro. Nature 328:649-52.
144. Inagaki M, Takahara H, Nishi Y, Sugawara K, Sato C. (1989) Ca2+-dependent deimination-induced disassembly of intermediate filaments involves specific modification of the amino-terminal head domain. J Biol Chem 264:18119-27.
145. Jacobson M. (1991) Developmental neurobiology, 3rd edition. New York: Plenum Press.
146. Jeftinija SD, Jeftinija KV, Stefanovic G. (1997) Cultured astrocytes express proteins involved in vesicular glutamate release. Brain Res 750:41-7
147. Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao M. (1986 Expression and structure of the human NGF receptor. Cell 47:545-54.
148. Jones KR, Reichardt LF. (1990) Molecular cloning of a human gene that is a member of the nerve growth factor family. Proc Natl Acad Sci U S A 87:8060-4.
149. Jones TA, Hawrylak N, Greenough WT. (1996) Rapid laminar-dependent changes in GFAP immunoreactive astrocytes in the visual cortex of rats reared in a complex environment. *Psychoneuroendocrinology*, 21:189-201.
150. Jope RS, Johnson GV. (1986) Quinacrine and 2-(4-phenylpiperidino)cyclohexanol (AH5183) inhibit acetylcholine release and synthesis in rat brain slices. Mol Pharmacol 29:45-51.
151. Juric DM, Carman-Krzan M. (2001) Interleukin-1 beta, but not IL-1 alpha, mediates nerve growth factor secretion from rat astrocytes via type I IL-1 receptor. Int J Dev Neurosci 19:675-83.
152. Kadhim HJ, Gadisseux JF, Evrard P. (1988) Topographical and cytological evolution of the glial phase during prenatal development of the human brain: histochemical and electron microscopic study. J Neuropathol Exp Neurol 47:166-88.
153. Kahle P, Hertel C. (1992) Nerve growth factor (NGF) receptor on rat glial cell lines. Evidence for NGF internalization via p75NGFR. J Biol Chem 267:13917-23.

154. Kaisho Y, Yoshimura K, Nakahama K. (1990) Cloning and expression of a cDNA encoding a novel human neurotrophic factor. FEBS Lett 266:187-91.
155. Kalman M, Ajtai BM (2001) A comparison of intermediate filament markers for presumptive astroglia in the developing rat cortex: immunostaining against nestin reveals more detail, than GFAP or vimentin. Int Dev Neurosci 19, 101-8.
156. Kang J, Jiang L, Goldman SA, Nedergaard M. (1998). Astrocyte-mediated potentiation of inhibitory synaptic transmission. *Nat. Neurosci.* 1:683–92
157. Kaplan DR (a), Hempstead BL, Martin-Zanca D, Chao MV, Parada LF. (1991) The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 252:554-8.
158. Kaplan DR (b), Martin-Zanca D, Parada LF. (1991) Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. Nature 350:158-60.
159. Kettenmann H, Ransom BR. (1995) Neuroglia. New York Oxford: Oxford University Press.
160. Klein R (a), Conway D, Parada LF, Barbacid M. (1990) The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. Cell 61:647-56.
161. Klein R (b), Martin-Zanca D, Barbacid M, Parada LF. (1990) Expression of the tyrosine kinase receptor gene trkB is confined to the murine embryonic and adult nervous system. Development 109:845-50.
162. Klein R, Jing SQ, Nanduri V, O'Rourke E, Barbacid M. (1991) The trk proto-oncogene encodes a receptor for nerve growth factor. Cell 65:189-97.
163. Klepper S, Naftolin F, Piepmeier JM. (1995) Verapamil treatment attenuates immunoreactive GFAP at cerebral cortical lesion site. Brain Res 695:245-9.
164. Kobayashi H, Watanabe T, Yasufuku T, Suzuki T, Saitoh S, Takeno K (1997) Effects of systemic administration of 2-(4-phenyl-piperidino)-cyclohexanol (vesamicol) and an organophosphate DDVP on the cholinergic system in brain regions of rats. Brain Res Bull 43:17-23.
165. Kobe B, Deisenhofer J. (1994) The leucine-rich repeat: a versatile binding motif. Trends Biochem Sci 19:415-21.
166. Koczyk D, Oderfeld-Nowak B. (2000) Long-term microglial and astroglial activation in the hippocampus of trimethyltin-intoxicated rat: stimulation of

NGF and TrkA immunoreactivities in astroglia but not in microglia. Int J Dev Neurosci 18:591-606.

