ISAS - INTERNATIONAL SCHOOL FOR ADVANCED STUDIES

Use of anti-NGF monoclonal antibodies to study synaptic plasticity in the developing and adult hippocampus.

Thesis submitted for the degree of

"Doctor Philosophiae"

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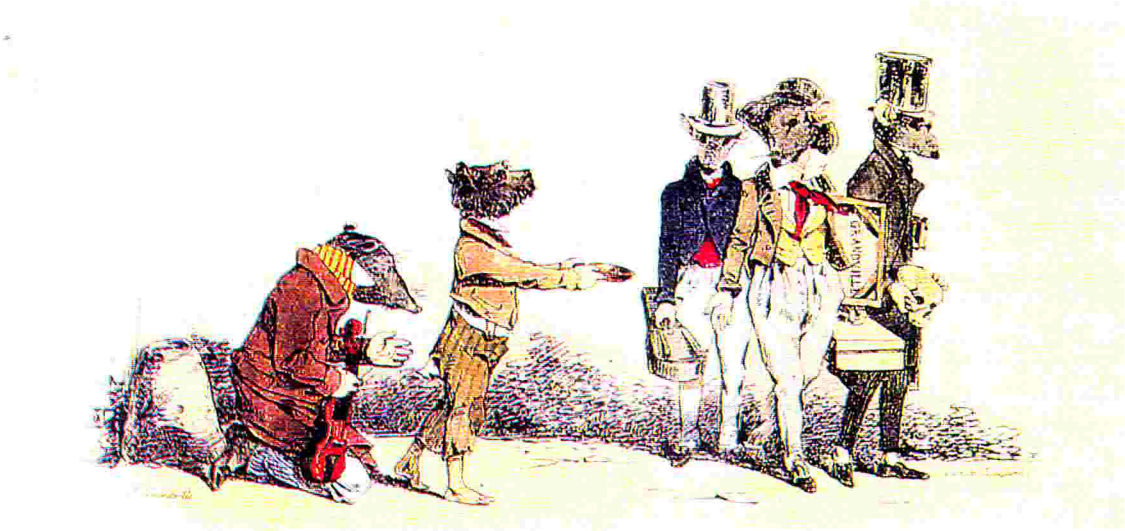

J.J. Grandville, *Les Métamorphoses du jour*.

Declaration

The work described in this dissertation was carried out at the International School of Advanced Studies , Trieste between October 92 and July 1996. All works reported, with the exceptions listed below, arise solely from my own experiments and this work has not been submitted in whole or in part to any other University

Chapter 2: The immunohistochemical experiments were carried out by Margherita Molnar. I thank Luciano Domenici for useful discussions.

Chapter 3: The electrophysiological experiments were carried out with the assistance of Nicola Berretta and the careful supervision of Prof. Enrico Cherubini.

Chapter 4: The implant of hybridoma or myeloma cells in adult Long Evans rats and the behavioural analysis of animals used for electrophysiological studies was performed by RosaMaria Costagliola in the laboratory of Nicoletta Berardi in Pisa. I am indebted to Nicoletta Berardi for the always lively discussions.

Chapter 5: Creation of transgenic mice from purified plasmid DNA supplied by myself was carried out by DNX.
Nociceptive tests on αD11 transgenic mice were carried out by myself in the laboratory of Prof. J. Donnerer , University of Graz.
SCG analysis was carried out with the assistance of Massimo Righi. Some RNase protection experiments were carried out with the assistance of Katia Gamel and Anna Rosati.
Histological analysis of testis was carried out by M. Russo, Universita' Tor Vergata, Roma.

Francesca Ruberti

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Chapter 1
Introduction

1.1 NGF and other Neurotrophins

Nerve Growth Factor (NGF) has an important role in the developing and mature nervous system (Levi Montalcini, 1987). It is now known to form part of the so called neurotrophin (NT) gene family. The neurotrophins are a family of currently five proteins: nerve growth factor (NGF), brain derived growth factor (BDNF) and neurotrophins 3 (NT-3), 4/5 (NT4/NT-5) and 6 (NT-6). These proteins share around 50% sequence identity. Each neurotrophin gene encodes for a mature secretory protein of 110 to 130 amino acids, expressed as a non covalent homodimer which is the biological active moiety. Heterodimers between different neurotrophins can form, both in vitro and in vivo (Radziejewski et al., 1992; Radziejewski and Robinson, 1993; Heymach and Shooter, 1995), but their biological significance, although intriguing, is still an open question.

The three dimensional structure of mouse NGF has been recently determined by X-ray crystallography (McDonald et al., 1991) and has given insights into the function of this protein (**fig.1.1**). The structure shows that NGF has a novel tertiary fold dominated by two pairs of anti-parallel ß-strands that define the elongated shape of the molecule. These ß strand are connected by a number of hairpin loops, three of which are located on the "upper" part of the molecule. Towards the opposite end of the protomer, the three disulphide bridges are clustered in a novel topological arrangement, subsequently found to be common to PDGF and TGFß1 proteins (McDonald and Hendrickson, 1993). Two of the disulphide bridges (Cys58-Cys108 and Cys68-Cys110) and their connecting residues form a ring structure through which the third disulphide bridge (Cys15-Cys80) passes, to form a cysteine knot motif. It is likely that all neurotrophins adopt the general fold shown in fig 1.1,

since the majority of invariant residues found in neurotrophins sequences play a critical structural role in the protomer or dimer.

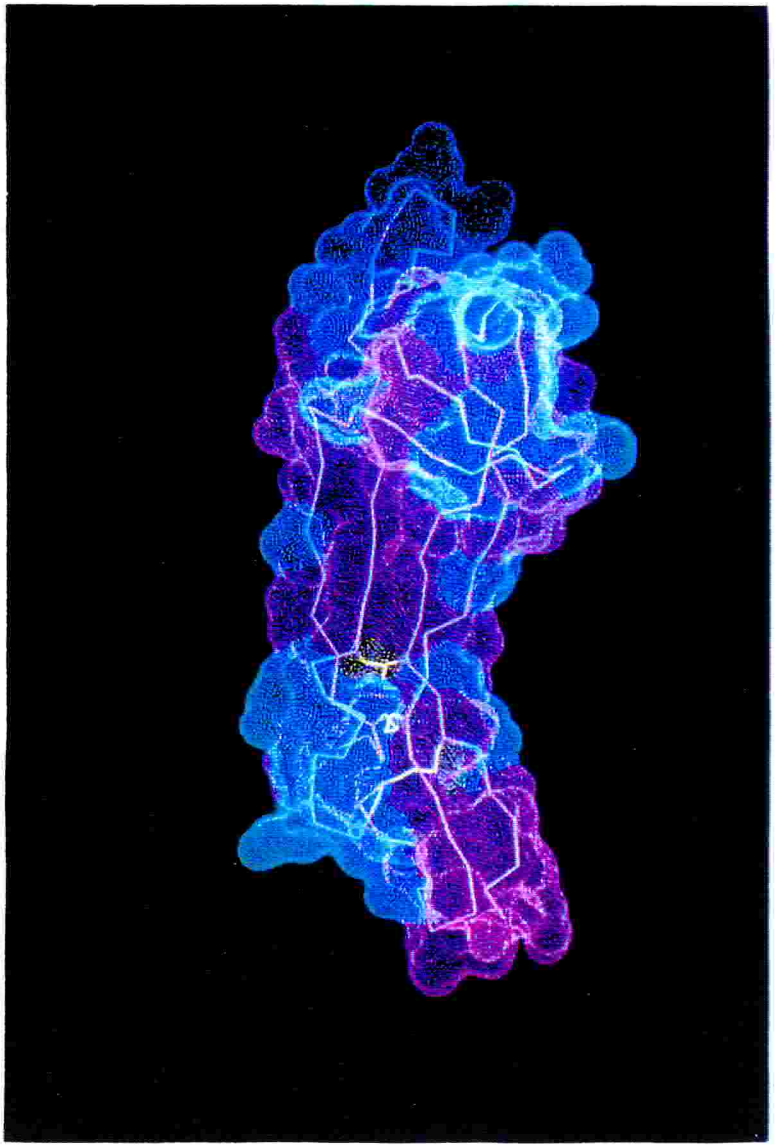


Fig.1.1 Three-dimensional structure of the NGF monomer. The N-terminal region (1-10 residues) is not seen in the crystal structure of NGF molecule, the variable regions (three β-hairpin loop and one reverse turn) are drawn in blue. The two disulphide bonds are shown in yellow.

This is confirmed by the recently determined crystal structure of a BDNF/NT-3 heterodimer (Robinson et al., 1995).

The neurotrophins interact with two classes of receptors: the p75 receptor (Chao, 1992) and the Trk tyrosine kinase receptor (TrkA,B, C) (Barbacid, 1993). Each neurotrophin binds preferentially to a specific form of Trk; BDNF and NT4 are preferred ligands for TrkB, whilst NGF and NT3 bind TrkA and TrkC, respectively. In contrast, p75 is able to interact with each neurotrophin with equal affinity, though with different rate constants (Rodriguez-Tebar et al., 1992) (**fig 1.2**). The Trk receptors trigger different signaling pathways, including Ras, phospholipase C-γ, and phosphoinositol-3-hydroxy-kinase. Inactivation of the Trk tyrosine kinase by inhibitors of tyrosine kinase, by mutagenesis of key tyrosine residues in the cytoplasmic domain or by disruption of the TrkA, TrkB and TrkC tyrosine kinase genes by homologous recombination in mice, results in the inhibition of neurotrophin signal transduction. The p75 receptor (Chao et al., 1986), originally described as the NGF receptor, was the first neurotrophin receptor to be identified at a molecular level, but its relevance in mediating the biological action of neurotrophins is still not yet fully understood, partly because of the lack of a "classical' signal transduction activity associated to it. The p75 receptor can increase the affinity and specificity of NGF binding to TrkA (Davies et al., 1993) and gene targeting studies (Lee et al., 1992) revealed that the absence of p75 affects the neurons that are more sensitive to NGF. Several reports suggest that p75 modulates TrkA tyrosine kinase activities (Verdi et al., 1994) and may also influence activation of TrkB and TrkC receptors. Only recently has it become clear that p75 may trigger cellular responses without participation of Trk receptors. In different transformed cell lines p75 was involved in promoting apoptosis (Rabizadeh et al., 1993) and in signalling through sphingomyelin turnover (Dobrowsky et al.,1994). In Schwann cells, a cellular system which does not express functional Trk receptors, while expressing substantial amounts of p75 receptors, there is evidence that NGF binding to p75 activates the transcription factor nuclear factor kappa B (NF kB) (Carter et al,

1996). In chick embryos it has been shown that endogenous NGF triggers the death of retinal neurons through its p75 receptor (Frade et al, 1996).

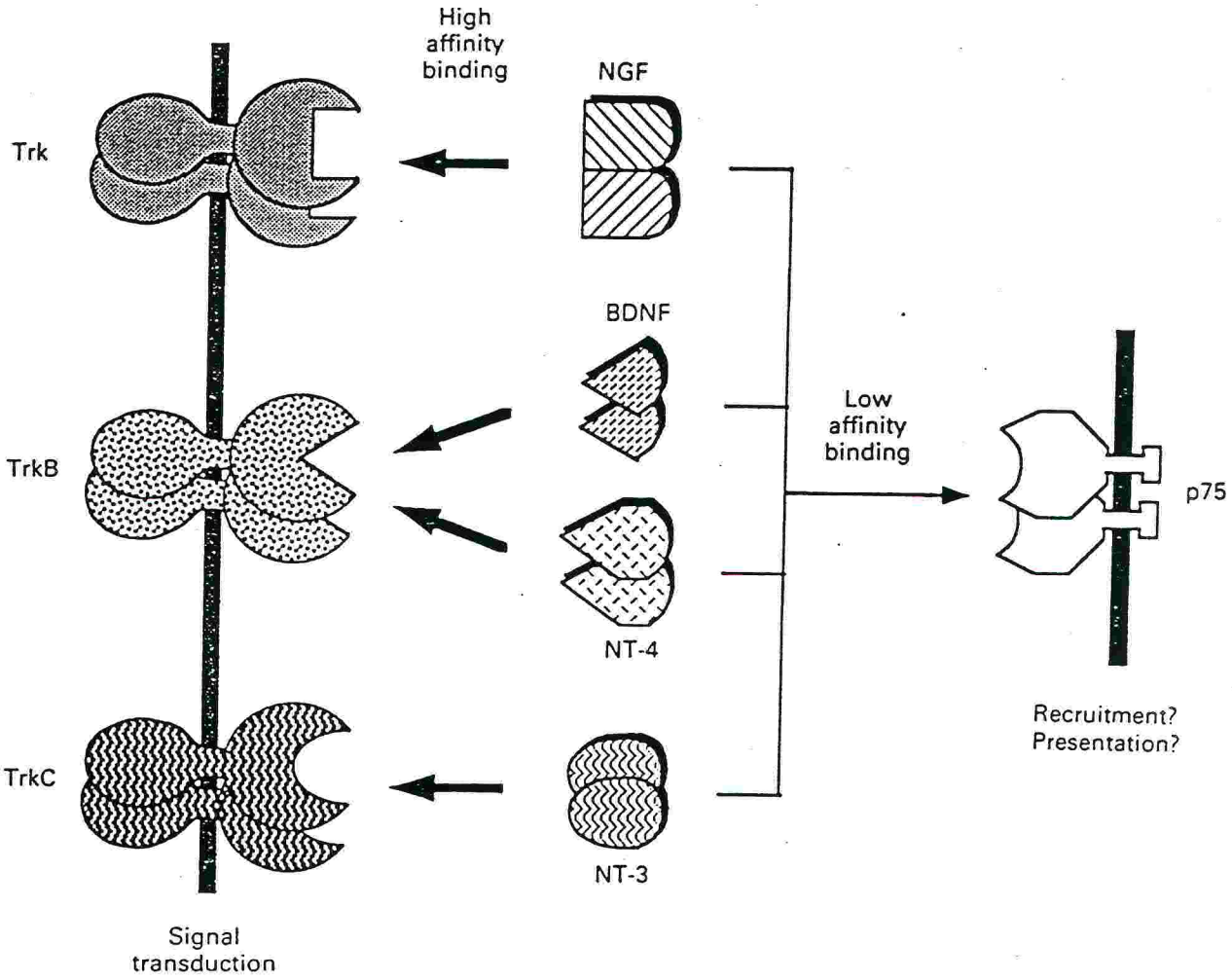


Fig1.2 The individual neurotrophins are assigned to individual Trk receptors, all neurotrophins have the same affinity to p75 receptor (from Barbacid, M., 1993).

The knowledge of the three-dimensional structure of NGF and mutagenesis experiments have been used to identify the role of the constant and variable regions of NGF in the interaction with their receptors (**fig.1.3**). The observation that all neurotrophins can bind to p75 suggested that conserved regions in the neurotrophins may be involved in p75 binding. Paradoxically however, positively charged residues K32 and K34 in the variable region I of NGF are required for the binding of NGF to p75. Mutation of these residues removed the ability to bind the low-affinity receptor, but had a minor effect on either TrkA binding or biological activity (Ibanez et al., 1992), establishing a functional dissociation between the two NGF receptors. However, mutation of K95, which is found on a different loop to K32 and K34, also affects binding to p75 and induces a considerable reduction of binding to TrkA. The interaction of NGF with TrkA (Ibanez, 1995) involves residues 3-9 at the NH2 terminus, I31 from region I, E41 and N45 from region II, Y79 and H84 from region IV and residues 94-98 from region V. Replacement experiments, in which all variable regions of NGF, but not the NH2 terminal, have been substituted by other neurotrophins, result in a chimeric molecule that still retains substantial binding to TrkA (**fig.1.4**).

Fig.1.3 Variable regions of the neurotrophins. (**A**) Alignment of the amino acid sequences (single-letter code) of rat nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). Variable regions are boxed and labelled. Note that, because of differences in the lengths of the N-termini of the different neurotrophins, homologous positions in different molecules do not have equivalent numbering. Dots indicate residues identical to NGF, and dashes represent gaps introduced for the sake of alignment.

(**B**) Schematic representation of three-dimensional structure of the NGF monomer. Amino acid residues in the different variable regions are indicated by their one-letter code. Variable residues within these domains are shaded. Thick lines represent disulphide bridges (from Ibanez, 1995)

A

	NH_2			I			II			
	1 9			23 35			40 49			
NGF	--SSTHPVFHM	GEFSVCDSVSVWV		G--DKTTATDIKGKE	VIVL		GEVNI-NNSVF	KQYFFETKC		
BDNF	----HSDPARR	..L.....I.E..		TAA..K..V.MS.GT	....		EK.PV-SKGQL	....Y....		
NT-3	---YAEHKS.R	..Y.....E.L..		T--..SS.I..R.HQ	....		..IKT-G..PV	....Y..R.		
NT-4	GV.E.A.ASRR	..LA...A..G..		T--.RR..V.LR.R.	.E..		...PAAGG.PL	R.....R.		

	III		IV		V		COOH
	59 66		79 88		94 98		111
NGF	RAPNPVES-------	GCRGIDSKHWSN	YCTTTHTFVK	ALTTD	DKQ-AA	WRFIRIDTACVC	VLSRKAARRG
BDNF	NPMGYTKE-------	.....KR....	Q.R..QSY.R	...M.	S.KRIG	........S...	T.TI.RG.--
NT-3	KEAR..KN-------	.....D...NS	Q.K.SQ.Y.R	...SE	NNKLVG	..W.....S...	A....IG.T-
NT-4	K.D.AE.GGPGAGGG	....V.RR..VS	E.KAKQSY.R	...A.	AQGRVG	..W.....	T.LSRTG.A-

B

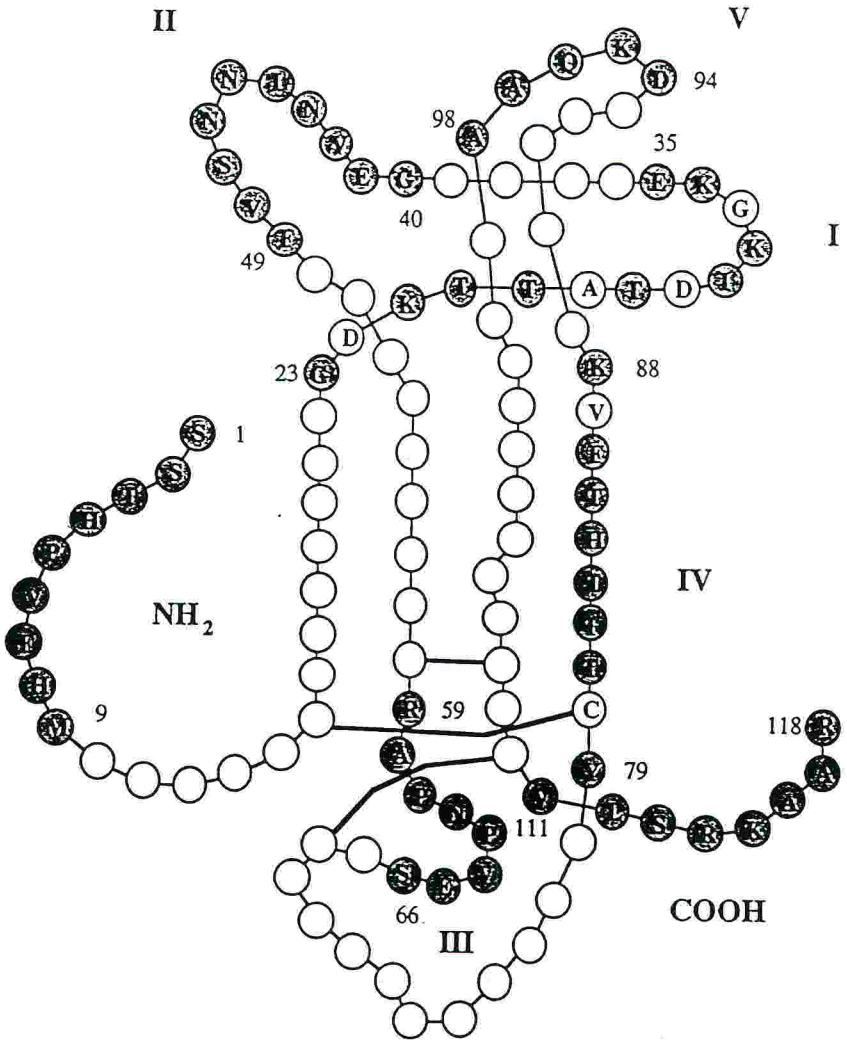
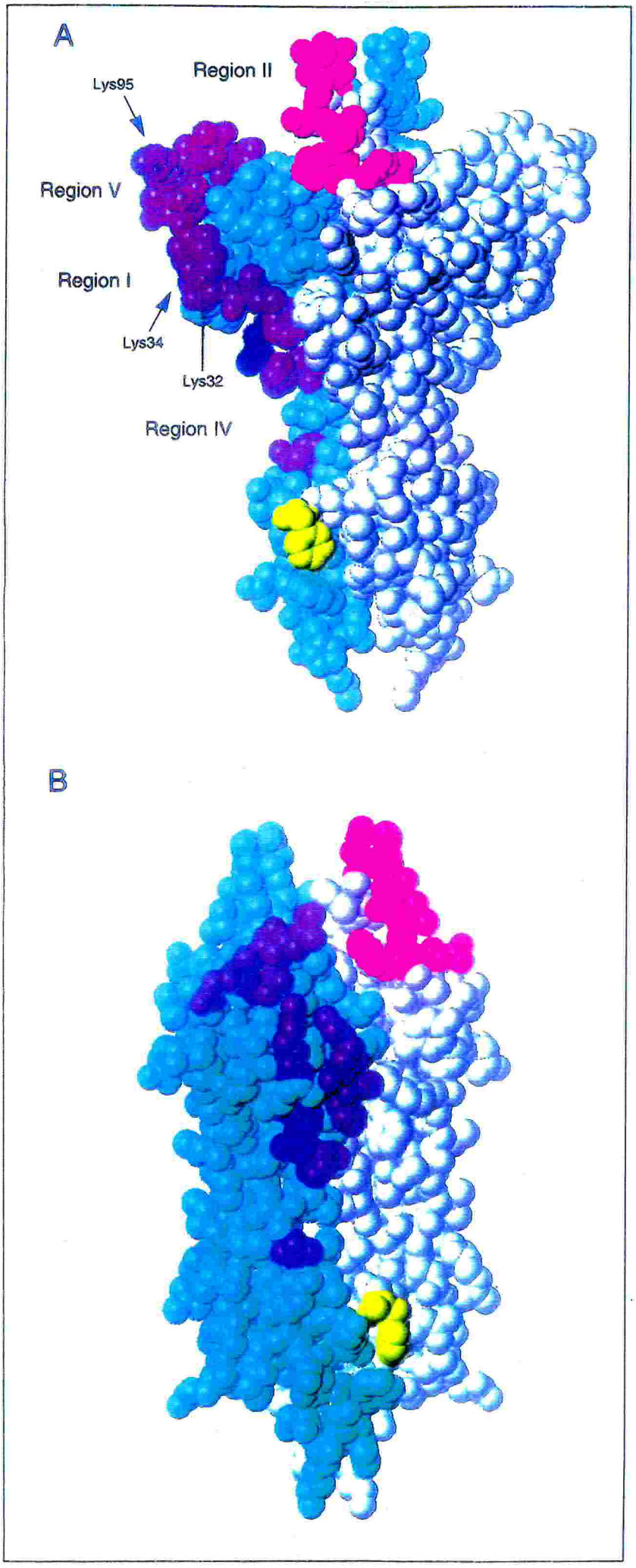


Fig.1.4 (A) Model of the NGF dimer viewed perpendicularly to the twofold axis. The first protomer is in white; the second protomer, like the first but rotated 180^{o} around the vertical axis, is in turquoise. Residues from the turquoise protomer that contribute to one TrkA binding site are in purple, and those from the 'white' protomer are in magenta. For simplicity , the symmetrically equivalent residues forming the second binding site are not coloured. Variable regions I, IV, and V from the turquoise protomer, and variable region II from the 'white' protomer are indicated. Each protomer begins at Phe 12 (shown in yellow in the 'white' protomer). The conformation of the remaining 11 N-terminal residues is unknown, but they may pack parallel to the axis that shows twofold symmetry, and extend the binding surface along the side of the dimer. In the turquoise protomer, Lys 32, Lys 34, and Lys 95, which mediate binding to $p75^{LNGFR}$, are indicated. Note that although these residues only make a minor contribution to TrkA binding, they are also exposed in the binding surface of this receptor.

B) Model of the NGF dimer rotated 90^{o} through the twofold axis as compared in (A). Residues contributing to TrkA binding, form a continuous surface that extends along the side of the dimer, and that contains elements from both protomers. At the bottom of the molecule, this surface may be extended by the N-terminus. Note that the conserved Arg 103 (dark blue), shown to be critical for binding of NT-3 to TrkC, is also exposed in the NGF binding surface (from Ibanez, 1995).


A
Region II
Lys95
Region V
Region I
Lys34
Lys32
Region IV
B

1.2 NGF regulates survival, differentiation and maintenance of specific neuronal populations in the peripheral nervous system

Competition for limited amounts of target derived survival signals is thought to underlie the death of many CNS and PNS neurons during development (and the survival of successful competitors!). Nerve Growth Factor (NGF) was discovered over 40 years ago as a diffusible substance capable of inducing neurite outgrowth in cell explants from dorsal root and sympathetic ganglia (Levi-Montalcini, 1987). In the PNS, NGF is produced by muscles and other cells which make up the target field towards which axons grow during development. Minute amounts of NGF control neuronal survival of specific cell populations during the critical period of development, when cellular death occurs in the nervous system. Classic experimental work, performed by manipulating the availability of NGF to developing neurons in vivo with blocking antibodies (Levi-Montalcini and Angeletti, 1966) clearly demonstrated the absolute dependence of neurons for survival factors, and showed that this changes dramatically with development and is limited to a narrow time window of neuronal development. During this time, and at later stages, a variety of other processes such as neurite outgrowth and synthesis of enzymes or production of neurotransmitters are also regulated by NGF. The classical paradigm of NGF action refers to NGF being supplied by the target innervated field, being retrogradely transported to the neural cell body, where it exerts its survival and differentiative actions. This classical paradigm of neurotrophic action by NGF, as developed by the seminal work of Rita Levi Montalcini and others, has been extended to the central nervous system, although, there, the action of neurotrophins may be related not just to neuronal survival but also to the modulation of synaptic plasticity (see below). In the CNS, high levels of NGF are found in the hippocampus and cortex. These represent target areas of the cholinergic Basal Forebrain (BF) system. In the CNS, NGF is believed to be essential for the development, normal function and survival of septohippocampal cholinergic

neurons. NGF supply prevents atrophy of axotomized forebrain cholinergic neurons in vivo. Transection of the fimbria, the main fiber bundle which connects septal cholinergic neurons to the hippocampus, results in the atrophy of cholinergic neurons, downregulation of the Acetylcholine (Ach) synthesising enzyme cholineacetyltransferase (ChAT) and ultimately, cell death (Hefti, 1986; Montero and Hefti, 1988). Infusion of NGF into the lateral ventricle prevents these changes, and, if provided in sufficient amounts, can induce hypertrophy in both damaged and undamaged cholinergic neurons. However, the role of NGF in the CNS, as opposed to that in the PNS, has not been conclusively established, also because of the difficulty the blood brain barrier poses to antibody studies. The proposed role of NGF as a survival factor for cholinergic neurons has, however, been challenged by recent knock-out experiments. Indeed, the number of forebrain cholinergic neurons in NGF-/- and TrkA-/- mice is apparently unchanged compared to normal controls (Smeyne et al., 1994; Crowley et al., 1994). It is quite clear that most of these neurons survive for as long as the animals do (1 month). The possibility that these cholinerigic neurons require NGF to survive into adulthood cannot be excluded, however. The forebrain cholinergic neurons of NGF -/- and NGF -/+ mice do, however, seem to be stained more lightly for choline acetyltransferase than wild type mice, which agrees with the previous demonstration that NGF increases and anti-NGF decreases ChAT expression in these neurons. There may, however, be potentially interesting differences in the extent to which the phenotype of BF cholinergic neurons is affected in NGF -/- and TrkA -/- mice compared with wild-type mice. AchE staining is apparently unaffected in the BF cholinergic neurons of NGF -/-. One possibility is that the ability of TrkA to mediate a response to high concentrations of NT-3, observed in fibroblasts ectopically expressing TrkA, might be physiologically relevant for BF cholinergic neurons and possibly other neurons (Soppet et al., 1991; Squinto et al., 1991).

The specificity of NGF action on a restricted number of neuronal populations and the generality of the phenomena of programmed cell death in the nervous system

suggested the existence of additional neurotrophic molecules. In 1982, Barde and coworkers reported the isolation of a second neurotrophic factor, designated brain derived neurotrophic factor (BDNF), capable of inducing neurite outgrowth and of supporting the survival of chicken embryonic sensory neurons (Barde et al., 1982). Molecular cloning of the gene encoding BDNF (Leibrock et al., 1989) revealed that this neurotrophic factor was very closely related to NGF, triggering the search for yet more members of this family. This opened a new period in neurotrophic factor research. PCR screening for homologous genes led to the identification of additional related molecules and to the definition of the neurotrophin family. Despite this high similarity, these molecules are dissimilar enough to show different neuronal specificities. The concomitant identification of receptor tyrosine kinases (TrkA, B, C) (reviewed by Barbacid, 1994) allowed the matching of ligand receptor specificities. Extensive work, localising neurotrophin and receptor gene expression, as well as pharmacological actions has been carried out and has led to changes in the restricted view of neurotrophins being involved only in neuronal survival (Thoenen, 1991).

Work localising NGF mRNA and the NGF receptor TrkA has been consistent with previously established functions for NGF in supporting the survival of sympathetic ganglion neurons and sub populations of sensory neurons in DRG and trigeminal ganglia and having no effect on the other peripheral neuronal classes. However, NGF and other neurotrophins have been shown to have overlapping patterns of gene expression and pharmacological action. Some neurons may switch the type of receptor expressed and their survival requirements may also switch from one set of neurotrophins to another during development. Therefore, several neurotrophins may be involved in regulating the survival of a particular population of neurons at any given time. For example, trigeminal neurons display a switch in neurotrophin requirement, from BDNF and NT-3 to NGF (Buchman and Davies, 1993). In the CNS, the highest levels of NGF mRNA are found in the hippocampus, neocortex and olfactory bulb (Maisonpierre et al., 1990). TrkA (mRNA and immunoreactivity)

expression was found mostly in cholinergic neurons in the BF and neostriatum (Holtzman et al., 1992). Interestingly, other non cholinergic nuclei also show TrkA expression (Holtzman et al., 1995a). TrkB receptors are present on BF neurons and the principal transmitter synthesising enzyme, ChAT, is regulated also by BDNF both in vitro and in vivo (Alderson et al., 1990; Venero et al.,1994). The idea that NGF is the exclusive survival factor for BF cholinergic neurons is therefore no longer tenable.

1.3 NGF and synaptic plasticity in the CNS

On the basis of early observations showing that the expression of NGF mRNA in the CNS is related to neuronal electrical activity, it was proposed, with considerable insight, that the role of NGF and of other neurotrophins in the CNS may be broader than in the PNS (Thoenen, 1991). This appealing hypothesis triggered numerous experiments and has been further confirmed and refined (Thoenen, 1995), leading to a new paradigm for the action of neurotrophins. Indeed, increasing evidence shows that, in the CNS, NGF, as well as the other neurotrophins, are involved in processes of synaptic plasticity, particularly mediating activity-dependent, prolonged functional changes, accompanied by corresponding biochemical and possibly morphological changes. This concept is particularly well exemplified by studies on the visual system and on the development of the geniculo-cortical system. This system has been for many years an ideal one to test hypotheses and ideas relating neuronal activity and development and has proved likewise fertile for providing insights into the activity dependent actions of neurotrophins. In addition to the initial results obtained with NGF (Maffei et al., 1992) and with anti-NGF antibodies (Berardi et al., 1994; Domenici et al., 1994) (see below, section 1.7), there are exciting observations with other neurotrophins, suggesting that competition for BDNF or NT-4/5 may be involved in activity-dependent plasticity in the developing visual cortex. Shatz and collegues reported that infusion of BDNF or NT-4/5, during the critical period, could prevent the formation of ocular dominance columns

(Cabelli et al., 1995). Local application of NT-4/5 rescues geniculate neurons from monocular deprivation-induced atrophy (Riddle et al., 1995). On the other hand, administration of NGF and of corresponding monoclonal antibodies dramatically influences the development of the rat visual cortex, as well as the critical period for cortical plasticity (see below). Although in this system the cellular and molecular components are not well identified yet and the mechanism of action of NGF is still not clear, complementary detailed studies on the activity dependent regulation of neurotrophins synthesis, the mechanism and site of neurotrophin release from neurons, and the presynaptic modulation of the release of classical neurotransmitters suggest a general role for neurotrophins as selective retrograde messengers that regulate synaptic plasticity, confirming the initial insights (Thoenen, 1991).

1.4 Expression of neurotrophins and their receptors is regulated by neuronal activity

In the CNS, in situ hybridisation experiments in the hippocampus and cortex demonstrated that NGF and BDNF are mostly expressed by neurons (Ernfors et al., 1990) and that their synthesis is modulated by specific neurotransmitters. Initially, rather extreme experimental conditions, such as seizures, were shown to modulate neurotrophin expression (Gall and Isackson, 1989). Cultured hippocampal neurons have been used to investigate the detailed mechanisms that determine neurotrophin expression in vitro. Production of NGF and BDNF is up-regulated by glutamate, via NMDA and non NMDA receptors, and by acetylcholine via muscarinic receptors, while their production is down regulated by gamma-aminobutyric acid (GABA) via GABA A receptors (Zafra et al., 1990; Zafra et al., 1991; Wetmore et al., 1994; reviewed by Lindholm et al., 1994). Also more subtle physiological stimuli, such as light input, have been shown to modulate BDNF synthesis in the visual cortex, suggesting a role for BDNF in the activity-dependent synaptic plasticity taking place during the development of the visual cortex (Castren et al., 1992). LTP (long term potentiation) has been widely considered as a neuronal

correlate for learning and memory. Induction of LTP in the synapses between Schaffer collaterals and CA1 pyramidal neurons increases the expression of BDNF but not NGF mRNA (Patterson et al., 1992). In another model, where LTP is induced in vivo in the synapses between perforant path and granule neurons in the dentate gyrus, BDNF and NGF mRNA are increased in dentate granule neurons (Castren et al., 1993). Activation of the septohippocampal and corticohippocampal afferent systems innervating the hippocampus evoke an increase in NGF and BDNF mRNA (Lindefors et al., 1992).

Less information is available on the activity dependent regulation of neurotrophin receptor expression. However, it has been shown that depolarisation leads to an increase in the expression of Trk receptors in the sympathoadrenal cell line MAH, and that this level of induction appears to be sufficient to confer NGF responsivity to the cells (Birren et al., 1992). Double labelling in situ hybridisation and histochemistry have showed that central neurons can coexpress a neurotrophin and its high affinity receptor, suggesting that neurotrophins might act locally via autocrine or paracrine mechanisms. Virtually all BDNF mRNA expressing neurons in the hippocampus, amygdala, piriform cortex and neocortex also express TrkB mRNA (Kokaia et al., 1993). Seizure activity increases the expression of BDNF and TrkB mRNA in the same cells. In the dentate gyrus, the subsequent increase of both BDNF protein and the functional TrkB receptor should lead to strongly enhanced BDNF signalling. Kindled seizure, ischemia and insulin-induced hypoglycaemic coma also increase the expression of TrkB mRNA (Merlio et al., 1993), this being accompanied by increases in the level of TrkB protein, in the case of kindled seizures. Moreover recent studies from our laboratory show that stimulation of hippocampal cultured neurons with high potassium increases the expression of TrkB mRNA, as well as that of BDNF (Tongiorgi, personal communication) .

1.5 Neurotrophins may modulate synaptic plasticity at fast and slow time scales enhancing synaptic transmission or inducing long term morphological changes

Evidence has accumulated that neurotrophins can modulate transmitter release and synaptic transmission. The first example was shown in developing neuromuscular synapses in Xenopus cell cultures, where treatment with BDNF and NT-3 increases the release of acetylcholine from Xenopus spinal neurons (Lohof et al., 1993). The potentiation effects were long lasting, but required the continuous presence of the neurotrophin. Exogenous applications of either BDNF or NT-4/5 has been shown to rapidly enhance spontaneous synaptic activity in primary cultures of hippocampal neurons. Unlike the effect found at the developing neuromuscular junctions, the effect at these hippocampal synapses was desensitised within 5-10 min (Leβmann et al., 1994). NGF and BDNF are able to enhance the release of acetylcholine from hippocampal synaptosome preparations of the rat hippocampus (Knipper et al., 1994). Consistent with these results on cell cultures, BDNF and NT-3 enhance the synaptic transmission in the Schaffer collateral-CA1 region of rat hippocampal slices (Kang and Schuman, 1995) and the observed decrease in paired pulse facilitation is consistent with a possible presynaptic modification.

From this ability of neurotrophins to modulate synaptic function, it was reasonable to think that neurotrophins may be involved in long term potentiation (LTP), one of the best studied forms of synaptic plasticity found within the hippocampus. Recently, Korte and colleagues demonstrated that hippocampal LTP was impaired in mice lacking BDNF (Korte et al., 1995). The magnitude of the potentiation, as well as the percentage of cases in which LTP could be induced successfully, was affected. This impairment was not restricted to homozygous mutant mice, but was also present in heterozygotes, in which BDNF mRNA amounts were reduced to 50%, suggesting a correlation between protein dosage and level of impairment. However, the possibility of developmental abnormalities precluded the definitive assignment of a direct role for BDNF in LTP. Rescue experiments can help to distinguish between developmental and acute requirements for the missing gene

product. Treatment of hippocampal slices from mice lacking BDNF by adenovirus-mediated BDNF gene transfer completely reverses the deficits in long term potentiation and significantly improves the deficits in basal synaptic transmission at the Schaffer collateral-CA1 synapse (Korte et al., 1996).

Among the most exciting experiments recently described are undoubtedly those in the developing visual system, in which the morphological effects of neurotrophins have been demonstrated. Infusion of neurotrophin-4/5 or BDNF into the cat primary visual cortex inhibited ocular dominance column formation (Cabelli et al., 1995). Injection of BDNF into the optic tectum of live Xenopus tadpoles increased the complexity of terminal arbors of retinal ganglion cell axons, whereas injection of specific neutralising antibodies to BDNF produced the opposite effect (Cohen-Cory and Fraser, 1995). Moreover, it has been shown that pyramidal cell dendrites respond differently to individual neurotrophins in different layers of the developing visual cortex, the exogenously applied neurotrophins specifically modulating distinct patterns of dendritic arborization (McAllister et al., 1995).

Thus, NTs may modulate synaptic plasticity at fast and slow time scales enhancing synaptic transmission or inducing long term morphological changes

1.6 Possible molecular mechanism for the action of neurotrophins in synaptic plasticity

Different mechanisms can be hypothesised for the way in which neurotrophins may be involved in synaptic plasticity. An intriguing possibility is that the release of neurotrophic factors is temporally controlled by synaptic activity, and that a restricted release site or distribution of Trk receptors in the pre or postsynaptic cell confer spatial specificity in their synaptic action. In the periphery, NGF is synthesised by a great variety of non neuronal cells. It has been firmly established that the regulation of NGF synthesis in the periphery is independent of the innervating neurons and that NGF secretion follows the constitutive calcium-independent pathway. In the CNS, NGF and BDNF are secreted by neurons by

both constitutive and activity dependent pathway. Surprisingly, in rat hippocampal slices and in cultures of hippocampal neurons, the release of NGF was triggered by high K^+, carbachol, glutamate or veratridin and it depended on extracellular Na^+, but was independent of extracellular Ca^{++} (Blochl and Thoenen, 1995). However, it does depend on intact intracellular calcium stores, as their depletion by thapsigargin, or blockade by dantrolene, inhibited NGF release. This is also blocked by the high affinity calcium chelator BAPTA-AM (1,2-bis[2-aminophenoxy] ethane-N,N,N',N'-tetracetic acid,tetra[acetoxymethyl]-ester). In addition, immunohistochemical localisation of NGF in hippocampal cultures by confocal microscopy has demonstrated that NGF is not only localised to the perikarion, but is also found in all neuronal processes, in particular in dendrites, and that constitutive secretion is confined to the neuronal soma and the very proximal parts of dendrites. In contrast, the activity-dependent secretion initiated by high potassium or glutamate also occurs all along the neuronal processes, in particular in dendrites (Blochl and Thoenen, 1996). The same characteristic secretion pattern has also been shown for BDNF (Goodman et al., 1996). Interestingly, recent studies form our laboratory on the subcellular localisation of neurotrophins and of their receptors (Tongiorgi et al., submitted) showed a dendritic localisation for TrkB mRNA but not for TrkA mRNA, in the hippocampus as well as in other brain regions. Within this context, a recent paper (Stoop and Poo, 1995) shows that in developing Xenopus neuromuscular synapses, local application of BDNF to the synapse, after the presynaptic neurites was severed from its soma, remained effective in inducing elevation of spontaneous neurotransmitter release. Thus, it appears that the intracellular target of BDNF can act autonomously at the nerve terminal itself and does not require the cell body or nucleus.