167. Koczyk D, Skup M, Zaremba M, Oderfeld-Nowak B. (1996) Trimethyltin-induced plastic neuronal changes in rat hippocampus are accompanied by astrocytic trophic activity. Acta Neurobiol Exp (Warsz) 56:237-41.
168. Kohama SG, Goss JR, Finch CE, McNeill TH. (1995) Increases of glial fibrillary acidic protein in the aging female mouse brain. Neurobiol Aging 16:59-67.
169. Kondou H, Inagaki N, Fukui H. (1994) Formation of inositol phosphates mediated by M3 muscarinic receptors in type-1 and type-2 astrocytes from neonatal rat cerebral cortex. Neurosci Lett 180:131-4.
170. Korr H, Schultze B, Maurer W. (1973) Autoradiographic investigations of glial proliferation in the brain of adult mice. I. The DNA synthesis phase of neuroglia and endothelial cells. J Comp Neurol 150:169-75.
171. Korr H, Schultze B, Maurer W. (1975) Autoradiographic investigations of glial proliferation in the brain of adult mice. II. Cycle time and mode of proliferation of neuroglia and endothelial cells. J Comp Neurol 160:477-90.
172. Kossmann T, Hans V, Imhof HG, Trentz O, Morganti-Kossmann MC. (1996) Interleukin-6 released in human cerebrospinal fluid following traumatic brain injury may trigger nerve growth factor production in astrocytes. Brain Res 713:143-52.
173. Kumanishi T, Usui H, Ichikawa T, Nishiyama A, Katagiri T, Abe S, Yoshida Y, Washiyama K, Kuwano R, Sakimura K, et al. (1992) Human glial fibrillary acidic protein (GFAP): molecular cloning of the complete cDNA sequence and chromosomal localization (chromosome 17) of the GFAP gene. Acta Neuropathol (Berl) 83:569-78.
174. Lamballe F, Klein R, Barbacid M. (1991) trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell 66:967-79.
175. Laming PR, Sykova E, Reichenbach A, Hatton GI, Bauer H (1998) Glial cells: their role in behaviour. Cambridge, UK: Cambridge UP.
176. Landry CF, Ivy GO, Brown IR. (1990) Developmental expression of glial fibrillary acidic protein mRNA in the rat brain analyzed by in situ hybridization. Neurosci Res 25:194-203.

177. Latov N, Nilaver G, Zimmerman EA, Johnson WG, Silverman AJ, Defendini R, Cote L. (1979) Fibrillary astrocytes proliferate in response to brain injury: a study combining immunoperoxidase technique for glial fibrillary acidic protein and radioautography of tritiated thymidine. Dev Biol 72:381-4
178. Lauterborn JC, Isackson PJ, Gall CM. (1994) Cellular localization of NGF and NT-3 mRNAs in postnatal rat forebrain. Mol Cell Neurosci 5:46-62.
179. Leibrock J, Lottspeich F, Hohn A, Hofer M, Hengerer B, Masiakowski P, Thoenen H, Barde YA. (1989) Molecular cloning and expression of brain-derived neurotrophic factor. Nature 341:149-52.
180. Levey AI, Hallanger AE, Wainer BH. (1987) Choline acetyltransferase immunoreactivity in the rat thalamus. J Comp Neurol 257:317-32.
181. Levey AI, Kitt CA, Simonds WF, Price DL, Brann MR. (1991) Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. J Neurosci 11:3218-26.
182. Levey AI. (1996) Muscarinic acetylcholine receptor expression in memory circuits: implications for treatment of Alzheimer disease. Proc Natl Acad Sci U S A 93:13541-6.
183. Levi-Montalcini and Booker (1960)
184. Levi-Montalcini R (1952) Effects of mouse tumor transplantation on the nervous system. Ann NY Acad Sci USA 55:330 –343.
185. Levi-Montalcini R. The nerve growth factor 35 years later. (1987) Science 237:1154-62.
186. Levitt P, Rakic P. (1980) Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain. J Comp Neurol 193:815-40.
187. Lewin GR, Mendell LM. (1993) Nerve growth factor and nociception. Trends Neurosci 16:353-9.
188. Lewin GR, Mendell LM. (1994) Regulation of cutaneous C-fiber heat nociceptors by nerve growth factor in the developing rat. J Neurophysiol 71:941-9.
189. Lewin GR, Rueff A, Mendell LM. (1994) Peripheral and central mechanisms of NGF-induced hyperalgesia. Eur J Neurosci 6:1903-12.
190. Lieberman AP, Pitha PM, Shin HS, Shin ML. (1989) Production of tumor necrosis factor and other cytokines by astrocytes stimulated with

lipopolysaccharide or a neurotropic virus. Proc Natl Acad Sci U S A 86:6348-52.