1.7 NGF is involved in the activity dependent plasticity of the developing visual cortex

The visual system offers unique possibilities to study synaptic plasticity, in that a fine modulation of the extent and pattern of neural activity along the visual pathways can be achieved by a modulation of the visual input. Manipulation of the visual environment during early postnatal life (critical period) of mammals leads to dramatic changes in the organisation of the visual cortex. For example, monocular deprivation (MD) by temporary closure of one eye renders neurons of the visual cortex, most of which are normally binocular, non responsive to stimuli presented to the deprived eye (ocular dominance shift). This synaptic plasticity is restricted to a time window during postnatal development (critical period): MD performed after the end of the critical period does not affect the visual cortex.

Visual cortical plasticity during the critical period has been attributed to the result of competition between afferent fibres from the lateral geniculate to the visual cortex. However, the nature of the competition at a molecular or electrical level has remained unknown for a long time. Exogenous supply of NGF, during the critical period of ocular dominance plasticity, prevented the effect of monocular deprivation in rats (Maffei et al., 1992), suggesting that visual afferents may be competing for NGF. This hypothesis is consistent with the presence of NGF and of other neurotrophins, in rat visual cortex areas and with the time course of their expression during postnatal development.

In order to gain insights into the role of endogenous NGF in the functional and anatomical development of the geniculo cortical system, the physiological action of NGF in the rat visual system was antagonised with an anti-NGF antibody which neutralises NGF (αD11). Anti-NGF treated rats show a shift in the ocular dominance distribution of visual cortical cells, a decrease in their visual acuity and a shrinkage of lateral geniculate neurons (Berardi et al., 1994). Moreover, anti-NGF treated and control rats were monocularly deprived at the end of the critical period. In anti-NGF treated rats, MD was still effective (Domenici et al., 1994). Therefore

loss of endogenous NGF prolongs the critical period, possibly by delaying the process of synapse consolidation in the visual cortex. The site of action of NGF in visual cortical plasticity has yet to be identified. Cholinergic neurons of the BF, that have terminals in neocortex, would represent a natural candidate to mediate the action of NGF in the visual cortex. However, pharmacological disruption of BF cholinergic neurons during the critical period does not reproduce the effects of anti-NGF on visual cortex cortical plasticity, even if one cannot exclude that surgical disruption may not be complete (Siciliano and Domenici, 1996). Experiments reported in chapter 2 of this thesis will likewise argue against a role for the cholinergic neurons of the BF in the NGF-mediated effects on the geniculo-cortical pathway. Another possibility to be considered is an uptake of NGF by geniculate terminals: this seems unlikely, because TrkA receptors are not present on geniculate neurons. In addition, a recent study failed to reveal a retrograde transport of iodinated NGF from the visual cortex to the geniculate nucleus during the critical period (Domenici et al., 1995). The action of NGF could be exerted directly at the level of intracortical connectivity. To validate this hypothesis, the expression of TrkA in the visual cortex must be shown. Some lines of evidence suggest that a subpopulation of cortical neurons may express functional NGF receptors (Domenici et al., 1995). Further indications which support this hypothesis include the demonstration that TrkA mRNA expression has been revealed in the rat visual cortex by the polymerase chain reaction (Cellerino and Maffei, 1996a), and that NGF modifies the morphology of pyramidal neurons in slices of visual cortex (McAllister et al., 1995). A p75-mediated effect, however, cannot be excluded. Whatever the underlying mechanism, these results indicate that NGF plays a physiological role in the activity-dependent development of the visual cortex.

1.8 Aim of the thesis

It is relevant that the hippocampal formation is the area of the brain where the expression of neurotrophins is the highest. In particular, NGF, the p75 receptor and, to a lesser extent, the TrkA receptor (Cellerino, 1996) are present in several parts of the hippocampus. This would suggest the possibility for an active role of this neurotrophin in the development and physiology of this region of the brain. The hippocampus has been extensively studied and, as a consequence, a great deal of information is available about its anatomy and physiology, and in particular about the most widely accepted model of synaptic plasticity, i.e. LTP and LTD (long term depression). From this point of view, the hippocampus represents a good model to study the possible role of NGF in synaptic plasticity. However, until now the effects of the acute application of exogenous NGF to synaptic processes within the hippocampus have yielded different and contradictory results. One group (Tancredi et al., 1993) reported that NGF delivered to hippocampal slices causes a concentration dependent reduction in the expression of long term potentiation in the CA1 region, without altering basal synaptic activity. An intriguing finding of this study was represented by the fact that chemically modified NGF, unable to interact with its receptors, also induced the same effect. However, the receptor specificity of the chemical modification of NGF was not tested (p75 versus TrkA). Another study showed that NGF enhanced spontaneous and evoked depolarisation-induced glutamate release from hippocampal synaptosomes, and increased glutamatergic EPSPs in the CA1 subfield of the hippocampus, an effect probably mediated by AMPA receptors (Knipper et al., 1994a). The transmitter releasing effect of NGF on glutamatergic nerve terminals parallels the action of NGF (and BDNF) on cholinergic nerve endings in the adult hippocampus (Knipper et al., 1994). In the papers described above, however, the conflicting results could be also due to differences in culture conditions and in dosage of NGF. Moreover, it was reported (but not analysed in detail) that 20% of cultured hippocampal cells show an increase in spontaneous synaptic activity after NGF administration (Levine et al., 1995).

It is therefore possible that the role of endogenous NGF in the hippocampus is not only that of a retrograde factor for the maintenance of septal cholinergic neurons. Further suggestions for a local action of NGF on hippocampal neurons, which, however, may or may not be related to an effect on synaptic transmission, come from the demonstration that the implant of genetically modified fibroblasts producing NGF protect rat hippocampal neurons from ischemic damage (Pechan. et al.1995). In order to gain insight into the role of endogenous NGF in synaptic plasticity processes in the hippocampus, a continuos application of blocking anti-NGF antibodies in the brain has been exploited in this thesis, using the hybridoma implant technique (chapter 2). The work described in this thesis was aimed at:

i) clarifying the role of endogenous NGF in the modulation of the developmental shift from LTD to LTP at the mossy fiber CA3 synapses (chapter 3);

ii) analysing LTP and LTD in the CA1 region of the hippocampus and at correlating the relationship between these forms of synaptic plasticity and an experimental paradigm for learning and memory processes (chapter 4);

iii) developing a transgenic model exploiting recombinant antibodies, complementary to models obtained so far by gene knock-out (chapter 5).

1.9 Anti-NGF antibodies in the CNS: experimental methods.

In order to investigate the role of endogenous NGF on synaptic plasticity in the developing and adult hippocampus I have exploited the availability of a hybridoma cell line producing the neutralising anti-NGF antibody αD11. I pursued two different methods to achieve a continuous release of antibody in the CNS: 1) implanting hybridoma cells into the lateral ventricle of rats and 2) creating transgenic mice harbouring αD11 recombinant antibodies as transgenes (neuroantibody approach).

To provide a continuous supply of antibodies in the CNS, injection of hybridoma cells into the ventricles has been recently performed with success (Schnell and Schwab, 1990). We have therefore followed this approach and implanted αD11

hybridoma cells into neonatal and adult rats (**fig.1.5A**). I have defined the conditions under which cells produce enough antibody to inhibit the action of endogenous NGF and characterised the diffusion of antibodies molecules in different brain areas (F. Ruberti, this thesis). This experimental method has been successfully used in a collaboration between our laboratory and the group in Pisa to study the role of NGF in the visual system (Berardi et al., 1994). I have exploited this approach to analyse the effect of NGF deprivation on synaptic plasticity in rat hippocampal slices. In particular, LTP or LTD was analysed at the mossy fiber-CA3 synapse during postnatal development (chapter 3) and at the Schaffer collateral -CA1 synapse (chapter 4) in adult rats. The second approach, the neuroantibody approach, is based on the ectopic expression of monoclonal antibodies by neuronal cells (Cattaneo and Neuberger, 1987; Piccioli et al., 1991). Functional and developmental studies on the mammalian CNS would greatly benefit from the ability to interfere with the function of selected neuronal subpopulations or pathways. The neuroantibody approach is based on the availability of hybridoma cell lines, which secrete monoclonal antibodies of interest. These cells, therefore, can be used as the source of the rearranged genes that encode for such antibodies . These genes can be manipulated with available gene transfer techniques, just as any other genes. To achieve antibody production by cells of the nervous system two complementary strategies can be pursued: 1) the creation of transgenic mice harbouring the recombinant mAbs as transgenes, under the transcriptional control of suitable promoters and 2) intracerebral grafting of cells engineered, with recombinant retroviral or viral vectors, to secrete the recombinant monoclonal antibody or an active derivative thereof, such as a single chain antibody (**fig.1.5B**). The feasibility of such an approach was demonstrated in vitro, by showing that immunoglobulins can be efficiently expressed as secreted proteins by mammalian non-lymphoid cells. In particular, the efficiency of antibody secretion by cells related to the nervous system was shown to be comparable to that of lymphoid cells (Cattaneo and Neuberger, 1987).

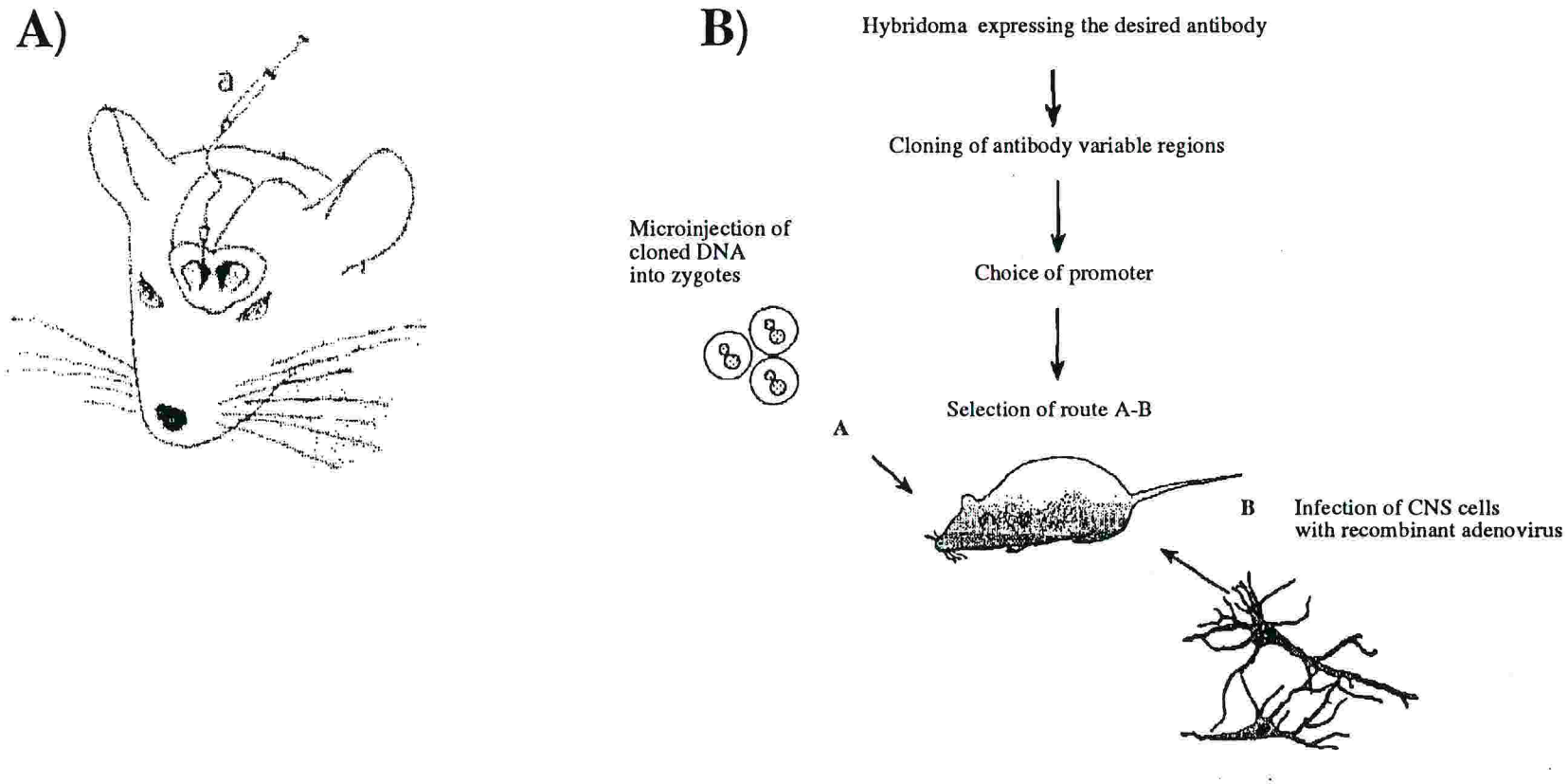


Fig.1.5 (A) The method used to implant hybridoma and myeloma cells into the right ventricle (a). **(B)** Schematic illustration of the neuroantibody approach: possible routes for expressing antibodies in cells of the nervous system

The neuroantibody approach could be used: i) to block synaptic transmission using antibodies against neurotransmitters or their receptors, ii) to facilitate synaptic transmission (for instance, using antibodies against acetylcholinesterase), iii) to eliminate or perturb specific cell populations with antibodies against growth factors or their receptors.

The range of proteins that can be studied by this approach may be extended to intracellular proteins. In fact recombinant antibodies, engineered with suitable targeting signals, have been successfully targeted to intracellular compartments, where they can compete with the corresponding antigen: cytoplasm, nucleus, endoplasmic reticulum, mitochondria (Biocca et al., 1990; Biocca et al., 1995). After the proof of principle, the neuroantibody approach has been successfully applied, to interfere in vivo with the actions of the tachykinin neuropeptide Substance P (Piccioli et al., 1995). This neuropeptide is involved in different functions in the peripheral nervous system, such as neurogenic inflammation, as well as in less characterised functions in the CNS. Ectopic expression of monoclonal antibodies against this peptide was achieved by producing transgenic mice expressing recombinant anti-SP antibodies under the control of the promoter of the neuronal gene vgf (Levi et al., 1985). The transgenic antibodies were shown to successfully inhibit some biological actions of the endogenous SP (Piccioli et al., 1995). These experiments demonstrated that the neuroantibody approach is a useful method to interfere with gene function in vivo, that may be complementary to the approach of gene knock-out by homologous recombination, for reasons discussed below (chapter 5). The neuroantibody approach should be of particular use to study the role of NGF in the developing and, particularly, the adult CNS. Furthermore, the neuroantibody approach is the only way to achieve inactivation of i) a molecule with biological activity which is not encoded by a gene, ii) single products of multiply processed preproteins, and iii) single variants derived from alternative splicing, providing, of course, that specific antibodies are available. However, it

should be borne in mind that the method will only be as effective as the antibodies used.

Chapter 2

A critical period in the sensitivity of basal forebrain cholinergic neurons to NGF deprivation

2.1 Introduction

The functional roles of NGF in the mammalian CNS and PNS are currently being investigated by a variety of experimental approaches, including the use of NGF antibodies and of NGF itself. However, a continuos delivery of antibodies or trophic factor to the nervous tissue poses several practical problems, mainly due to limited diffusion and half-life of the injected substance. Moreover, in view of the fact that NGF is a member of the NT family, an antibody specific for a selected NT should be used.

In order to study the role of NGF in the developing and adult CNS it is of importance to achieve control over timing, locale and degree of gene function. To this aim we have established a way for locally delivering antibodies to the CNS in a continuous fashion. This chapter is devoted to a description of the hybridoma implant technique and of the successful use of this technique to investigate in more detail the role of NGF on BF cholinergic neurons, using hybridoma cells secreting anti-NGF antibodies.

2.2 *Results*

2.2.1 Properties of αD11 monoclonal antibody

The mAb αD11 (Cattaneo et al., 1988) is a rat IgG2a antibody which binds NGF with an affinity greater than 10^{-9}M and which neutralises very efficiently the biological action of NGF both in vitro (on PC12 cells as well as on sympathetic neurons) and in vivo, as judged by a successful immunosympathectomy (A.C. unpublished).

This neutralising activity is exerted by preventing the binding of NGF to its receptors (see below). In order to be able to use this antibody unambiguously in vivo, it was mandatory to characterise the specificity of the antibody with respect to the other members of the neurotrophin family. Thus, the crossreactivity of Mab αD11 with the other known members of the neurotrophin family has been analysed (in collaboration with S.Gonfloni) with a bioassay based on embryonic chick dorsal root ganglia. As illustrated in figure **2.1**, mAb αD11 blocks the biological activity of NGF and does not crossreact with other protein members of the neurotrophin family BDNF, NT3 and NT4. mAb αD11 recognises the NGF protein from a wide spectrum of species, ranging from Xenopus laevis to man, thus suggesting that the epitope recognised is highly conserved across evolution. The epitope mapping of the αD11 antibody has been obtained in the laboratory (Gonfloni, 1995), by testing the binding activity of the αD11 antibody against a high number of rat NGF mutant proteins (kindly provided by C. Ibanez). αD11 recognises the loop region 41-49 of NGF and is also sensitive to the substitution of K95, which is not surprising, due to its close proximity to this 41-49 loop (**fig. 2.2**). The aminoacidic residues N43, I44, N45 are highly conserved between NGF proteins derived from different species. As described in the introduction, this 41-49 loop (**fig.1.4**) is involved in the interaction surface of the NGF molecule with the cognate receptor TrkA. Thus, mAb αD11 appears to exert its inhibitory activity by a direct competition between NGF itself and the TrkA receptor. This functional and molecular characterisation of the MAb αD11 suggest it to be an ideal reagent to study the role of NGF in vivo.

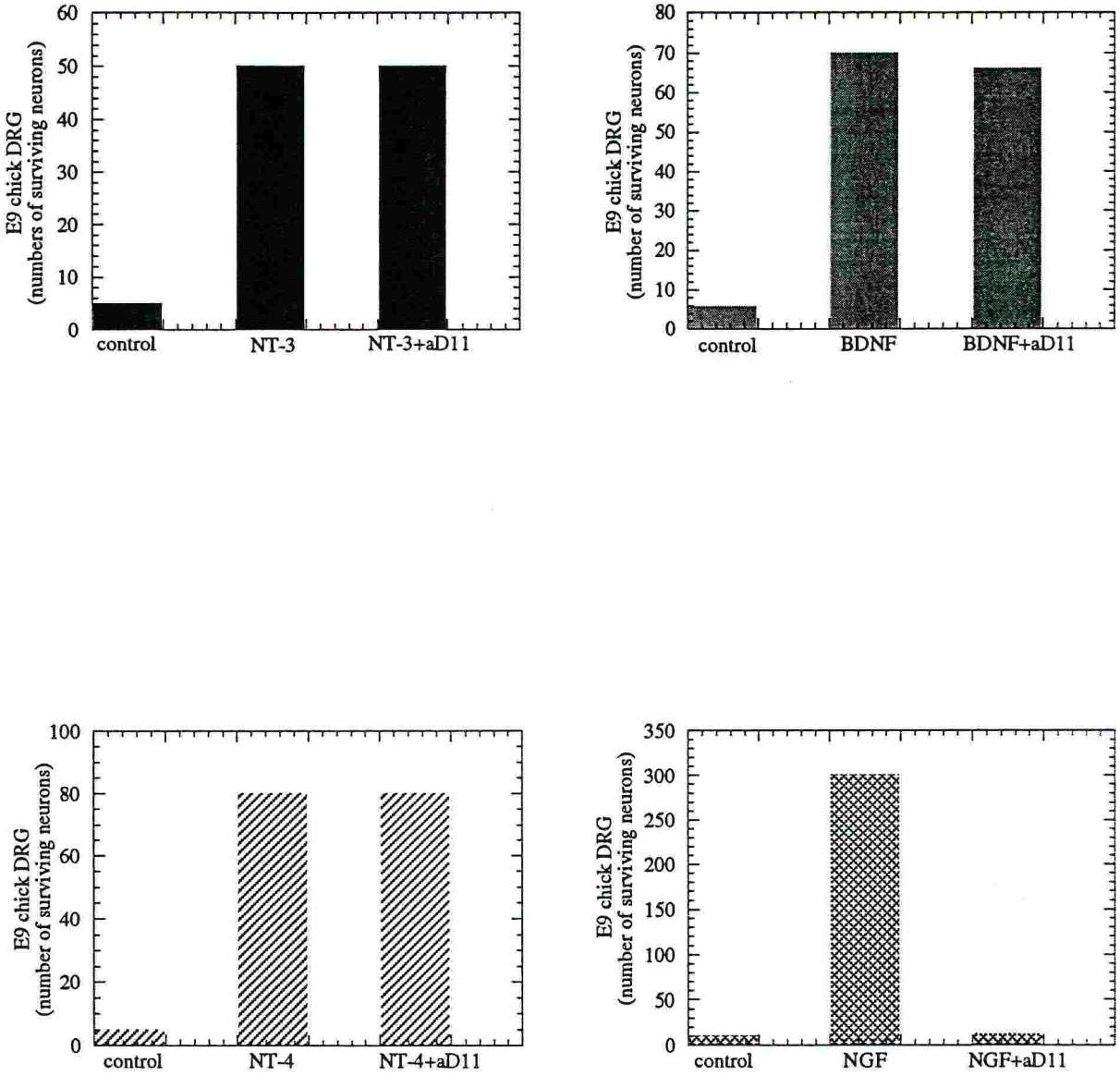


Fig.2.1Survival of chick dorsal root ganglion neurons after 48 hr in culture.Neurotrophins (1ng/ml) were added either alone or togheter with mAb aD11. Note that aD11 has no effect when used with BDNF, NT-3 or NT-4.

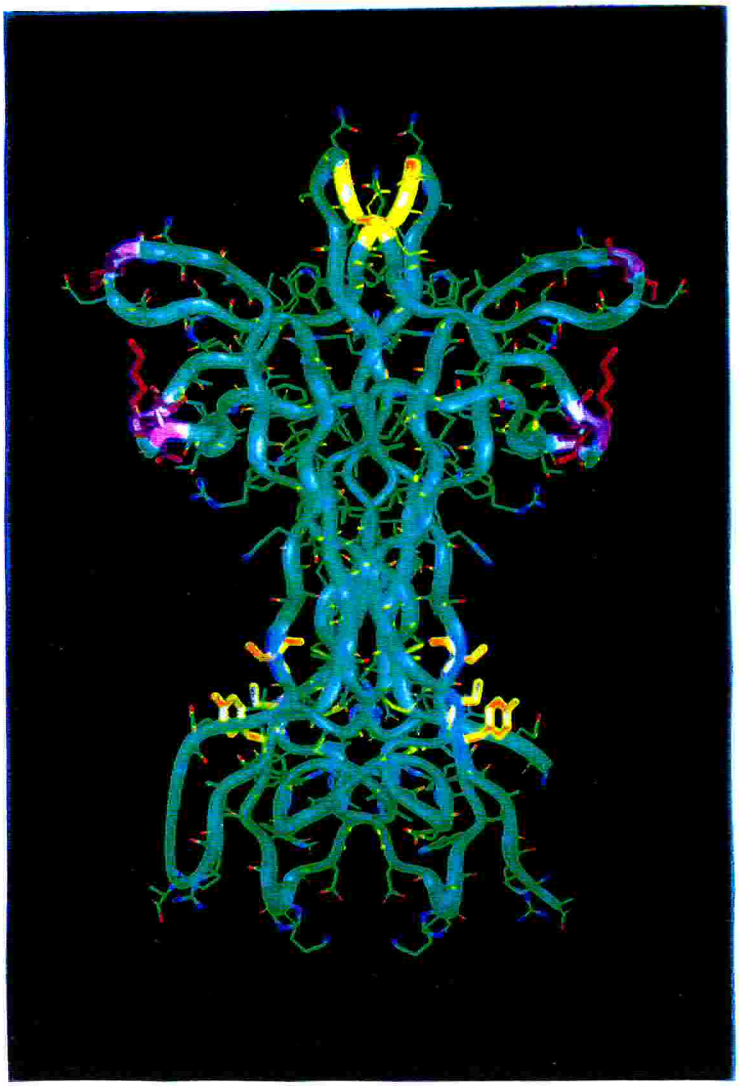


Fig.2.2 Structure-based model of the dimer of the NGF molecule. Epitope mapping of three monoclonal antibodies to NGF, αD11 (yellow, top), 4C8 (Cattaneo et al., 1988) (yellow left and right), 27/21 (kind gift of Prof. H. Thoenen), (Korsching and Thoenen, 1987) (magenta, left and right). (from Gonfloni, 1995)

2.2.2 Intraventricular implant of αD11 hybridoma cells to neutralize the action of endogenous NGF

In order to achieve a continuous supply of antibodies to the CNS, αD11 hybridoma cells were injected into the lateral ventricle of rats at different postnatal ages (P2, P8, P15). Control rats were injected with the parental myeloma cell line P3U, to control for possible non specific effects due the transplant per se. In some experiments, controls also included the implant of hybridoma cell line secreting a non relevant antibody. In a series of preliminary experiments the fate of the injected cells was assessed by labelling hybridoma cells with the lipophilic tracer 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) prior to their injection. Labelled cells were found after 1 and 2 weeks in the lateral ventricle at the level of the injection site **fig. 2.3.**

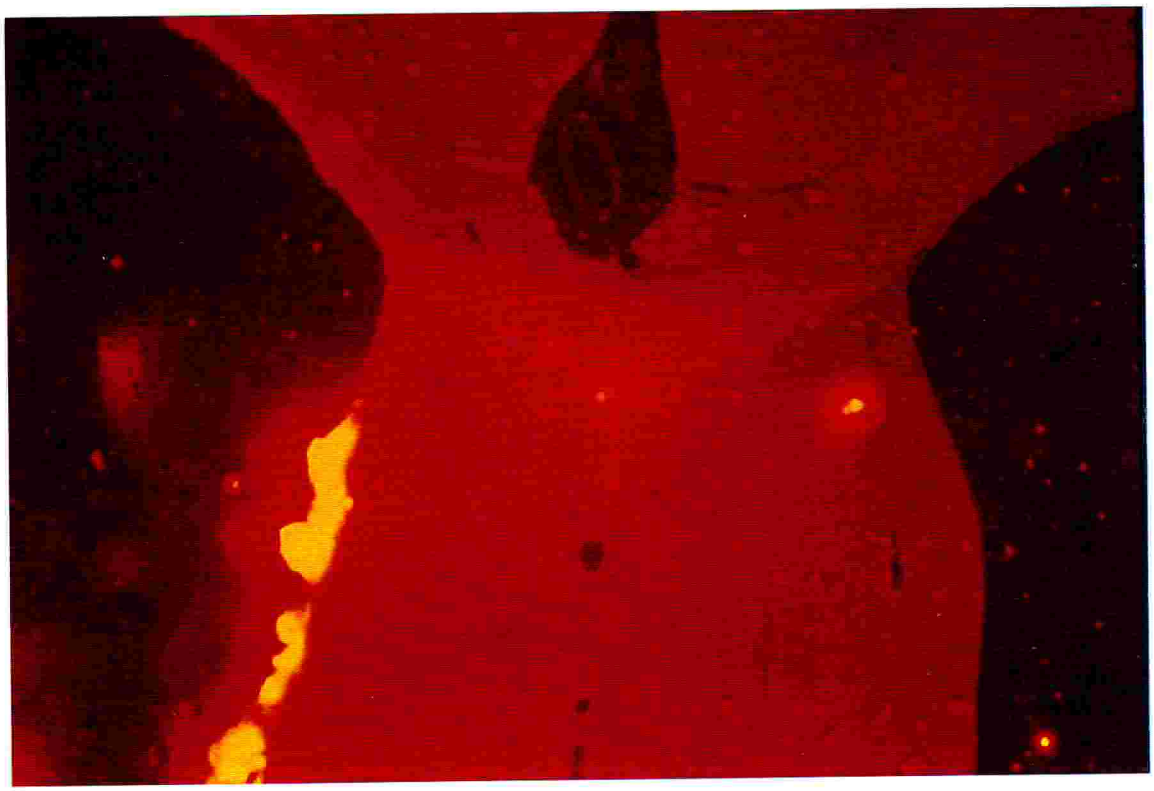


Fig.2.3 Microphotographs showing the distribution of hybridoma cells 15 days after injection. Hybridoma cells previously labelled with Dil were injected at P2. Labelled cells were found lining in the lateral ventricle (VL) at the level of the injection sites.

The measure of antibody levels secreted by the injected cells was evaluated as a function of time after injection and anatomical location. Anti NGF antibodies were detected both in the CSF as well as in specific brain structures: cortex and hippocampus, ipsilateral and controlateral to the injection site. One week after the injection, αD11 antibody was present in a range of about 2ng/μl of cerebrospinal fluid. The levels of αD11 in the hippocampus and cortex was found to be in a range of 2-3ng/mg, regardless of the distance from the injection site (**Tab. 2.1**).

	CSF ng/μl	hippocampus ng/mg	cortex ng/mg
P9 inj. P2	2.4 ± 0.24	2.3 ± 1.1	3.5 ± 1.5
P15 inj. P8	2.3 ± 0.22	2.6 ± 1.4	2.4 ± 1.1
P22 inj. P15	2.3 ± 0.22	7 ± 1.1	2.5 ± 1.3
P15 inj. P2	1 ± 0.23	4 ± 1.2	3.5 ± 1.4

Table 2.1: levels of αD11 antibody in cerebrospinal fluid (CSF), hippocampus and cortex of rats one week after the injection, at the indicated ages. Antibody levels were determined as described in material and methods; the number of injected rats (n) was n=6 for CSF; n=4 for hippocampus and cortex.

Two weeks after the injection, high levels of αD11 antibodies are still present.
The comparison between the levels of circulating αD11 antibodies and the levels of NGF present in the brain (Large et al., 1986) shows that the concentration of antibodies is in a vast molar excess over endogenous NGF. Moreover, it should be noted that while the value for antibody concentration refers mainly, if not exclusively, to extracellular molecules, the figure for NGF levels refers mainly to the pool of NGF stored intracellularly. Thus, it is safe to assume that most of the extracellular NGF activity is potentially neutralised by the antibodies secreted by the implanted cells. The technique has been successfully applied, in a collaboration

between our laboratory and the group in Pisa, to the study of the development of the geniculo-cortical synapse in the rat visual system (Berardi et al., 1994). In the following paragraph, I describe experiments aimed at investigating the sensitivity of BF cholinergic neurons to NGF deprivation, during postnatal development.

2.2.3 A critical period in the sensitivity of basal forebrain cholinergic neurons to NGF deprivation

BF cholinergic neurons constitute a group of nuclei localised at different levels in the forebrain region of CNS. These neurons send projections to the hippocampus and several cortical areas (Dutar et al., 1995). They have been considered the prototypical NGF-responsive neurons in the CNS. A great number of previous studies have reported that cholinergic neurons of BF express receptors for NGF and that limiting amounts of NGF are produced in target areas receiving projections from BF. Exogenous supply of NGF increases many cholinergic markers, including cholinacetyltransferase (ChAT) activity, choline uptake and acetylcholine release. BF cholinergic neurons express both classes of NGF receptors, TrkA and p75 (Koh and Higgins, 1991; Holtzman et al., 1992). Surgical transection of fibres connecting BF cholinergic neurons to target neurons induces the shrinkage of the cell body and finally cell death of parent cells (Hefti et al., 1986). These effects are completely prevented by the contemporaneous NGF exogenous supply. Recent data (Vantini et al., 1989; Li et al., 1995) showed that antibody-mediated blockade of endogenous NGF results in the reduction of ChAT immunoreactive neurons in newborn but not in adult rats, suggesting that endogenous NGF acts on developing BF cholinergic neurons to enhance cellular differentiation. Consistent with this hypothesis are the findings that the uptake/transport of NGF (Domenici, 1995) and the expression of NGF and NGF receptors are developmentally regulated during the postnatal life (Large et al., 1986; Maisonpierre et al., 1990; Li et al., 1995; Ringstedt et al., 1993).

The results from NGF and TrkA knock-out mice appear to be somewhat inconsistent with the view that NGF is the only differentiation and/or survival factor for BF neurons. In order to shed some light on this point, and to investigate whether a critical period for NGF sensitivity is present, hybridoma cells producing the monoclonal antibody anti-NGF were injected into the lateral ventricle of rats at different postnatal ages (P2, P8 and P15).

The injection of hybridoma cells at P2, resulted in a marked decrease of ChAT positive cells after one week (**fig. 2.4**). The effect was stronger in the medial septum than in the diagonal band.

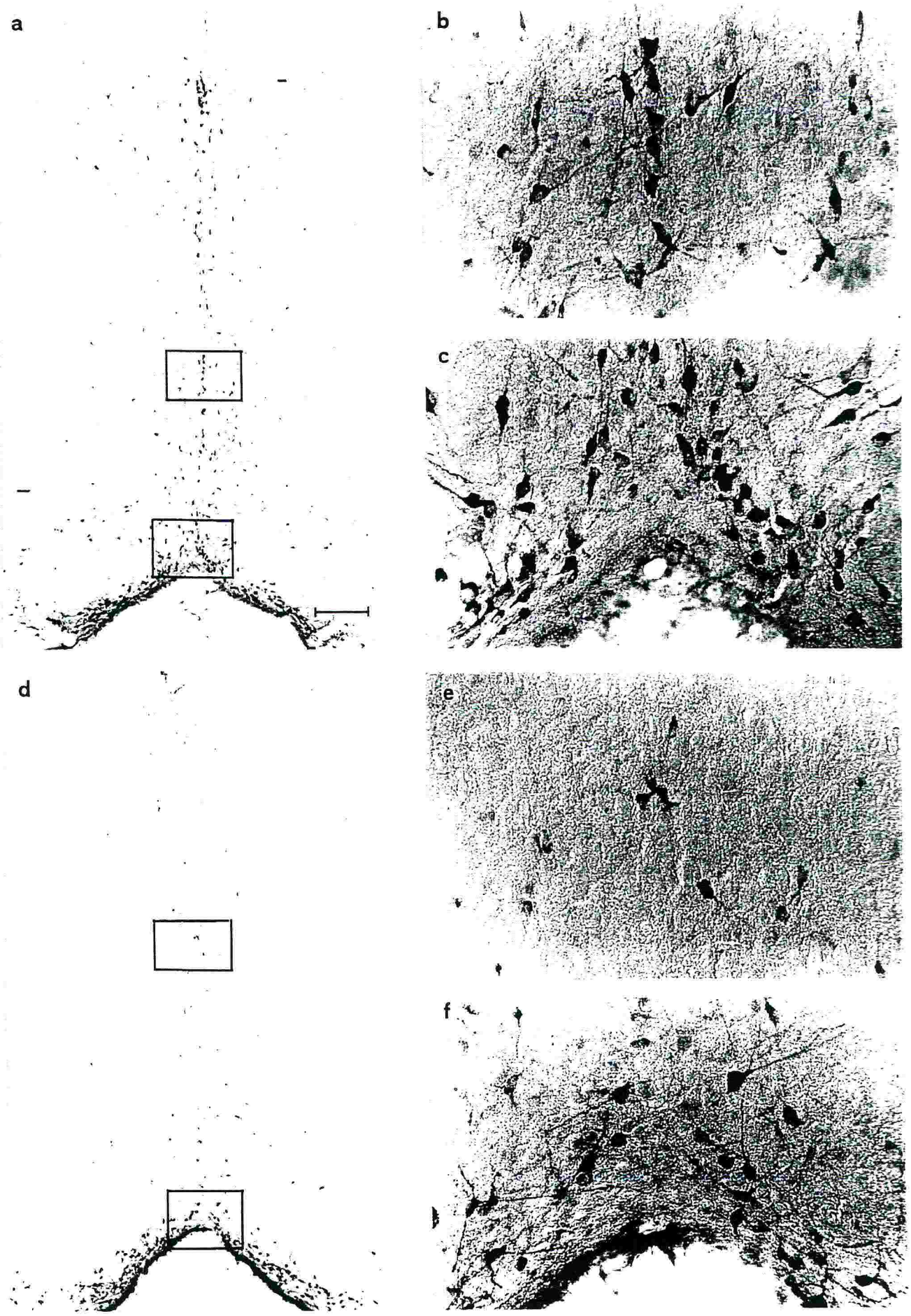
Fig. 2.4 ChAT immunoreactive cells in the septal complex of 9 day old rats implanted with myeloma or hybridoma cells at P2.

a) labelled neurons in control rats implanted with myeloma cells;

b) and c) higher magnification of the areas framed in panel a.

d) labelled neurons implanted with hybridoma.

e) and f) higher magnification of the areas framed in panel d; note a marked decrease of ChAT neurons in rats implanted with anti NGF producing cells in respect to the control situation.(scale bar 400 μm panels a and b, 66 μm panels b, c, and f).


a
b
c
d
e
f

Different results were obtained implanting the cells in 8 or 15 days old rats. As shown in **fig. 2.5,** no difference in the number of labelled cells was detected in both regions studied. Therefore, ChAT-positive cholinergic neurons of BF are drastically reduced in number only when endogenous NGF is antagonised during the first postnatal week.

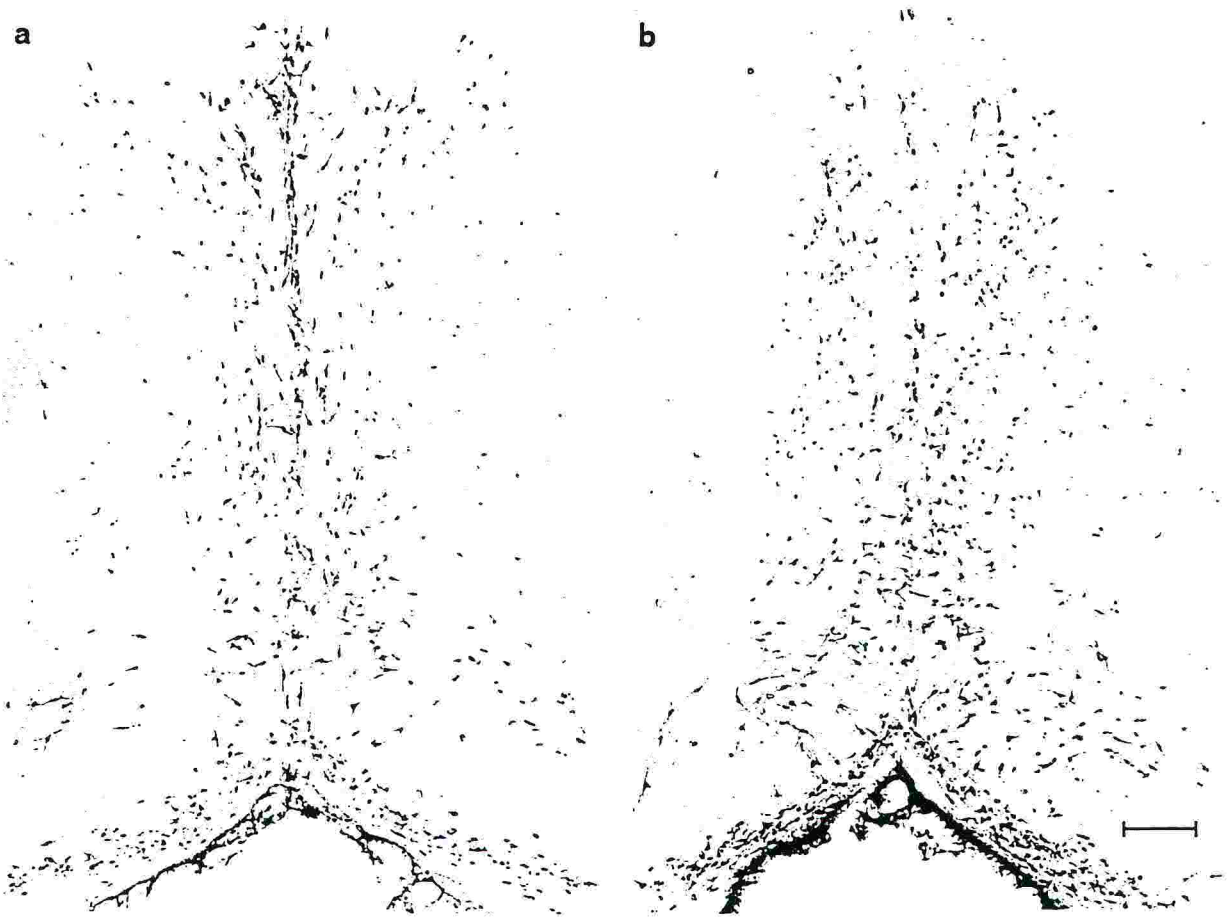


Fig. 2.5 ChAT immunoreactive cells in the septal complex of 15 days old rat implanted with myeloma cells (a) or hybridoma cells (b) at P8. Note the absence of differences in the pattern of ChAT positive neurons. (scale bar 400 μm)

2.3 *Discussion*

The αD11 mAb is a useful tool to perform in vivo studies on the role of NGF in the CNS. In fact, αD11 mAb, as shown in bioassay experiment on DRG ganglia, selectively inhibits NGF biological activity and does not crossreact with the other known neurotrophins. The implantation of hybridoma cells in the ventricle allows one to antagonise NGF action during a selected time period and to remove endogenous NGF activity completely, as shown from the doses of αD11 antibody, which are in large excess over the amount of NGF likely to be present in the extracellular environment of the brain. The effectiveness of implanted hybridoma cells to antagonise endogenous NGF was shown by the reduction of ChAT staining in BF cholinergic neurons, a population of cells known to be sensitive to NGF.