191. Liu T, McDonnell PC, Young PR, White RF, Siren AL, Hallenbeck JM, Barone FC, Feurestein GZ. (1993) Interleukin-1 beta mRNA expression in ischemic rat cortex. Stroke 24:1746-50.
192. Lugaro E. (1907) Sulle funzioni della nevroglia.. Riv. Patol. Nerv. Ment., 12:225-233.
193. Lund RD, Mustari MJ. (1977) Development of the geniculocortical pathway in rats. J Comp Neurol 173:289-306.
194. Malatesta P, Hartfuss E, Gotz M. (2000) Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. Development 127:5253-63.
195. Manuelidis L, Tesin DM, Sklaviadis T, Manuelidis EE. (1987) Astrocyte gene expression in Creutzfeldt-Jakob disease. Proc Natl Acad Sci U S A 84:5937-41.
196. Marien MR, Richard JW, Allaire C, Altar CA (1991) Suppression of in vivo neostriatal acetylcholine release by vesamicol: evidence for a functional role of vesamicol receptors in brain. J Neurochem 57:1878-83.
197. Marin-Padilla M. (1995) Prenatal development of fibrous (white matter), protoplasmic (gray matter), and layer I astrocytes in the human cerebral cortex: a Golgi study. J Comp Neurol 357:554-72.
198. Mathewson AJ, Berry M. (1985) Observations on the astrocyte response to a cerebral stab wound in adult rats. Brain Res 327:61-9.
199. Matsuoka Y, Nishizawa K, Yano T, Shibata M, Ando S, Takahashi T, Inagaki M. (1992) Two different protein kinases act on a different time schedule as glial filament kinases during mitosis. EMBO J 11:2895-902.
200. McCall MA, Gregg RG, Behringer RR, Brenner M, Delaney CL, Galbreath EJ, Zhang CL, Pearce RA, Chiu SY, Messing A. (1996) Targeted deletion in astrocyte intermediate filament (Gfap) alters neuronal physiology. Proc Natl Acad Sci U S A. 25:6361-6.
201. McCarthy JB, Barker-Gibb AL, Alves SE, Milner TA. (2002) TrkA immunoreactive astrocytes in dendritic fields of the hippocampal formation across estrous. Glia 38:36-44.

202. McGehee DS, Role LW. (1995) Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annu Rev Physiol 57:521-46.

203. McMillian MK, Thai L, Hong JS, O'Callaghan JP, Pennypacker KR. (1994) Brain injury in a dish: a model for reactive gliosis. Trends Neurosci 17:138-42.

204. Messamore E, Bogdanovich N, Schroder H, Winblad B. (1994) Astrocytes associated with senile plaques possess muscarinic acetylcholine receptors. Neuroreport 5:1473-6.

205. Middlemas DS, Lindberg RA, Hunter T. (1991) trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. Mol Cell Biol 11:143-53.

206. Minami M, Kuraishi Y, Satoh M. (1991) Effects of kainic acid on messenger RNA levels of IL-1 beta, IL-6, TNF alpha and LIF in the rat brain. Biochem Biophys Res Commun 176:593-8.

207. Misson JP, Austin CP, Takahashi T, Cepko CL, Caviness VS Jr. (1991) The alignment of migrating neural cells in relation to the murine neopallial radial glial fiber system. Cereb Cortex 1:221-9.

208. Misson JP, Edwards MA, Yamamoto M, Caviness VS Jr. (1988) Identification of radial glial cells within the developing murine central nervous system: studies based upon a new immunohistochemical marker. Brain Res Dev Brain Res 44:95-108

209. Molnar M, Tongiorgi E, Avignone E, Gonfloni S, Ruberti F, Domenici L, Cattaneo A (1998) Eur J Neurosci 10:3127-40.

210. Monzon-Mayor M, Alvarez M, Arbelo-Galvan J, Romero-Aleman M, Yanes C, Plaza ML, Rodriguez JR, Rodriguez JJ, Toledano A. (2000) Long-term evolution of local, proximal and remote astrocyte responses after diverse nucleus basalis lesioning (an experimental Alzheimer model): GFAP immunocytochemical study. Brain Res 865:245-58.

211. Mossakowski MJ, Weinrauder H (1986) Glial fibrillary acidic protein and S100 protein in abnormal astrocytes in Wilson's disease. Neuropatol Pol 24:365-76.

212. Mower GD, Berry D, Burchfiel JL, Duffy FH. (1981) Comparison of the effects of dark rearing and binocular suture on development and plasticity of cat visual cortex. Brain Res14: 255-67.

213. Mrzljak L, Levey AI, Goldman-Rakic PS. (1993) Association of m1 and m2 muscarinic receptor proteins with asymmetric synapses in the primate cerebral cortex: morphological evidence for cholinergic modulation of excitatory neurotransmission. Proc Natl Acad Sci U S A 90:5194-8.

214. Muller CM. (1990) Dark-rearing retards the maturation of astrocytes in restricted layers of cat visual cortex. Glia 3:487-94.

215. Muller CM. Astrocytes in cat visual cortex studied by GFAP and S-100 immunocytochemistry during postnatal development. (1992) J Comp Neurol 317:309-23.

216. Nakamura Y, Takeda M, Aimoto S, Hojo H, Takao T, Shimonishi Y, Hariguchi S, Nishimura T. (1992) Assembly regulatory domain of glial fibrillary acidic protein. A single phosphorylation diminishes its assembly-accelerating property. J Biol Chem 267:23269-74.

217. Nakamura Y, Takeda M, Angelides KJ, Tada K, Hariguchi S, Nishimura T. (1991) Assembly, disassembly, and exchange of glial fibrillary acidic protein. Glia 4:101-10.

218. Nase G, Weishaupt J, Stern P, Singer W, Monyer H. (1999) Genetic and epigenetic regulation of NMDA receptor expression in the rat visual cortex. Eur J Neurosci 11:4320-6.

219. Nawashiro H, Brenner M, Fukui S, Shima K, Hallenbeck JM. (2000) High susceptibility to cerebral ischemia in GFAP-null mice. J Cereb Blood Flow Metab 20:1040-4.

220. Nishio T, Furukawa S, Akiguchi I, Oka N, Ohnishi K, Tomimoto H, Nakamura S, Kimura J. (1994) Cellular localization of nerve growth factor-like immunoreactivity in adult rat brain: quantitative and immunohistochemical study. Neuroscience 60:67-84.

221. Niu H, Hinkle DA, Wise PM. (1997) Dexamethasone regulates basic fibroblast growth factor, nerve growth factor and S100beta expression in cultured hippocampal astrocytes. Brain Res Mol Brain Res 51:97-105.

222. Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR. (2001) Neurons derived from radial glial cells establish radial units in neocortex. Nature 409:714-20.

223. Obata S, Obata J, Das A, Gilbert CD. (1999) Molecular correlates of topographic reorganization in primary visual cortex following retinal lesions. Cereb Cortex 9:238-48.

224. O'Callaghan JP, Miller DB. (1991) The concentration of glial fibrillary acidic protein increases with age in the mouse and rat brain. Neurobiol Aging 12:171-4.

225. Oderfeld-Nowak B, Zaremba M, Micera A, Aloe L. (2001) The upregulation of nerve growth factor receptors in reactive astrocytes of rat spinal cord during experimental autoimmune encephalomyelitis. Neurosci Lett 308:165-8.

226. Oh TH, Markelonis GJ, Von Visger JR, Baik B, Shipley MT. (1995) Acidic pH rapidly increases immunoreactivity of glial fibrillary acidic protein in cultured astrocytes. Glia 13:319-22.

227. Ohta M, Mizuta I, Ohta K, Nishimura M, Mizuta E, Hayashi K, Kuno S. (2000) Apomorphine up-regulates NGF and GDNF synthesis in cultured mouse astrocytes. Biochem Biophys Res Commun 272:18-22.

228. Panter SS, McSwigan JD, Sheppard JR, Emory CR, Frey WH 2nd (1985) Glial fibrillary acidic protein and Alzheimer's disease. Neurochem Res 10:1567-

229. Patapoutian A, Reichardt LF. (2001) Trk receptors: mediators of neurotrophin action. Curr Opin Neurobiol 11:272-80.

230. Paxinos J &Franklin KBJ. (1997) The Mouse Brain in Stereotaxic Coordinates. Academic Press, London.

231. Pechan PA, Chowdhury K, Seifert W. (1992) Free radicals induce gene expression of NGF and bFGF in rat astrocyte culture. Neuroreport 3:469-72.

232. Pekny M, Leveen P, Pekna M, Eliasson C, Berthold CH, Westermark B, Betsholtz C. (1995) Mice lacking glial fibrillary acidic protein display astrocytes devoid of intermediate filaments but develop and reproduce normally. EMBO J 14:1590-8.