When the antibody secreting cells are implanted at P8 or at P15, no reduction in the number of ChAT positive cells was found. The period of maximal sensitivity of BF cholinergic neurons to NGF deprivation thus coincides with the period of maturation of these neurons. It is not clear what distinguishes BF neurons after P8, from their counterparts at P2, making them insensitive to NGF withdrawal. It is unlikely to be the absence of the NGF receptors p75 and TrkA, since these are expressed in BF cholinergic neurons during the entire postnatal period. Interestingly, the expression of TrkA mRNA and protein in the BF increases sharply around P8-P10 (Li et al., 1995). Whatever the mechanism(s) underlying the existence of a critical period for sensitivity to NGF withdrawal, these data show that BF neurons appear to become NGF-independent after the first postnatal week. One may speculate that, after P8, TrkA receptors become transiently activated in the absence of ligand, possibly due to an increase in the number of receptors per cell. It is unlikely that the insensitivity to NGF withdrawal after P8 is due to lower NGF doses being needed to activate TrkA receptors, similarly to what found in transfected cells overexpressing these receptors (Hempstead, 1992), since our data show that the doses of antibody present are in large excess over the amount of NGF likely to be present in the BF. Alternatively, BF neurons may switch their dependence towards another neurotrophin, for

example BDNF. Further experiments are needed to address these issues. These results lay the ground for understanding the mechanisms underlying the sharp developmental transition that turns NGF sensitive neurons into insensitive ones.

These results are also important to understand the mechanisms of NGF action on the developing visual system. Recent data obtained using hybridoma cell implants showed that the rat geniculo-cortical system is altered when endogenous NGF was antagonised, starting from the beginning of the rat critical period for visual cortical plasticity (P15) (Berardi et al., 1994). One could argue that these effects are not due to the direct action of NGF on the geniculo-cortical system, but that they could be secondary to an alteration of BF cholinergic neurons, which send a well defined projection to the visual cortex. This hypothesis is not supported by the results of these experiments, showing that BF cholinergic neurons are not affected by endogenous NGF neutralisation started at P15.

2.4 *Material and Methods*

2.4.1 Bioassay on chick dorsal root ganglia

Bioassays for neurotrophin activity have been performed on chick embryonic ganglions (E9). The collection, cleaning, trypsinization, and dissociation of ganglia was carried out as described previously (Barde et al. 1980). The dissociated cell suspension was enriched for neurons by the preplating technique. Cultures were maintained in F-14 medium supplemented with 10% heat-inactivated horse serum (GIBCO). Neuron enriched suspensions were plated at 2000 cells/well. Prior to plating of neurons, cell culture dishes were coated with the basement membrane protein laminin (10μg/ml, SIGMA). Neurotrophins were used at a concentration of 1 ng/ml and the αD11 monoclonal antibody at 1 μg/ml. The number of surviving neurons was determined at least 48 hr after plating. NGF was kindly provided by D. Mercanti. BDNF, NT-3 and NT-4 were purchased from Preprotech.

2.4.2 Cell culture, intracerebroventricular injection and antibody detection.

Endogenous NGF was antagonised by implanting hybridoma cells producing αD11, a monoclonal antibody selective for NGF, into the lateral ventricle. The parental myeloma cell line (P3-X63Ag8 or P3U, which produces IgG molecules which are not secreted) was implanted into control rats. The implants were performed at different postnatal ages: P2, P8 and P15. Hybridoma and myeloma cells were cultured and injected as follows. Cells were grown in Dulbecco modified Eagle's medium supplemented with 10 % calf serum. Before being injected, cells were washed three times in Hank's balanced salt solution (HBSS) and finally resuspended at a concentration of $2x10^5$. Hybridoma and myeloma cells were injected into the lateral ventricle and rats were treated with cyclosporin A (Sandoz) (15mg/kg) to prevent implant rejection.

The presence of αD11 antibodies produced by the implanted cells in the intracerebroventricular liquor was detected by means of ELISA analysis. NUNC

Maxi Sorb plates were coated with NGF (5µg/ml, a gift from D. Mercanti). Samples of liquor collected before the perfusion, were diluted in 2% milk in phosphate-buffered saline (PBS) and incubated in the coated plates. The presence of αD11 antibodies was detected utilising a secondary antibody peroxidase conjugated, and the reaction developed with 1-step MB Turbo ELISA (PIERCE). The absorbance was measured at 450 nm. The absorbance values obtained for the CSF samples were compared to those of a standard curve of αD11 antibody.

2.4.3 Immunohystochemistry

Animals were deeply anaesthetised (chloral hydrate, 6 ml/kg, 10% in saline) and perfused transcardially with saline solution followed by 4% paraformaldehyde in phosphate buffer 0.1 M (PB). After cryoprotection with 30% Sucrose in PB 0.1 M, the brains where cut at 30 µm and incubated with a mouse monoclonal anti ChAT antibody.(Umbriaco et al., 1994). Sections were then incubated with a biotinylated secondary antibody. The reaction was revealed with the ABC KIT (Vector Laboratories).

Chapter 3

Developmental shift of mossy fibre LTD to LTP in the CA3 region: NGF dependence?

3.1 Introduction

The experiments described in the previous chapter have demonstrated that the method of injection of hybridoma cells secreting anti NGF monoclonal antibodies is effective to achieve a long lasting neutralisation of endogenous NGF. We have demonstrated that during the first postnatal week there is a critical period during which BF neurons are more sensitive to NGF.

During this time, which coincides with the period in which the septal fibers start innervating the hippocampus, we have performed experiments to ask whether blocking endogenous NGF would affect, either directly or indirectly, through the cholinergic system, some paradigms of synaptic plasticity. In particular, we have focused our attention on the CA3 hippocampal region, that is heavily innervated by cholinergic fibres.

3.1.1 Synaptic plasticity LTP and LTD in the hippocampus

Long Term Potentiation (LTP) and Long Term Depression (LTD) of excitatory synaptic transmission in the mammalian brain are long lasting, activity-dependent changes in synaptic efficacy. Such mechanisms may be crucial to establish the appropriate connections between developing neurons and in the modification of circuits underlying forms of learning and memory (for review and discussion see Bear, 1994). LTP was first reported in the perforant path-dentate granule cell synapses in the hippocampus, where brief trains of high frequency stimulation caused a sustained and long-lasting increase in the efficacy of synaptic transmission. Subsequent studies established that a similar potentiation could be elicited at the Schaffer collateral/commisural synapses in the CA1 region of the hippocampus as

well as at the mossy fibres synapses in the CA3 region of hippocampus. In particular, the pyramidal cells of the CA3 hippocampal region receive two main, anatomically distinct excitatory synaptic inputs: the associative-commissural and the mossy fibre pathways (Bliss and Collingridge, 1993) (**fig. 3.1**). The former contains fibers coming mainly from the contralateral hippocampus, whereas the latter is made of the axons of the granule cells. Both pathways exhibit LTP. All types of excitatory synapses in the hippocampus use glutamate as their neurotransmitter. Glutamate is known to bind to both ionotropic and metabotrobic receptors. The ionotropic receptors can be further subdivided into at least two types, AMPAr and NMDAr, based on the selective glutamate agonists used to distinguish them. LTP at the synapses made by hippocampal mossy fibers onto pyramidal neurons of the CA3 area does not require activation of NMDAr while at the other sites, including the synapse between the associative-commissural pathway and the CA3 pyramidal neurones, blockade of NMDAr blocks the induction of LTP (Nicoll and Malenka, 1995).

Most of the studies of LTP have been performed in the CA1 region of hippocampal slices where induction of LTP is input specific and associative, that is, modifications are restricted to activated inputs, and synapses are potentiated only if they are active while the respective postsynaptic dendrite is sufficiently depolarised. These characteristics are present in the majority of cases of LTP, also in other brain regions.

More recently, evidence has been obtained that synaptic transmission can also undergo Long Term Depression. LTD, in contrast to LTP, has been observed to occur both at active and inactive synapses. When LTD occurs at inputs whose activation contributes to the induction of the modification, it is called "homosynaptic' LTD. When it manifest itself at inputs that had been inactive during induction, it is addressed as "heterosynaptic". Some experimental protocols result in a depression of synaptic transmission that has a shorter duration (5-20 min)

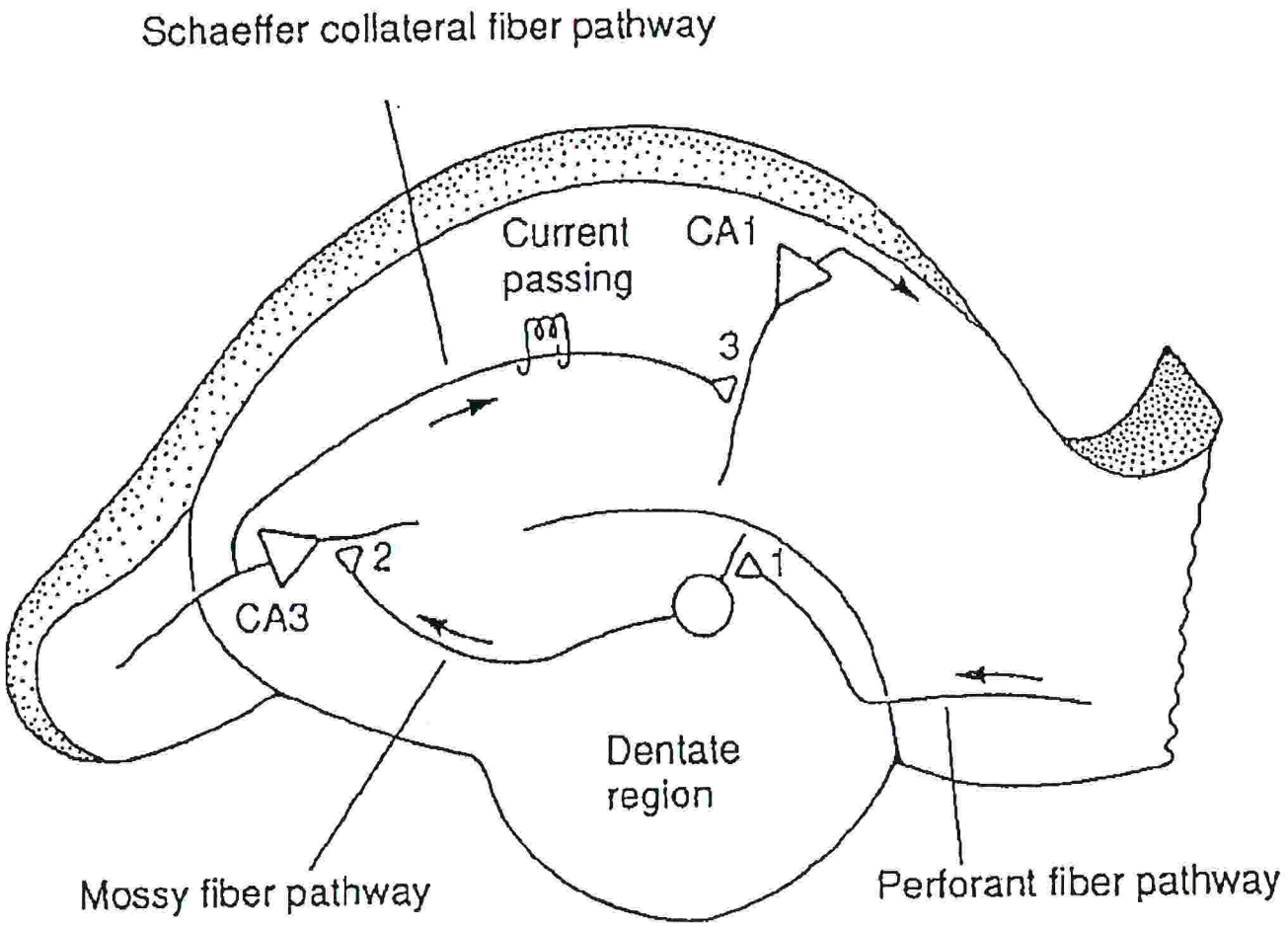


Fig.3.1 Main anatomically distinct synaptic pathways of the hippocampus

than LTD. This more transient change can be referred to as short-term depression (STD). The fact that STD can be elicited even when LTD is blocked suggests that STD and LTD may depend on different mechanisms.

3.1.2 Critical period plasticity

During development, LTP and LTD play a crucial role in the strengthening of synaptic connections, thus contributing to the final tuning of the neural circuitry (Fox, 1995). In other brain regions, most notably in the visual system, both activity-dependent synaptic plasticity and NMDA receptor activity may contribute to shape neuronal networks during brain maturation (Scheetz and Constantine-Paton, 1994). On theoretical and experimental grounds, LTP and LTD of excitatory synaptic transmission have been proposed to contribute to development of normal ocular dominance and the ocular dominance shift due to Monocular Deprivation in the visual cortex (Singer, 1995). In support of this proposition, both LTP and LTD can be induced at excitatory synapses in visual cortex in vitro (Artola, 1990; Kirkwood et al., 1993). More recently, it has been shown that LTP exhibits a critical period in the visual cortex (Kirkwood et al., 1995), that correlates with the falling phase of the critical period for ocular dominance plasticity (Fagiolini et al., 1994).

In the hippocampus, the expression of LTP or LTD changes markedly during development: LTD, which consists in a long lasting use-dependent decrease in synaptic efficacy, is more prominent in early postnatal life (Dudek and Bear, 1993). In the CA1 hippocampal region, both a non NMDA and a NMDA-dependent type of LTD have been described (Bolshakov and Siegelbaum, 1994). The former is usually present during the first postnatal week and is induced by repetitive stimulation (5 Hz for 3 min) of the Schaffer collaterals whereas the latter occurs during the second postnatal week and is produced by low frequency stimulation of the afferent pathway maintained for a prolonged period of time (1 Hz for 5-15 min.).

This form of LTD appears to be synaptic specific and saturable, and is prevented by loading the cell with the calcium chelator BAPTA (Mulkey and Malenka, 1992). LTD precedes the developmental onset of LTP, which reaches its maximal expression towards the end of the second postnatal week (Harris and Teyeler, 1984; Jackson et al., 1993).

Information concerning the developmental changes in activity-dependent processes in the CA3 hippocampal region has been obtained only recently, in our laboratory (Battistin and Cherubini, 1994). These experiments, which represent the background for the experiments described in this chapter, are reported in more detail in the following paragraph.

3.1.3 Mossy fiber CA3 synapses show a critical period for LTD induction

Important morphological changes take place in the hippocampus during the first two weeks of post-natal life. These are characterised by migration of the granule cells, by maturation of their axons, the mossy fibers, and by the development of thorny escrescences and giant synapses of the mossy fibres-CA3 level. At P4-P5 synaptic spines start to appear on the dendrites of CA3 pyramidal neurones and reach adult levels by P20 (**fig. 3.2**), concomitantly with the maturation of the mossy fibres (Gaiarsa et al., 1992). It is therefore possible that, because of the delayed maturation of the mossy fibres, LTP at the mossy fibre-CA3 synapse undergoes a critical period. This was the rationale behind the experiments carried out in this region during an early stage of development (Battistin, T., E. Cherubini, 1994). As already mentioned, the pyramidal cells of the CA3 hippocampal region receive two main anatomically distinct excitatory synaptic inputs: the associative-commissural and the mossy fibre pathways. In the adult hippocampus, in the CA3 region, two distinct forms of LTP have been characterised, depending on the pathways which are activated. An HFS train to the associative-commissural pathway induces LTP, which requires activation of NMDA receptors, whereas the

same stimulus, to the MF pathway, produces an LTP which is NMDA-independent.

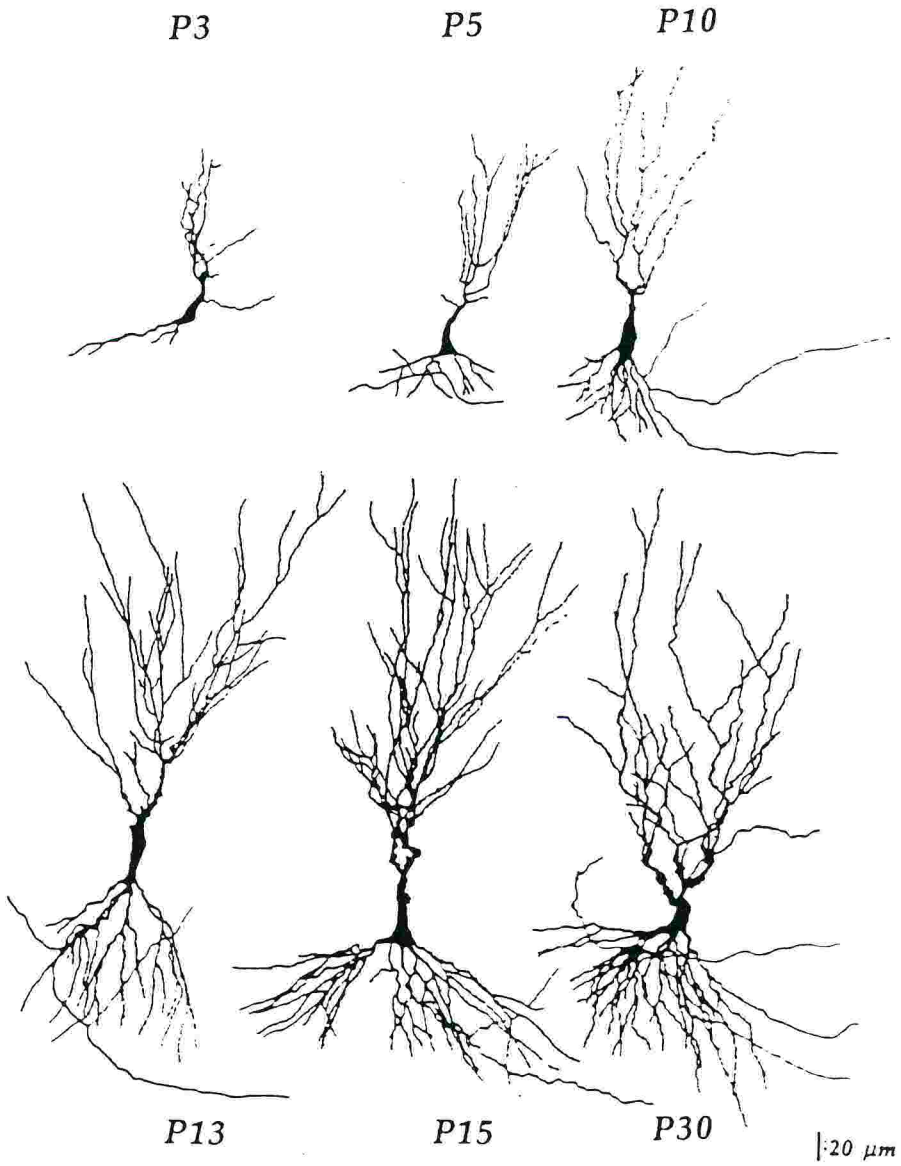


Fig. 3.2 Postnatal maturation of CA3 pyramidal cells. Camera lucida reconstruction of representative CA3 pyramidal cells stained by intracellular peroxidase injection at different stages of development (from Gaiarsa. et al., 1992)

To avoid contamination with the NMDA-dependent associative-commissural pathway LTP, all the experiments were performed in the presence of the NMDA receptor antagonist CPP or APV. At P11 a high frequency stimulation train to the mossy fibers (100 Hz 1s) produced a stable and long lasting depression of the field excitatory postsynaptic potential (EPSP).

The depression attained its maximum immediately after the train, then slowly (10-15min) declined to a stable value. This form of LTD was homosynaptic. In fact, when two independent fibre bundles synapsing on the same population of postsynaptic cells were alternatively stimulated, depression of synaptic strength occurred only in the synapse receiving the HFS train, whereas the control synapse was unaffected. LTD was obtained in 67% of cases in slices from P6-P14 rats. The mean reduction of the field EPSP measured 40-60 min. after the HFS was 20-30%. In 22% of cases HFS of the mossy fibers induced only a short term depression of the field EPSP. Immediately after the train, the EPSP was reduced to 30% of the control response, then slowly returned to the control level in 20-30 min. In some cases, no changes in synaptic strength occurred after an HFS.

There was a tendency for LTD to shift to LTP with age. As shown in figure **3.3** between P6 and P11 an HFS train to the mossy fibres induced LTD in 60% of cases. After the second week of age, the same high frequency stimulation train induced LTP as in adult neurons. The depression or potentiation of the synaptic transmission was not due to enhancement of presynaptic axon excitability, since the curve relating the stimulation intensity and the amplitude of the afferent volley was unchanged after HFS.