233. Pekny M, Stanness KA, Eliasson C, Betsholtz C, Janigro D. (1998) Impaired induction of blood-brain barrier properties in aortic endothelial cells by astrocytes from GFAP-deficient mice. Glia 22:390-400.

234. Pesavento E, Capsoni S, Domenici L, Cattaneo A (2002) Acute cholinergic rescue of synaptic plasticity in the neurodegenerating cortex of anti-nerve-growth-factor mice. Eur J Neurosci 15:1030-6.

235. Pesavento E, Margotti E, Righi M, Cattaneo A, Domenici L. (2000) Blocking the NGF-TrkA interaction rescues the developmental loss of LTP in the rat visual cortex: role of the cholinergic system. Neuron 25:165-75.

236. Peters A, Palay SL, Webster H (1991) The fine structure of the nervous system: the neurons and supporting cells. Philadelphia: W. B. Saunders.

237. Piccioli P, Di Luzio A, Amann R, Schuligoi R, Surani MA, Donnerer J, Cattaneo A. (1995) Neuroantibodies: ectopic expression of a recombinant anti-substance P antibody in the central nervous system of transgenic mice. Neuron 15:373-84.

238. Pixley SK, de Vellis J. (1984) Transition between immature radial glia and mature astrocytes studied with a monoclonal antibody to vimentin. Brain Res 317:201-9.

239. Poltorak M, Herranz AS, Williams J, Lauretti L, Freed WJ. (1993) Effects of frontal cortical lesions on mouse striatum: reorganization of cell recognition molecule, glial fiber, and synaptic protein expression in the dorsomedial striatum. J Neurosci 13:2217-29.

240. Porter JT, McCarthy KD. (1996) Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. *J. Neurosci.* 16: 5073–81

241. Prior C, Marshall IG, Parsons SM. (1992) The pharmacology of vesamicol: an inhibitor of the vesicular acetylcholine transporter. Gen Pharmacol 23:1017-22.

242. Quinlan EM, Olstein DH, Bear MF. (1999) Bidirectional, experience-dependent regulation of N-methyl-D-aspartate receptor subunit composition in the rat visual cortex during postnatal development. Proc Natl Acad Sci U S A 96:12876-80.

243. Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM. (1987) Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325:593-7.

244. Rakic P. (1981) neuronal-glial interaction during brain development. Trends Neurosci 4:184-187.

245. Rataboul P, Vernier P, Biguet NF, Mallet J, Poulat P, Privat A. (1989) Modulation of GFAP mRNA levels following toxic lesions in the basal ganglia of the rat. Brain Res Bull 22:155-61.

246. Reichenbach A, Siegel A, Rickmann M, Wolff JR, Noone D, Robinson SR. (1995) Distribution of Bergmann glial somata and processes: implications for

function. Paul Flechsig Institute for Brain Research, Leipzig University, F.R.G. J Hirnforsch 36:509-17

247. Ridet JL, Malhotra SK, Privat A, Gage FH. (1997) Reactive astrocytes: cellular and molecular cues to biological function. Trends Neurosci 20:570-7.

248. Rogers GA, Parsons SM (1993) Photoaffinity labeling of the vesamicol receptor of cholinergic synaptic vesicles. Biochemistry 32:8596-601.

249. Rogers GA, Parsons SM, Anderson DC, Nilsson LM, Bahr BA, Kornreich WD, Kaufman R, Jacobs RS, Kirtman B (1989) Synthesis, in vitro acetylcholine-storage-blocking activities, and biological properties of derivatives and analogues of trans-2-(4-phenylpiperidino)cyclohexanol (vesamicol) J Med Chem 32:1217-30.

250. Rossner S, Schliebs R, Hartig W, Perez-Polo JR, Bigl V. (1997) Selective induction of c-Jun and NGF in reactive astrocytes after cholinergic degenerations in rat basal forebrain. Neuroreport 8:2199-202.

251. Rouach N, Glowinski J, Giaume C. (2000) Activity-dependent neuronal control of gap-junctional communication. J Cell Biol 149:1513-26

252. Ruberti F, Bradbury A, Cattaneo A. (1993) Cloning and expression of an anti-nerve growth factor (NGF) antibody for studies using the neuroantibody approach. Cell Mol Neurobiol 13:559-68.

253. Ruberti F, Capsoni S, Comparini A, Di Daniel E, Franzot J, Gonfloni S, Rossi G, Berardi N, Cattaneo A (2000) Phenotypic knockout of nerve growth factor in adult transgenic mice reveals severe deficits in basal forebrain cholinergic neurons, cell death in the spleen, and skeletal muscle dystrophy. J Neurosci 20:2589-601.

254. Rudge JS, Silver J. (1990) Inhibition of neurite outgrowth on astroglial scars in vitro. J Neurosci 10:3594-603.

255. Sancho-Tello M, Valles S, Montoliu C, Renau-Piqueras J, Guerri C. (1995). Developmental pattern of GFAP and vimentin gene expression in rat brain and in radial glial cultures. Glia 15:157-66.

256. Sargent PB. (1993) The diversity of neuronal nicotinic acetylcholine receptors. Annu Rev Neurosci 16:403-43.

257. Schliebs R, Rossner S, Kumar A, Bigl V. (1994) Muscarinic acetylcholine receptor subtypes in rat visual cortex--a comparative study using quantitative receptor autoradiography and in situ hybridization. Indian J Exp Biol 32:25-30.

258. Schuldiner S, Shirvan A, Linial M (1995) Vesicular neurotransmitter transporters: from bacteria to humans. Physiol Rev 75:369-92.

259. Schwartz JP, Nishiyama N. (1994) Neurotrophic factor gene expression in astrocytes during development and following injury. Brain Res Bull 35:403-7.

260. Searl T, Prior C, Marshall IG. (1990) The effects of L-vesamicol, an inhibitor of vesicular acetylcholine uptake, on two populations of miniature endplate currents at the snake neuromuscular junction. Neuroscience 35:145-56.

261. Seguela P, Wadiche J, Dineley-Miller K, Dani JA, Patrick JW. (1993) Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. J Neurosci 13:596-604.

262. Selmaj K, Shafit-Zagardo B, Aquino DA, Farooq M, Raine CS, Norton WT, Brosnan CF. (1991) Tumor necrosis factor-induced proliferation of astrocytes from mature brain is associated with down-regulation of glial fibrillary acidic protein mRNA. J Neurochem 57:823-30.

263. Selmaj KW, Farooq M, Norton WT, Raine CS, Brosnan CF. (1990) Proliferation of astrocytes in vitro in response to cytokines. A primary role for tumor necrosis factor. J Immunol 144:129-35.

264. Semba K, Fibiger HC. (1988) Time of origin of cholinergic neurons in the rat basal forebrain. J Comp Neurol 269:87-95.

265. Shelton MK, McCarthy KD. (2000) Hippocampal astrocytes exhibit Ca2+-elevating muscarinic cholinergic and histaminergic receptors in situ. J Neurochem 74:555-63.

266. Sheng JG, Mrak RE, Bales KR, Cordell B, Paul SM, Jones RA, Woodward S, Zhou XQ, McGinness JM, Griffin WS (2000) Overexpression of the neuritotrophic cytokine S100beta precedes the appearance of neuritic beta-amyloid plaques in APPV717F mice. Neurochem 74:295-301.

267. Sheng JG, Mrak RE, Rovnaghi CR, Kozlowska E, Van Eldik LJ, Griffin WS (1996) Human brain S100 beta and S100 beta mRNA expression increases with age: pathogenic implications for Alzheimer's disease. Neurobiol Aging 17:359-63.

268. Shibata T, Yamada K, Watanabe M, Ikenaka K, Wada K, Tanaka K, Inoue Y. (1997) Glutamate transporter GLAST is expressed in the radial glia-astrocyte lineage of developing mouse spinal cord. J Neurosci 17:9212-9.

269. Shibuki K, Gomi H, Chen L, Bao S, Kim JJ, et al. (1996). Deficient cerebellar longterm depression, impaired eyeblink conditioning, and normal motor coordination in GFAP mutant mice. Neuron 16:587–99

270. Shoeman RL, Traub P. (1993) Assembly of intermediate filaments. Bioessays 15:605-11.

271. Sirevaag AM, Greenough WT. (1991) Plasticity of GFAP-immunoreactive astrocyte size and number in visual cortex of rats reared in complex environments. Brain Res 540:273-8.

272. Sloane JA, Hollander W, Rosene DL, Moss MB, Kemper T, Abraham CR. (2000) Astrocytic hypertrophy and altered GFAP degradation with age in subcortical white matter of the rhesus monkey. Brain Res 862:1-10.

273. Smith ME, Somera FP, Eng LF. (1983) Immunocytochemical staining for glial fibrillary acidic protein and the metabolism of cytoskeletal proteins in experimental allergic encephalomyelitis. Brain Res 264:241-53.