This form of LTD was NMDA-independent since it was induced in the presence of CPP. It required ionotropic non-NMDA receptor activation since it was blocked by CNQX. Results from experiments using nifedipine allowed us to exclude the possibility that nifedipine-sensitive calcium channels were involved in the induction of LTD. There is general agreement that LTD in several brain structures is a postsynaptic phenomenon. To test whether the expression of HFS LTD at the

mossy fibre-CA3 synapse involved a postsynaptic mechanism, paired pulse facilitation, which is a clear presynaptic phenomenon (Zucker, 1989), was monitored before and after the induction of LTD.

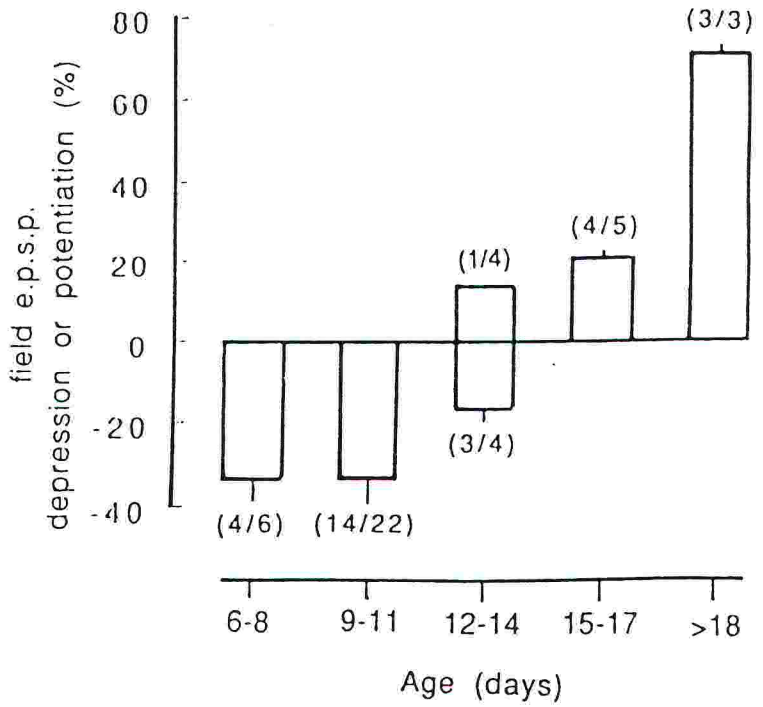


Fig.3.3 Each column in the graph represents the mean depression or potentiation of the amplitude of the field EPSP measured 40-60 min. after the HFS train in different age groups. Bars represent the SD. On the top of each column is the number of slices depressed or potentiated/number of slices tested (from Battistin and Cherubini, 1994).

The magnitude of paired pulse.during LTD was not affected, implying that LTD expression occurred at a site different from that involved in paired pulse facilitation. Also it was found that a rise in intracellular calcium, most likely (even if not exclusively) through voltage dependent calcium channels, is essential for the induction of mossy fibre LTD, since LTD was blocked when calcium was chelated into the postsynaptic cell with BAPTA (Gyori et al., 1996).

3.2 *Results*

3.2.1 Implant of αD11 cells into the lateral ventricle of neonatal rats inhibits LTD induction at the mossy fibre-CA3 synapse.

High levels of NGF are present in the hippocampus (Ernfors et al., 1990; Ayer-LeLievre et al., 1988) and the p75 NGF receptor is also present in this brain region (Pioro and Cuello, 1990). The TrkA NGF receptor is present on BF nerve terminals, but its expression on hippocampal neurons has also been reported (Cellerino, 1996), a finding, however, of some controversy. In addition, NGF has been shown to protect hippocampal neurons against hypoglycaemic damage, an effect which involves modulation of calcium currents (Cheng et al., 1993). After an initial experimental survey of different paradigms of synaptic plasticity in CA1 and CA3 regions of hippocampal slices of αD11 implanted rats, we have focused our attention on the mossy fibre-CA3 pathways. In particular, cholinergic innervation from BF to the hippocampus reach adult level around P20 (Milner et al., 1983), concomitantly with the full development of the mossy fibre-CA3 synapse (Amaral and Dent, 1981).

Selective blockade of endogenous NGF at early stages of postnatal development in other systems, such as the visual system, has revealed that NGF modulation could be considered one mechanism to modulate the critical period for synaptic plasticity. For instance, the group of Pisa showed that the presence of αD11 antibodies at early stages of postnatal development maintains visual cortical connections sensitive to

Monocular Deprivation beyond the expected end of the critical period (Domenici et al., 1994). Total deprivation of the visual experience by rearing animals in the dark has so far been the only manipulation reported able to prolong neural plasticity in the visual cortex.

Focusing, on the hippocampus, it seems plausible to hypothesise that endogenous NGF directly or indirectly through the cholinergic system may influence the developmental shift from LTD to LTP occurring at the mossy fibre-CA3 synapse. As shown previously (chapter 2), cholinergic neurons of BF are sensitive to endogenous NGF deprivation during the first postnatal week. In order to investigate whether NGF deprivation modulates the developmental shift from LTD to LTP at the mossy fibres-CA3 synapse, αD11 hybridoma cells were injected into the lateral ventricle of rats at postnatal ages P2. As a control, rats were injected with the parental cell line P3U. Electrophysiological analysis on hippocampal slices started one week after cell injection. Field EPSPs were recorded in CA3 dendritic region after stimulation of mossy fibres in the proximity of the granule cell layer. HFS of mossy fibres was induced in 13 slices from P3U (control) rats and in 16 slices from αD11 (treated) rats, after at least 10 min of stable recording of CA3 field EPSPs. All the experiments were performed in the presence of the NMDA receptor antagonist CPP to block the NMDA-dependent associative commissural pathway LTP. HFS induced LTD in 4/16 slices from αD11 and 6/13 slices from P3U implanted rats. In two slices from both group of animals HFS produced short term depression (STD). In the remaining 10/16 αD11 and 5/13 P3U slices no changes in synaptic strength occurred after an HFS. Examples of LTD and STD recording are shown in **fig. 3.4.**

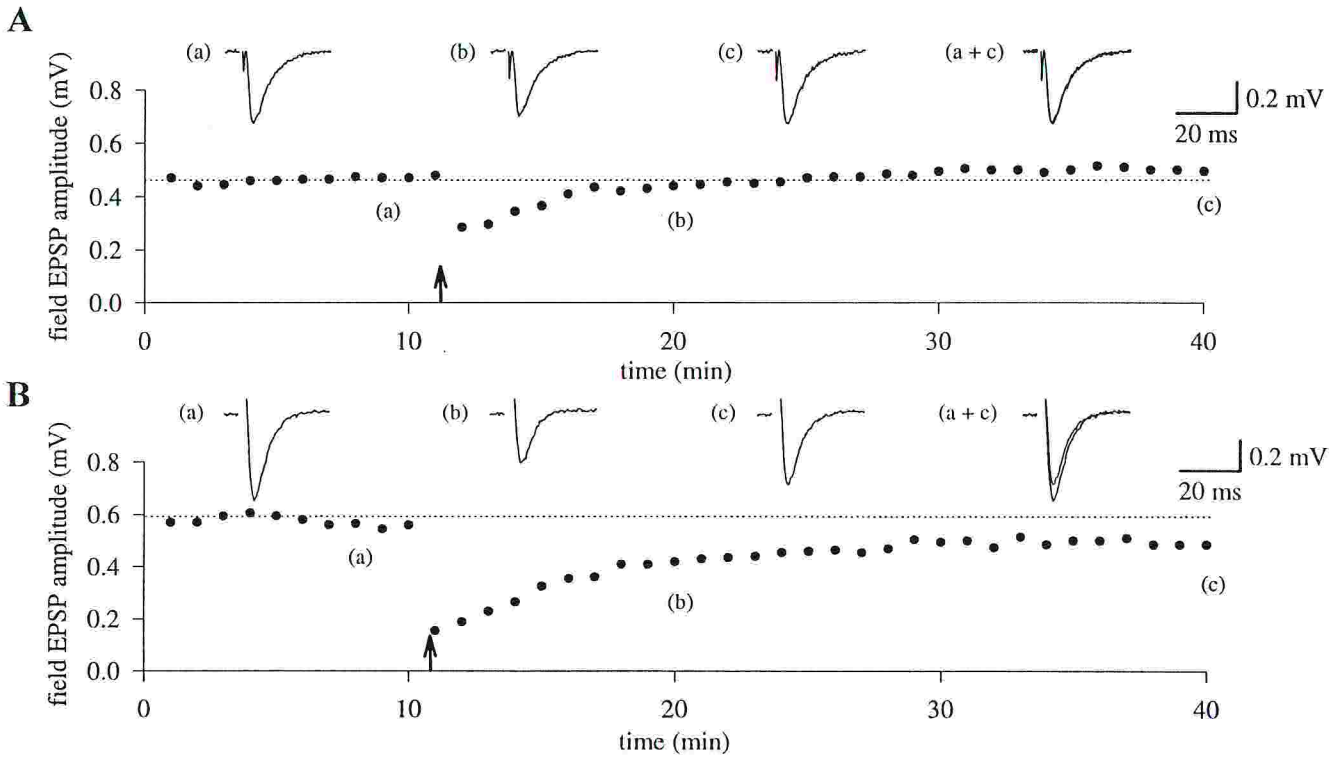


Fig.3.4 LTD and STD of the field potential induced by a train of HFS to the mossy fibers in hippocampal slices from P9 rats. (A) Example of STD induced by HFS, at P9-P12. (B) Example of LTD. Insets shows the average of three responses taken at the times indicated by the letters above the graph. (a+c) indicates superposition of typical responses before and after HFS. (a+c) is the same in A (no LTD) and different in B (example of LTD).

Considering all cases in which LTD lasted at least 30 min after a HFS train, its magnitude and time course were not significantly different in αD11 slices, with respect to control slices (**fig. 3.5**). However, it was noticed that a HFS failed to elicit any change at all in efficacy of response in a proportion of αD11 slices significantly higher than control (**fig. 3.6**).

Thus, these data suggest that NGF deprivation, during the first postnatal week, may inhibit the induction of MF-LTD, at least as observed between postnatal days P9 to P12. Experiments are in progress to determine the developmental dependence of this effect, over the time period in which the transition between prevalent LTD to LTP is observed, in order to verify whether the anti-NGF treatment determines a shift of the LTD to LTP transition curve.

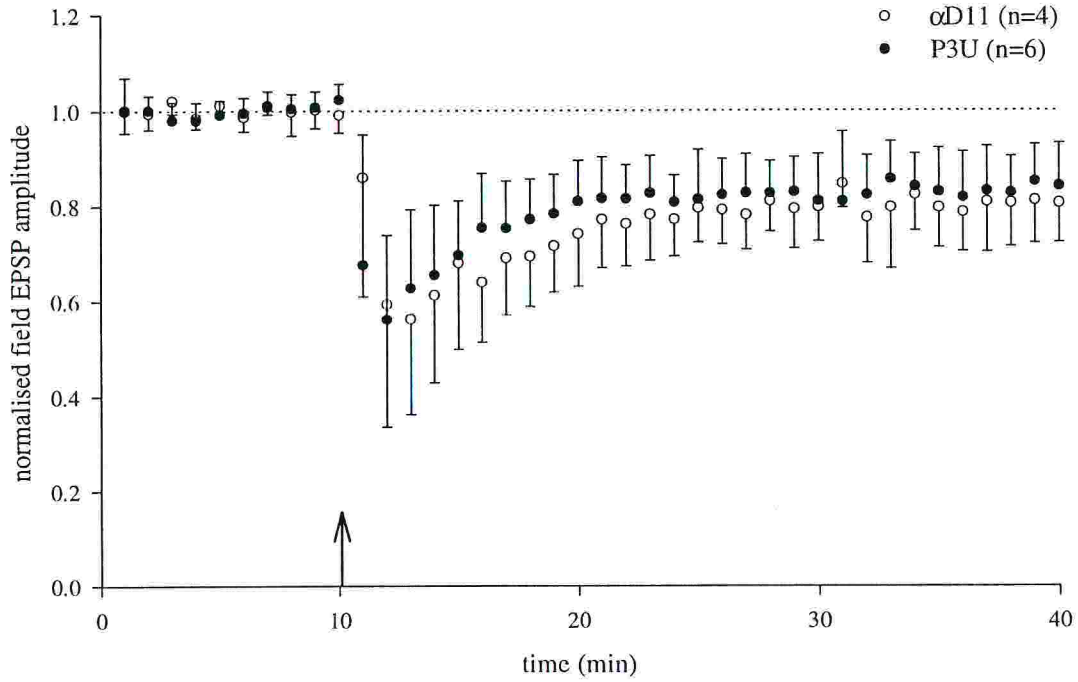


Fig.3.5 Pooled data from 6 slices of P3U and 4 slices of αD11 treated rats displaying LTD of the fEPSP in the CA3 region. Synaptic modification is expressed as a percentage (mean ± s.e.m.) of the fEPSPs amplitude before and after HFS (arrow). The difference in the amplitude of the fEPSPs does not reach statistical significance, using the t test.

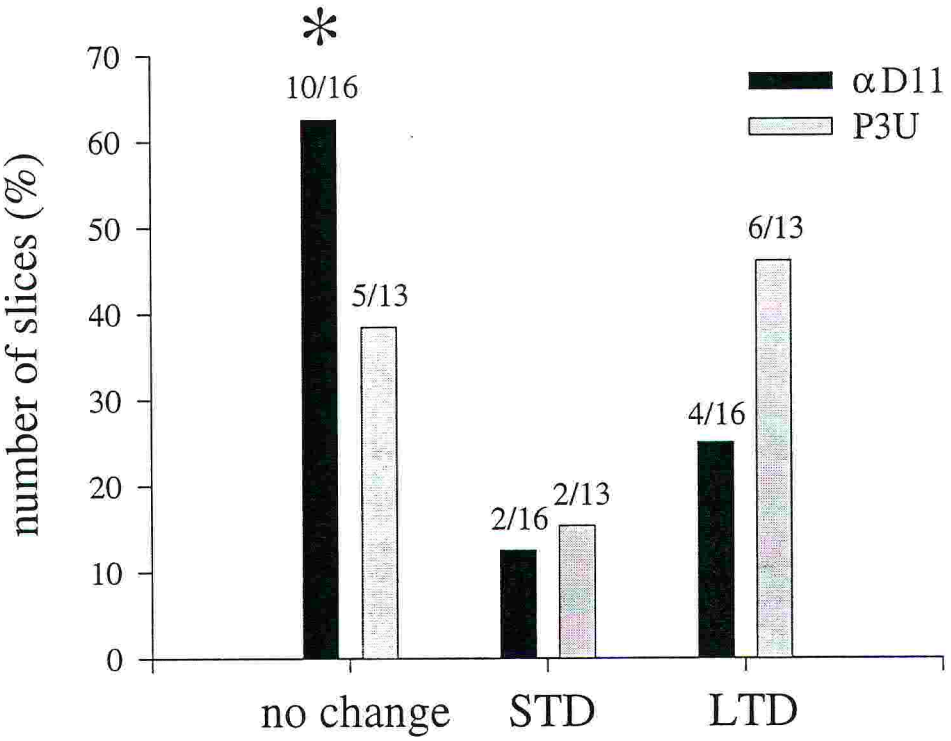


Fig.3.6 Each column is the number of cases, in percentage, in which a HFS produces LTD or STD or no change in slices of αD11 and P3U rats. The proportion of slices in which HFS failed to elicit any change in synaptic strength is significantly higher for αD11 than for P3U slices using the binomial analysis ($P<0.05$).

3.3 *Discussion*

The MF-LTD is NMDA-independent and is therefore different from that observed in the CA1 region following sustained low frequency stimulation of the afferent pathway (Dudek and Bear, 1992; Mulkey and Malenka, 1992). Glutamatergic ionotropic non-NMDA receptor activation is necessary for the induction of LTD. Like other forms of LTD, a rise in intracellular calcium is responsible for the induction of MF-LTD (Gyori et al., 1996). It is currently held that, in different experimental paradigms of synaptic plasticity (including CA1 and visual cortex), the levels of calcium rise/entry in the postsynaptic cell determine the transition between LTD and LTP (Artola and Singer, 1993; Bear and Malenka, 1994) (**fig. 3.7**). It is tempting to draw a parallel between the transition from LTD to LTP observed in many systems as a function of the frequency of conditioning stimulation (Bear, 1995) (**fig. 3.8**), and the developmentally regulated transition from LTD to LTP observed at the mossy fibre-CA3 synapses (Battistin and Cherubini, 1994). One could argue that the developmentally regulated relevant parameter for that transition to occur is related to Calcium influx/rise in the postsynaptic cell, in response to the same electrical stimulation. Of course, different mechanisms could be responsible for this Calcium influx/rise in the CA1 and the CA3 regions.

The MF-LTD resembles that described in the visual cortex, where the same tetanic stimulation can induce either LTP or LTD depending on the level of depolarisation of postsynaptic neurons (Artola et al., 1990). Functional changes in synaptic connections may limit depolarisation and calcium fluxes during development. In the CA3 region, dendritic spines start appearing at P4-P5 and reach adult levels at P20, concomitantly with the maturation of the mossy fibres (Gaiarsa et al., 1992). The latter may have an inductive role in spine formation. Synaptic activity, in turn, would regulate protein phosphorylation and intracellular calcium (Mulkey and Malenka, 1993) and this may be the critical point leading to the development of LTD or LTP in early and late postnatal life respectively.

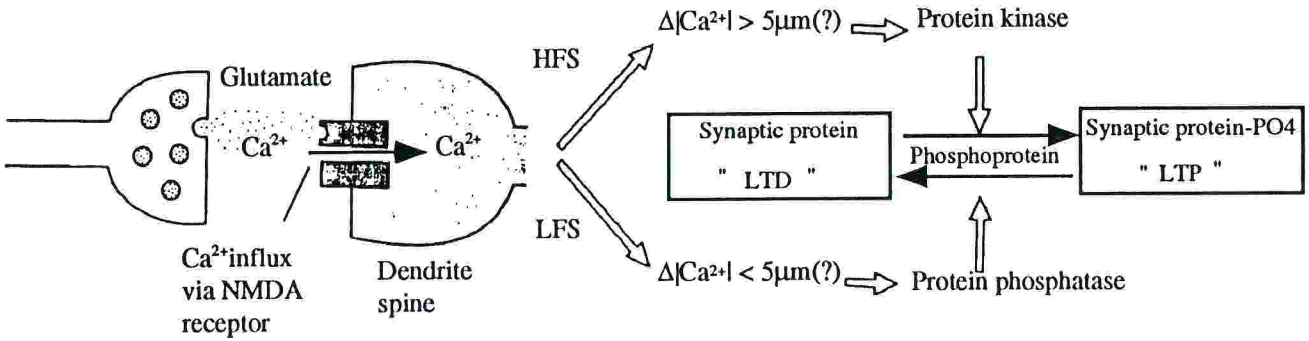


Fig.3.7 Model for the induction of LTP and LTD at CA1 synapses.

During afferent activity Ca^{2+} enters dendritic spines through NMDA receptors. During high-frequency stimulation (HFS), Ca^{2+} reaches high levels and preferentially activates a protein kinase. During low-frequency stimulation (LFS), lower Ca^{2+} levels are achieved and this preferentially activates a protein phosphates. Both the kinase and phosphatase act on a common synaptic phosphoprotein, the phosphorylation state of which controls synaptic strength (from Bear and Malenka, 1994)

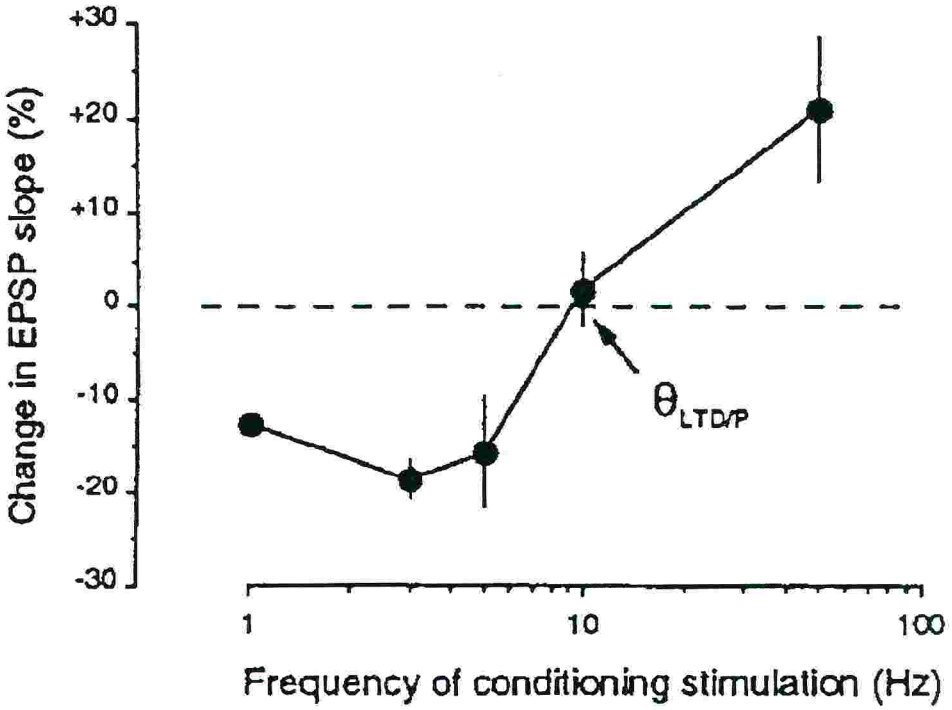


Fig.3.8 The mean (+/- SEM) effect of 900 pulses of conditioning stimulation delivered at various frequencies to the Schaffer collaterals on the synaptic response measured in rat CA1 30 min postconditioning (replotted from Dudek and Bear, 1992)

The results described in this chapter suggest that NGF deprivation, during the first postnatal week, may inhibit the induction of MF-LTD, at least as observed between postnatal days P9 to P12. Our experiments do not allow to conclude whether the observed inhibition of LTD derives from a direct effect of NGF deprivation in the hippocampus proper or to an indirect effect, secondary to a reduction of cholinergic input to the hippocampus. More data are needed, at different time points, to distinguish between the two possibilities and also to investigate if NGF interferes selectively with the induction of MF-LTD and does not affect LTP at later ages.

It could be hypothesised that αD11 antibody could modify mossy fibre development, or the density of synaptic contacts made by these fibres, and that these, in turn, may differentially affect the postsynaptic level or entry of Calcium. The possibility that anti NGF treatment may affect the development of mossy fibres in CA3 is being experimentally addressed, by performing a Timm histochemistry procedure to stain the zinc containing mossy fibres. In this respect, it is interesting to note that intraventricular administration of anti-NGF antibodies in adult rats blocks mossy fibre sprouting induced by kindling (Van der Zee et al., 1995; Rashid et al., 1995), but not the mossy fiber sprouting induced by pilocarpine induced seizures (Holtzman et al., 1995). A direct effect of NGF on mossy fibres would require that these cells express either the Trk or the p75 receptors (or both). While p75 is expressed in the hippocampus, and in the CA3 region in particular (Pioro and Cuello, 1990), the presence of TrkA in hippocampus is more controversial (Cellerino, 1996). The second possible explanation for the effect of anti-NGF treatment on mossy fibre LTD is that the effect is achieved indirectly, by a reduction i) in the synthesis of acetylcholine in the BF cholinergic neurons (see chapter 2) or ii) in the release of acetylcholine from BF neuronal terminals in the hippocampus (Knipper et al., 1994). A reduced synthesis/release of acetylcholine would result in a reduced cholinergically mediated synaptic drive to the target neurons, principally granule cells of the dentate gyrus. It is known that granule cell activation upregulates the mRNA for BDNF (Lindefors et al., 1992) and that disruption of the cholinergic

innervation causes a decrease in BDNF mRNA in the hippocampus (Berzaghi et al., 1993; Lapchak et al., 1993). The result of anti NGF treatment, therefore, could result in a reduced release of BDNF from the granule cells, with an ensuing reduced effect on the density of mossy fibre innervation. Experiments aimed at studying the levels and the localisation of the BDNF mRNA and protein in the hippocampus of αD11 treated rats will be performed to address this issue.

3.4 Materials and Methods

Experiments were performed on hippocampal slices obtained from P6-P12 Wistar rats. The standard method for preparing and maintaining the slices was as follows: the brain was quickly removed from the skull and the hippocampi were dissected free. Transverse 500-600 μm thick slices were cut with a tissue chopper and incubated at 33-34 °C in oxygenated artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl, 126; KCl, 3.5; NaH2PO4, 1.2; MgCl2, 1.3; CaCl2, 2; NaHCO3, 25; glucose, 11. The ACSF was equilibrated with 95% O2 and 5% CO2 (ph 7.3). Following a recovery period (1-3 h) one slice was transferred to a recording chamber in which it was continuously superfused at 33-34°C with oxygenated ACSF at a rate of 3 ml/min.

Extracellular field potentials were recorded with 2 M NaCl-filled microelectrodes (resistance 2-5 MΩ) positioned in the stratum radiatum. The mossy fibres were stimulated at 0.05 Hz using bipolar twisted NiCr-insulated electrodes (50 μm o.d.) which were positioned on the mossy fibres tract, close to the recording electrode. Due to the complexity of the dentate gyrus CA3 circuitry (Claiborne et al., 1993) and to the small number of mossy fibres present in young animals, a site that produced a synaptic response with a minimal stimulation current was searched. Recording sites close to the dentate gyrus were preferred (Regeher et al., 1991).

Drugs were dissolved in ACSF and applied through a three-way tap system by changing the superfusion solution to one which differed only in its content of drug(s). The ratio of flow rate to bath volume ensured complete exchange within 1 min. Drugs used were (+)-3-(2-carboxypiperazin-4-xl)-propyl-1-phosphonic acid (CPP; gift of Dr. P.L. Herrling, Sandoz).

If not otherwise stated, results are presented as mean +/- SD.

Chapter 4

Blocking endogenous NGF: how does it interfere with memory?

4.1 Introduction

4.1.1 Monoclonal antibodies to NGF affects learning of radial maze in adult rats

The first suggestion of a link between cognitive functions and NGF was acquired from experiments in which a supply of exogenous NGF was shown to ameliorate the spatial memory impairment found in aged rats (Fischer et al., 1987; Fischer et al., 1991). These studies provided a correlation between the amelioration in the spatial memory impairment and a significant reversal of the cholinergic neural atrophy. Recently, it has been shown that memory deficits associated with ageing can be reversed, both in terms of acquisition and retention, by several members of the neurotrophin family and that this effect may be mediated through activation of multiple neurotrophin receptors associated with both the cholinergic and non cholinergic systems (Fischer et al., 1994).

A direct test for the possible involvement of endogenous NGF in the learning of adult animals in vivo has been recently attempted by studying the learning performance of mice heterozygous for deletion of the NGF gene (Chen et al., 1993), and in rats treated with anti NGF antisera, in the Morris water maze (Van der Zee et al., 1995a). In both cases, the deficits found in the spatial learning behaviour of aged rats are not reproduced. Moreover, in the first study, the transgenic approach makes it difficult to disentangle the possible effects of NGF shortage on the development of the nervous system from those on neural plasticity per se, as well as the possible role of compensatory mechanisms which may become operative during the development of knock-out mice.

The group of Nicoletta Berardi in Pisa has attempted to study the role of endogenous NGF in neural plasticity in normal adult animals, by using the hybridoma implant

approach described in chapter 2. Thus, hybridoma cells secreting specific anti-NGF antibodies (αD11) were implanted into the ventricles of adult rats, and performance in a radial maze was assessed before and after intraventricular transplant of αD11 cells. 14 adult Long Evans rats were intraventricularly implanted with αD11 cells. 13 littermates were implanted with parental P3U myeloma cells as controls. The animals were tested in an 8 arm radial maze, where both the short term memory (working memory) and long term memory (reference memory) can be evaluated. All animals were tested before the cell transplant (first test) and after transplant (second test). The antibody titre was determined in samples of CSF collected at different intervals from the time of implant. The antibody titre remains at fairly constant levels for up to 21 days after implant. This level, around 0.4 ng/μl, is in molar excess with respect to the maximal estimated concentration of NGF in the CNS of adult rats. The second test was therefore carried out under conditions in which we would expect extracellular NGF to be blocked by the antibodies. Before the implant the performance of the two groups of animals, (αD11 and P3U), are indistinguishable (**fig. 4.1A**).

The effect of cell transplant on the learning ability is clear cut (**fig. 4.1B**): the learning curve of αD11 rats becomes shallower, and levels off at a higher level of daily errors with respect to that of P3U rats (the difference is significant, $p<0.001$). In all αD11 implanted rats, post implant performance was significantly impaired in comparison to their pre-implant performance, both in terms of acquisition rate (**fig.4.2**) and of final level of accuracy (**fig. 4.3**). Analysis of individual cases showed that the impairment in performance correlated well with αD11 titre in CSF.

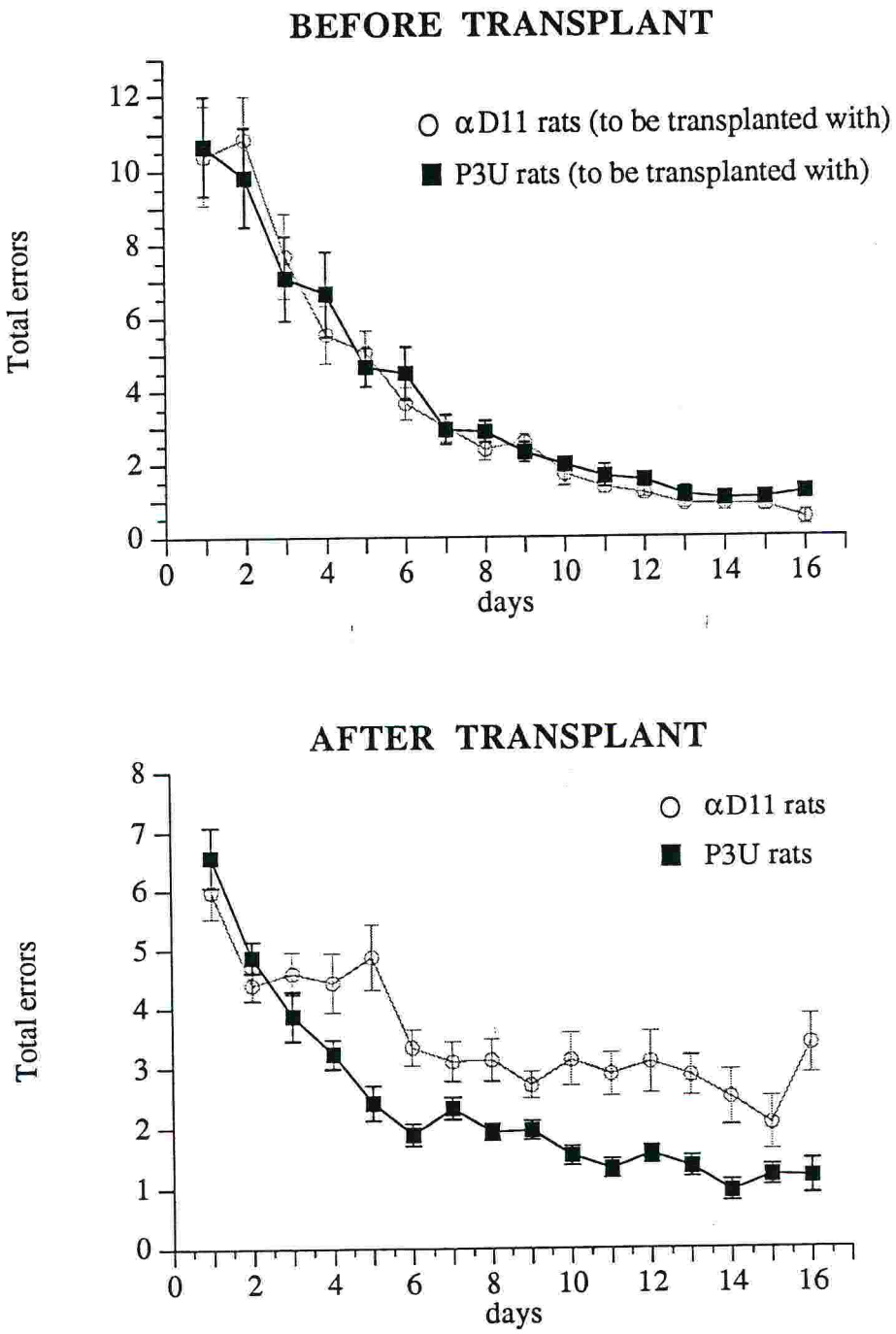


Fig.4.1 Learning curves for total errors (mean total errors as a function of time) for rats aD11 (n=14) and rats P3U (n=13) before (A) and after cell implant (B). Learning curves for P3U and aD11 rats do not differ significantly before cell implant, while they do after it ($p<0.001$) (from Costagliola et al., 1995).

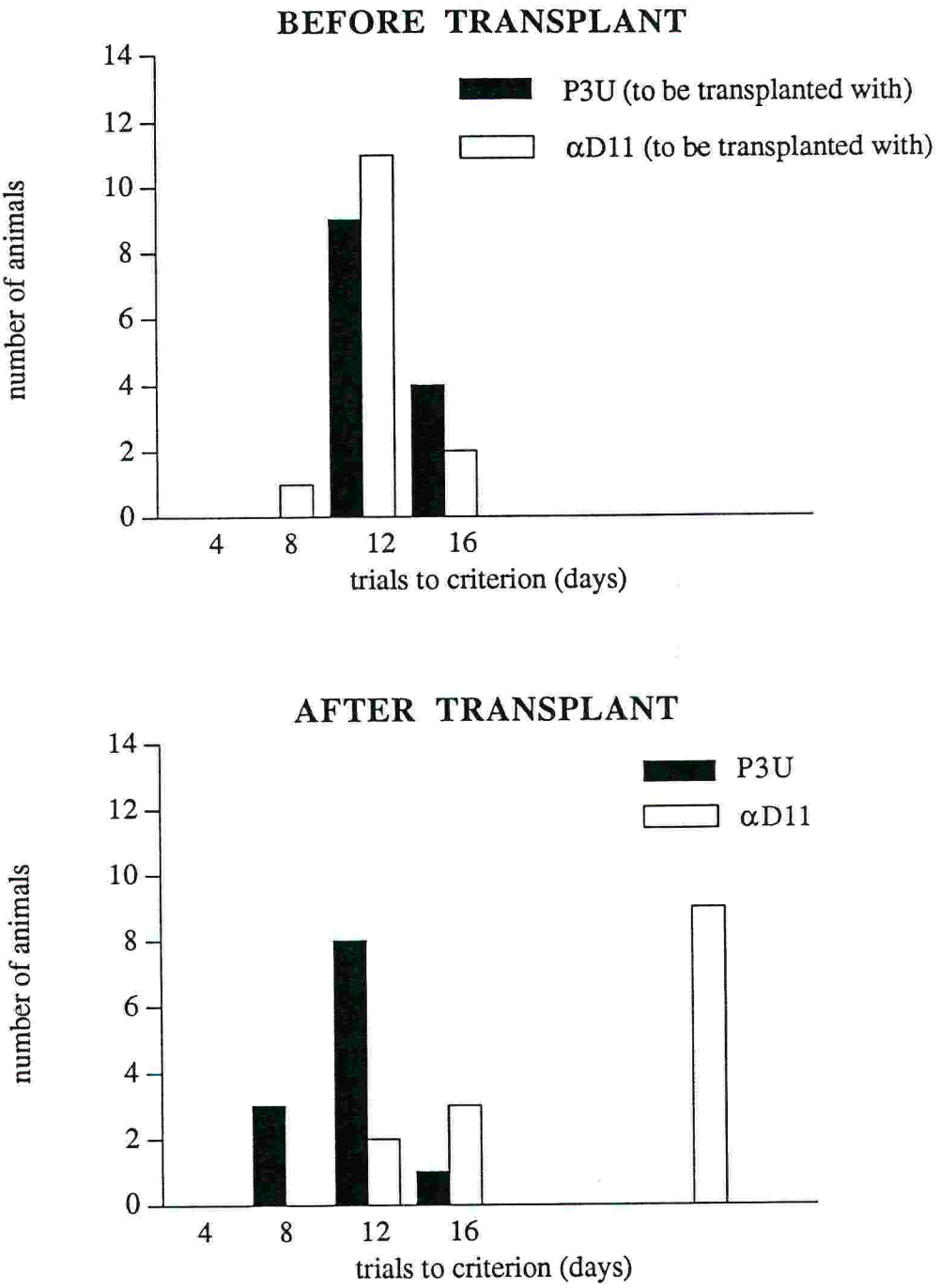


Fig.4.2 Distribution of numbers of trials required to reach criterion for aD11 (n=14) and P3U (n=13) rats before and after transplant. The two groups do not differ before transplant while they do significantly after it, with aD11 animals exhibiting slower acquisition rates. 9 aD11 rats never reach criterion within the 16 days of testing (from Costagliola et al., 1995)

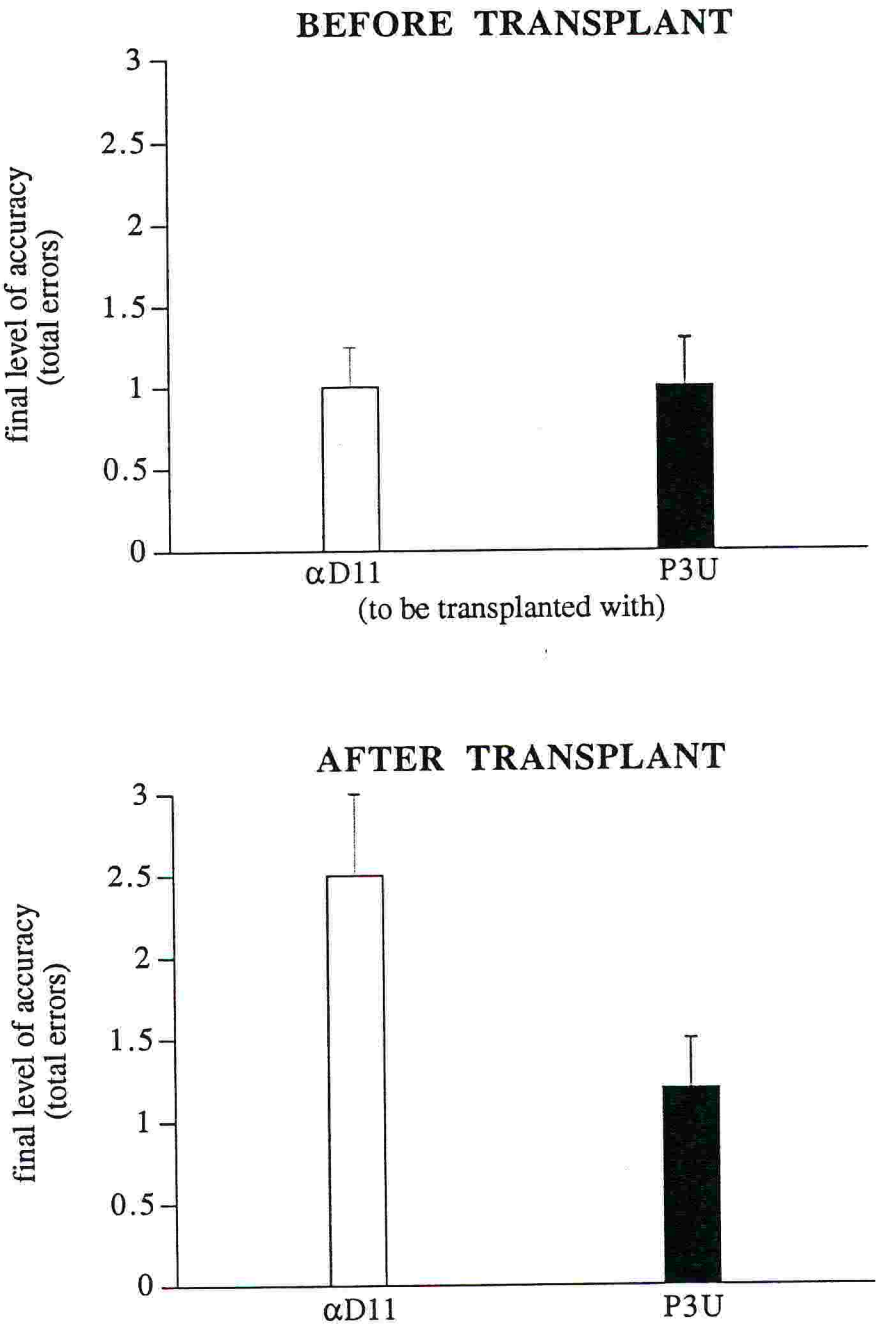


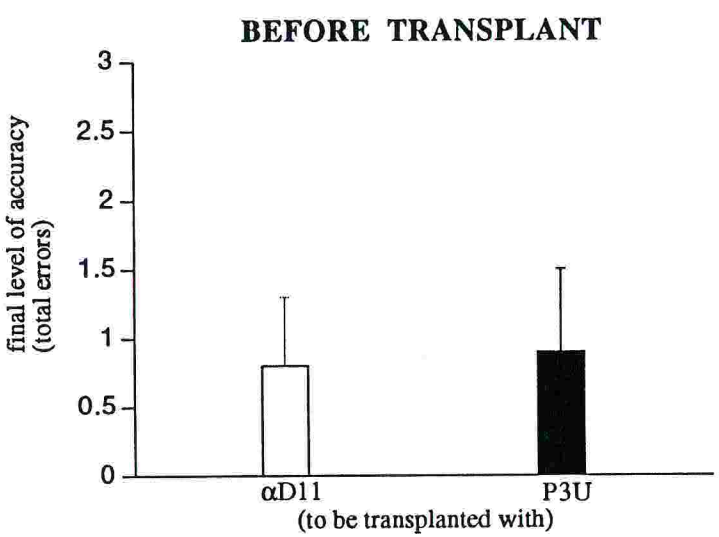
Fig.4.3 Final level of accuracy (mean total errors in the last three days of testing) for aD11 (n=14) and P3U (n=13) rats before and after transplant. The difference after transplant is significant (Costagliola et al., 1995)

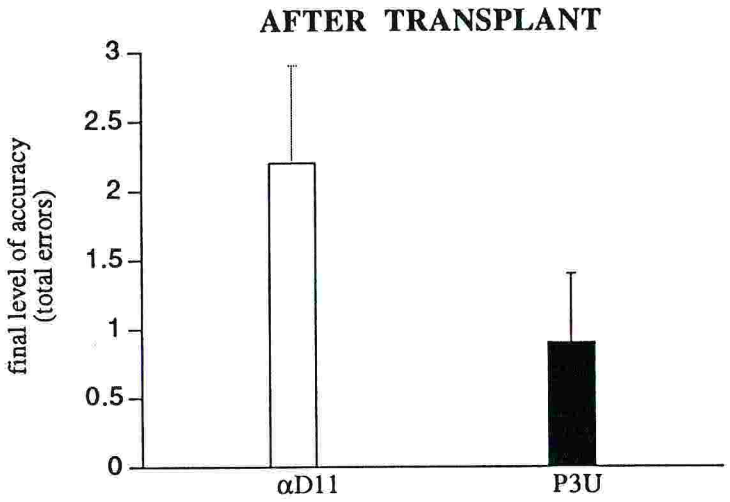
In order to assess whether the impairment brought about by αD11 cell implant was specific for spatial memory, a subgroup of αD11 and P3U rats were tested in a visually cued version of the maze. The difference in the level of accuracy of performance in the maze between αD11 and P3U rats disappeared when visual cues were put at the end of each baited arm. Finally, alterations in motor activity and exploratory behaviour were excluded, testing the animals in an Open Field motor test. A further analysis allowed to evaluate whether the changes found in learning performance were due to differences in reference memory, working memory or both. Therefore, the mean reference memory errors (entries in never baited arms) and mean working memory errors (re-entries in already visited arms) were determined for P3U (n=13) and αD11 rats (n=14) before and after transplant . In αD11 rats both types of errors were significant increased in αD11 rats after transplant.