274. Soellner P, Quinlan RA, Franke WW. (1985) Identification of a distinct soluble subunit of an intermediate filament protein: tetrameric vimentin from living cells. Proc Natl Acad Sci U S A 82:7929-33.

275. Spillmann AA, Bandtlow CE, Lottspeich F, Keller F, Schwab ME. (1998) Identification and characterization of a bovine neurite growth inhibitor (bNI-220). J Biol Chem 273:19283-93.

276. Squinto SP, Stitt TN, Aldrich TH, Davis S, Bianco SM, Radziejewski C, Glass DJ, Masiakowski P, Furth ME, Valenzuela DM, et al. (1991) trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell 65:885-93.

277. Steward O, Kelley MS, Torre ER. (1993) The process of reinnervation in the dentate gyrus of adult rats: temporal relationship between changes in the levels of glial fibrillary acidic protein (GFAP) and GFAP mRNA in reactive astrocytes. Exp Neurol 124:167-83.

278. Stewart M, Quinlan RA, Moir RD. (1989) Molecular interactions in paracrystals of a fragment corresponding to the alpha-helical coiled-coil rod portion of glial fibrillary acidic protein: evidence for an antiparallel packing of molecules and polymorphism related to intermediate filament structure. J Cell Biol 109:225-34.

279. Stewart MG, Bourne RC, Gabbott PL. (1986) Decreased levels of an astrocytic marker, glial fibrillary acidic protein, in the visual cortex of dark-reared rats: measurement by enzyme-linked immunosorbent assay. Neurosci Lett 63:147-52.

280. Stichel CC, Muller CM, Zilles K. (1991) Distribution of glial fibrillary acidic protein and vimentin immunoreactivity during rat visual cortex development. J Neurocytol 20:97-108.

281. Strauss S, Otten U, Joggerst B, Pluss K, Volk B. (1994) Increased levels of nerve growth factor (NGF) protein and mRNA and reactive gliosis following kainic acid injection into the rat striatum. Neurosci Lett 168:193-6.

282. Sulston JE, Schierenberg E, White JG, Thomson JN. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. Dev. Biol. 100:64–119

283. Takemura M, Gomi H, Colucci-Guyon E, Itohara S. (2002) Protective role of phosphorylation in turnover of glial fibrillary acidic protein in mice. J Neurosci 22:6972-9.

284. Tanaka H, Katoh A, Oguro K, Shimazaki K, Gomi H, Itohara S, Masuzawa T, Kawai N. (2002) Disturbance of hippocampal long-term potentiation after transient ischemia in GFAP deficient mice. J Neurosci Res 67:11-20.

285. Tanaka J, Tada K, Takeda M, Hariguchi S, Nishimura T. (1989). Effect of metallic cations and pH on reassembly of glial fibrillary acidic protein. Neurochem int 15:421:428.

286. Tardy M, Fages C, Riol H, LePrince G, Rataboul P, Charriere-Bertrand C, Nunez J. (1989) Developmental expression of the glial fibrillary acidic protein mRNA in the central nervous system and in cultured astrocytes. J Neurochem 52:162-7

287. Thoenen H. (1995) Neurotrophins and neuronal plasticity. Science 270:593-8.

288. Tigges M, Tigges J, Rees H, Rye D, Levey AI. (1997) Distribution of muscarinic cholinergic receptor proteins m1 to m4 in area 17 of normal and monocularly deprived rhesus monkeys. J Comp Neurol 388:130-45.

289. Timney B, Mitchell DE, Giffin F. (1978) The development of vision in cats after extended periods of dark-rearing. Exp Brain Res 31:547-60.

290. Tongiorgi E, Cattaneo A, Domenici L. (1999) Co-expression of TrkB and the N-methyl-D-aspartate receptor subunits NR1-C1, NR2A and NR2B in the rat visual cortex. Neuroscience. 90:1361-1369.

291. Urfer R, Tsoulfas P, O'Connell L, Hongo JA, Zhao W, Presta LG. (1998) High resolution mapping of the binding site of TrkA for nerve growth factor and TrkC for neurotrophin-3 on the second immunoglobulin-like domain of the Trk receptors. J Biol Chem 273:5829-40.

292. van Der Zee EA, De Jong GI, Strosberg AD, Luiten PG (1993) Muscarinic acetylcholine receptor-expression in astrocytes in the cortex of young and aged rats. Glia 8:42-50.

293. van der Zee EA, Matsuyama T, Strosberg AD, Traber J, Luiten PG (1989) Demonstration of muscarinic acetylcholine receptor-like immunoreactivity in the rat forebrain and upper brainstem. Histochemistry 92:475-85.

294. Ventura R, Harris KM (1999) Three-dimensional relationships between hippocampal synapses and astrocytes. J Neurosci 19:6897-906.

295. Vikstrom KL, Borisy GG, Goldman RD (1989) Dynamic aspects of intermediate filament networks in BHK-21 cells. Proc Natl Acad Sci U S A 86:549-53.

296. Virchow R. (1846) Uber das granulierte Ansehen der Wandungen der Gerhirnventrikel. Allg Z Psychiatr. 3: 242.

297. Voigt T. (1989) Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes. J Comp Neurol 289:74-88.

298. Volknandt W. (2002) Vesicular release mechanisms in astrocytic signalling. Neurochem Int 41:301

299. Wallach D. (1997) Apoptosis. Placing death under control. Nature 388:123, 125-6.

300. Weber K, Geisler N. (1985) Intermediate filaments: structural conservation and divergence. Ann N Y Acad Sci 455:126-43.

301. Wiesel TN, Hubel DH. (1965) Comparison of the effects of unilateral and bilateral eye closure on cortical unit responses in kittens. J Neurophysiol 28:1029-40.

302. Wiesel TN. (1982) Postnatal development of the visual cortex and the influence of environment. Nature 299:583-91.

303. Woodhams PL, Basco E, Hajos F, Csillag A, Balazs R. (1981) Radial glia in the developing mouse cerebral cortex and hippocampus. Anat Embryol (Berl) 163:331-43.

304. Woolf CJ, Safieh-Garabedian B, Ma QP, Crilly P, Winter J. (1994) Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. Neuroscience 62:327-31.

305. Wu VW, Nishiyama N, Schwartz JP. (1998) A culture model of reactive astrocytes: increased nerve growth factor synthesis and reexpression of cytokine responsiveness. J Neurochem 71:749-56.

306. Yamakuni T, Ozawa F, Hishinuma F, Kuwano R, Takahashi Y, Amano T. (1987) Expression of beta-nerve growth factor mRNA in rat glioma cells and astrocytes from rat brain. FEBS Lett 223:117-21.

307. Yokoyama M, Black IB, Dreyfus CF. (1993) NGF increases brain astrocyte number in culture. Exp Neurol 124:377-80.

308. Yong VW, Moumdjian R, Yong FP, Ruijs TC, Freedman MS, Cashman N, Antel JP. (1991) Gamma-interferon promotes proliferation of adult human astrocytes in vitro and reactive gliosis in the adult mouse brain in vivo. Proc Natl Acad Sci U S A 88:7016-20.

309. Zilles K. (1985). The cortex of the rat. Spriner, Berlin.

Acknowledgments

THANKS

First of all thanks to the reader, who, patiently, arrived at the end of this thesis. I hope you appreciated my work and enjoyed your mind.

Thanks to my supervisor Luciano Domenici, for scientific and human support and for collecting my scattered ideas and transforming them into scientific works.

Thanks to Simona, her help has been fundamental in conducting my experiments.

Of course thanks to my family that supported me with love during these years, and supported my choices that pushed and will push me so far from my home.

Thanks to all my colleagues in the Bellavista's lab for the friendly support and for creating a joyful atmosphere, that allowed me to tolerate also the worst periods, and for the small but precious suggestions they gave me every day, and to colleagues of the "pollaio", and especially to Annalisa. Special thanks to Kevin and Daniela for lessons of English.

However, all my work has not been possible without the support of my friends. First of all, a huge thanks is for the ones who has been my "family in Trieste" and overall to Biri and Lorenzo, with whom I shared my first flat in Trieste, then, as tey appared, thanks to Anna and Laura (the lighthouse in the Milonga), to Silvia and Giovanni, to Nonno Mario and to "I Pagnotti" (Fabio, my "pusher of music", and Sara, "compagna di merende") and the small one. Thanks to all my other friends, that in these years have given me the most and have let me know many interesting aspects of the life. In scattered order: Thanks to Guido, Franceschina, LauraMaggi, Marcelo, Barbara, Marta, Corrado, Valerie, Damir, Andrea Delfino-Mammachioccia, Jennifer, Lamatta, Mleone, il Latitanzi, Ras, Mohinish, il Bezzi and il Capriotti and Filippo, Nicola, Padhu and Eugenio and..... many, many others. Probably I missed a lot of people, sorry but that's only for my chronic forgetfulness.

Grazie

Cia'

Gi