It should be underlined that the learning performance recovered in αD11 rats when monoclonal antibodies were no longer detectable in the CSF (**fig.4.4**). This suggests that a continuous presence of antibodies is needed to produce an impairment in spatial memory and, as a correlate, it could be inferred that a continuos availability of NGF is required to allow normal performance in spatial learning (Costagliola et al., 1995).

Various neurotransmitters, neuromodulators and neuropeptides have each had their moment in the limelight with respect to a possible role in cognitive function. First and foremost among these is acetylcholine, which, on the basis of clinical and neuropathological data, as well as on work in a variety of animal models, has long been implicated in learning and memory. The analysis of ChAT immunohistochemistry in αD11 implanted rats did not show a major modification in BF staining. Furthermore a quantitative test of ChAT activity also showed no reduction in either cortex or hippocampus from αD11 rats. These results provided further proof that the sensitivity of BF cholinergic neurons to NGF deprivation is limited to a critical period for NGF sensitivity of BF neurons during the first

postnatal week (chapter. 2) and that the memory loss observed here may be independent of cholinergic activity.





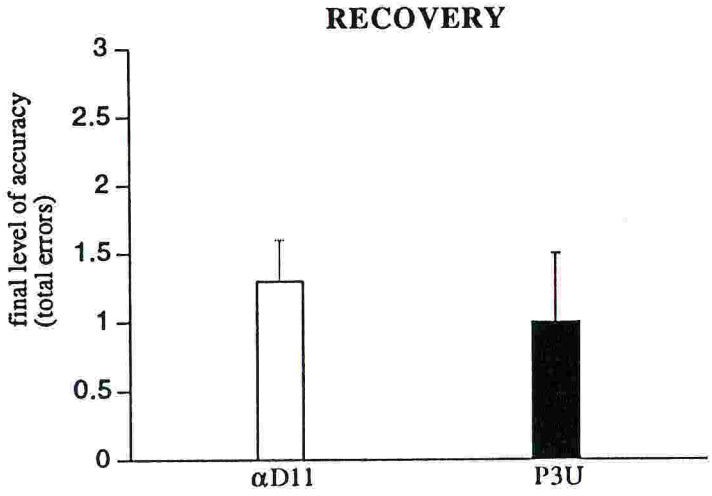


Fig.4.4 The difference in final level of accuracy (and acquisition rate also, not shown) introduced by cell transplant between learning performances of aD11 (n=8) and P3U (n=7) rats is no longer apparent in the third learning test (recovery) run after disappearence of aD11 antibody from CSF (Costagliola et al., 1995)

Analysis of acetylcholine release is in progress to further pursue this point. However, the impairment in spatial learning of αD11 implanted rats seems not to be mediated through changes in the forebrain cholinergic nuclei. This is in line with recent data which challenge the view that the BF system is crucial for rodent spatial navigation (Fibiger, 1991; Dunnet, 1991) . Moreover, the recent development of an immunolesioning technique using 192-IgG-saporin (a monoclonal antibody to the low-affinity NGF receptor, p75, coupled to a cytotoxin) shed light on the issue, as 192 IgG-saporin can selectively kill cholinergic neurons within the region of injection, while leaving neighbouring cells unaffected. Interestingly, the behavioural results obtained from animals with 192 IgG-saporin lesions to their BF cholinergic system reveal that, despite cholinergic cell loss comparable to that which can occur in an aged population, no learning or memory impairment is observed (Berger-Sweeney et al., 1994; Torres et al., 1994). This confirms the conclusion above that BF cholinergic neurons do not play a role in this form of memory.

We were interested to investigate further how NGF block interferes with spatial learning of αD11 implanted adult rats. A tempting hypothesis is that αD11 antibodies exert their action in the hippocampus. We are trying to verify this hypothesis by analysing the characteristics of LTP and LTD in the CA1 region of the hippocampus (Ruberti et al., 1996). The initial results are described in the results section of this chapter.

4.1.2 The role of LTP, LTD and Metaplasticity in Spatial Learning

The hippocampal structure has long been shown to have a clear role in learning and memory, as indicated by lesion experiments (see Squire, 1987). Hippocampal lesions have served to define two classes of associative learning: a complex class of learning, termed declarative or configural, that is sensitive to hippocampal lesions, and a simple class of learning, termed procedural, that is insensitive to hippocampal lesion. Hippocampus dependent learning tasks commonly involve association

among multiple sets of cues, whereas hippocampus-independent tasks commonly involve a simple association.

LTP, a form of synaptic strengthening, and homosynaptic LTD (discovered more recently), which involves the weakening of synaptic connections, are two forms of synaptic plasticity present in many areas of the brain, but most usually studied in the hippocampus (Bear, 1994). Both conform to rules that Hebb initially postulated to be the basis of the associative learning and memory: a) coincident presynaptic and postsynaptic neuronal activity is required for its induction; b) the modulation is specific for the activated synapse; and c) it is long lasting.

In the past few years many attempts have been made to answer the question whether LTP and/or LTD in the hippocampus provide the neural underpinning for spatial learning in rats. One of the studies that has been most influential in promoting this perception is the saturation experiment of Castro et al. (1989), in which repeated high frequency trains were delivered to the perforant path to drive LTP in the dentate gyrus to a ceiling and thus, for a time, to remove the potential for further plastic change (Castro et al., 1989). Performance in the water maze was found to be significantly impaired in this condition; and furthermore, several days later, following the decay of LTP, performance had recovered to control levels.

Moreover, many experiments interfering with molecular components of synaptic plasticity have been performed to show the relationship between hippocampal LTP/LTD and spatial learning. A pioneering experiment in this field was that of Morris which examined the spatial learning capability of rats whose hippocampal LTP had been blocked by AP5, an antagonist for glutamate receptors of the NMDA class. Since these rats were defective in both LTP and spatial learning, at least under certain conditions, these data suggest that LTP is the cellular mechanism for this type of learning (Morris et al., 1986; Davies et al., 1992).

More recently, the approach of gene knockout was applied to proteins that are clearly implicated in synaptic plasticity, to examine further the correlation between LTP/LTD deficiency and the severity in spatial learning task impairment. Knock out

of either the α calcium/calmodulin kinase II (αCaMKII) (Silva et al., 1992) or the fyn tyrosin kinase gene (Grant et al., 1992) gives rise to mice that have a defect in LTP and show concomitant deficits in spatial memory. However, both blockade of the NMDA receptor and the knock out of CaMKII or fyn interfere with LTD as well as LTP. Indeed, when LTD is spared (as in mice with knock out of the PKCγ gene) there is only a minor learning impairment even though high frequency LTP is blocked completely (Abeliovich et al., 1993; Abeliovich et al., 1993a). Since in these mice the block of LTP can be overcome by priming stimulation at frequencies that produce LTD, Tonegawa and his collegues have suggested that LTD may serve as a primary mechanism for the acquisition of spatial information.

Until recently, the - no LTP, poor learning - pattern of results was strongly sustained. However, these experiments do not unequivocally prove the role of LTP or LTD in learning. In the cases in which synaptic plasticity has been disrupted (i.e., NMDA blockade, LTP saturation, and transgenics), there has not been adequate demonstration that the effect of the treatment is restricted to plasticity mechanisms. Interpretation of these experiments depend on the assumption that the effect is restricted to a blockade of, or interference with, LTP mechanisms, and that normal mechanisms of information transmission are intact. In the transgenic experiments, the effects of developmental adaptations confuse the issue. Moreover, this approach shares the interpretation difficulties encountered by other types of systemic treatments in which altered plasticity in brain regions other than the one under study cannot be ruled out as participants in the behavioral change.

Furthermore, many studies have complicated the picture by weakening the correlation between the brain's ability to exhibit LTP/LTD and the animal's spatial learning facility. Following the first demonstration that LTP saturation leads to the expected deficits in spatial behaviour, other laboratories conducted experiments that failed to replicate the early findings (Korol et al., 1993; Jeffery and Morris, 1993; Sutherland et al., 1993). There is now ample evidence that these failures to find behavioural disruption were primarily due to a failure to achieve adequate saturation

of hippocampal synaptic weights, and that the proportion of saturated synapses necessary to produce a behavioural deficit may be different for different behavioural tasks (Barnes et al., 1994). Recently Morris and his colleges (Bannerman et al., 1995) have provided evidence that, under some circumstances , LTP may not be necessary for spatial memory . They used the Morris swim task in which rats learn the location of a submerged escape platform in a circular pool of water. Normally, acquisition of this task is prevented by hippocampal NMDA receptor blockade; however, if rats are pretrained in the same apparatus in a different room, acquisition is normal under NMDA receptor blockade but it is nevertheless prevented by hippocampal lesions. One might conclude that hippocampal LTP was unnecessary for this form of learning. Another explanation includes the possibility that the rats do not always use a spatial strategy to solve the problem. The Bannerman study may thus be explained by inertial navigation within the already familiar framework of the pool itself, which may not require learning any new metric relationship between landmarks and the goal. Finally a number of knock out mice do not display the expected relationship between LTP/LTD and spatial learning. The two strains of PKA (cAMP dependent protein kinase A) mutants and the Thy-1 (a glycosiyphosphatidylinositol-anchored neuronal cell surface protein) mutant mice all display deficiency in at least one of the forms of synaptic plasticity at various points in the hippocampal circuitry, yet they perform normally in hippocampus-dependent learning (Huang et al., 1995; Nosten-Bertrand et al., 1996).

Activity dependent modifications of synaptic efficacy are fundamental to the storage of information in the brain. Altought LTP or LTD have the right properties for learning mechanisms, they may not be the only way by which synaptic activity can leave a lasting trace. A step forward may be to consider LTP and LTD as two equally necessary elements for the storage of information. For example: LTD could prevent saturation of LTP by actively resetting potentiated synapses and so making the synapse more responsive and temporally flexible than it would be with passive decay alone, and LTD of a previously potentiated synapse could serve as a

forgetting mechanism for information stored by increasing synaptic strength. In a network of synaptic contacts, LTD could serve to accentuate the signal from neighbouring potentiated synapses, in a manner conceptually analogous to the way lateral inhibition functions to promote edge detection in the visual system.

Theoretical studies have suggested that the critical level (threshold) of synaptic activity needed to induce LTD versus LTP of synaptic transmission should vary as a function of previous synaptic activity. The Bienenstock-Cooper-Munro theory (Bienenstock, E., et al., 1982) was originally proposed to account for aspects of experience-dependent visual-cortical plasticity. It assumes that active synapses undergo LTP or LTD depending on the level of postsynaptic response. Bienestock called the critical level of postsynaptic response at which the sign of the synaptic modification reverses from negative to (LTD) to positive (LTP) the modification threshold (θ_m). Another important assumption of the BCM theory is that the value of θ_m is not fixed, but rather slides as a function of the history of postsynaptic activity. This plasticity of synaptic plasticity, also called metaplasticity, may play an important role in the modification of neural networks, both in development and during learning (Artola, and Singer, 1993; Abraham and Bear, 1996).

Now it has been shown that the effectiveness of particular synapses can be turned up and down through successive episodes of LTP and LTD. In the CA1 region of hippocampus and in layer III of visual cortex, repetitive low frequency stimulation (LFS) (Dudek, 1993) of excitatory afferents produces LTD, and HFS produces LTP. Both forms of synaptic plasticity depend on NMDA receptor activation and postsynaptic Ca entry. Available data support a model in which the state of correlation of pre and postsynaptic activity is converted by the voltage-dependent NMDA receptor channel into a graded postsynaptic Ca signal: schematically, Ca triggers LTD at low concentrations and triggers LTP at high concentrations (see chapter 3). The smooth transition from net LTD to net LTP may be demonstrated by systematically varying the frequency of conditioning stimulation. There is now physiological evidence that the threshold for induction of LTP and LTD is regulated

by the previous history of synaptic activity both in hippocampus and neocortex. The hippocampal LFS-induced LTD is more robust early in postnatal life and declines with increasing age in CA1 region (Dudek and Bear, 1993). However, following establishment of LTP with HFS, conditioning stimulation at the same low frequency results in far greater depression (Fujii et al., 1991). Perhaps, 1 Hz stimulation in addition to producing LTD de novo, also causes depotentiation by interfering with the mechanisms of LTP consolidation. Alternatively, a tetanus that fails to produce lasting LTP can still promote LTD following 1Hz stimulation. The latter interpretation is supported by evidence that a tetanus that fails to produce lasting LTP can still promote LTD following 1 Hz stimulation (Wexler and Stanton, 1993). Moreover previous tetanic stimulation can also inhibit subsequent induction of LTP (Huang et al., 1992). These data suggest that HFS shifts $\theta_{LTD/LTP}$ to the right.

4.2 Results

4.2.1 LTP is normal in αD11 adult rats

We have examined LTP in hippocampal slices from P3U and αD11 rats at 20-27 days after cell implant, after they have carried out behavioural tests. Because of the interindividual variability between different experiments, ELISA was performed on the CSF of each αD11 implanted rat two days prior to recording to control for antibody levels. All αD11 animals impaired in the radial maze had antibody levels significantly high to be likely to inhibit endogenous NGF. Analysis of electrophysiological experiments was accomplished on slices from αD11 rats which were impaired in radial maze and with abundant αD11 antibody level in their CSF. Field excitatory postsynaptic potentials were recorded in stratum radiatum of CA1 after stimulation of Schaffer collaterals. LTP was elicited by 2 trains (100Hz for 1s) of tetani with an interval of 10 sec. We found that in the majority of slices from αD11 animals LTP was inducible and its early phase (up to 1 hours after tetanus) was essentially the same as that found in slices prepared from P3U rats. An example

is shown in **fig.4.5 A, B**. The probability distribution of the LTP slopes was the same for 7 αD11 and 10 P3U slices (**fig.4.5,C, left**).

In order to analyse the electrophysiological properties of the hippocampus during exactly the same period in which the behavioural tests had been carried out, i.e. 7-21 days after implant, an independent group of animals was examined at 13-17 days after implant (without behavioural testing). Also in this case, we found that in the majority of slices from 15 αD11 animals LTP was inducible and its early phase was essentially the same as that found in slices prepared from 7 P3U rats (data not shown) .

In these animals we have also tried to analyse the late phase of LTP. In NGF -/+ mice it has been shown that the late phase of LTP is abnormal (Shinsky et al., 1995), pointing to deficits in the consolidation of short term to long term plasticity. Therefore we have analysed the late phase of LTP (3 hours after the tetanus) in hippocampal slices from αD11 animals at 14-17 days after implant. In order to rule out generalised postsynaptic changes, which might change synaptic field potentials in a non selective manner, we performed control recordings from a second input to the same postsynaptic cells. Two independent fibre bundles synapsing on the same population of postsynaptic cells were alternatively stimulated. Potentiation of synaptic strength occurred only in the synapse receiving the HFS train, whereas the control synapse was unaffected and the response at the control input was stable during the period of the experiment (**fig 4.6**). We were unable to observe differences in late phase of LTP between slices from 7 αD11 and 3 P3U rats.

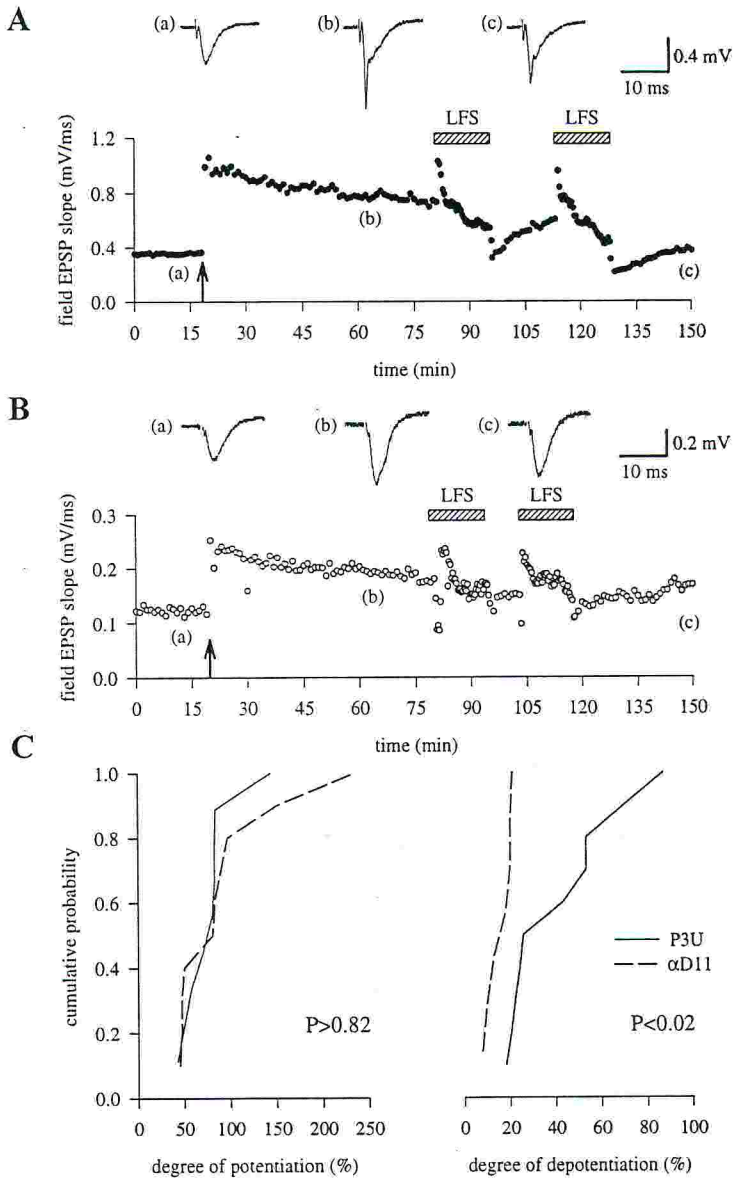


Fig.4.5 (**A/B**) Plots of fEPSP slope from slices of a P3U (**A**) and a αD11 (**B**) treated rat. LTP could be elicited in both slices by HFS delivered at the time indicated by the arrow.Two sets of LFS reduced the degree of potentiation in the P3U slice to a greater extent than in the αD11 slice. Insets show the average of 4 responses taken at the time indicated by the letters on the graph.

(C) Cumulative probability distributions of fEPSP slope potentiation by HFS (left) and depotentiation by LFS (right) in 10 slices from P3U (solid line) and 7 slices from (dashed line) treated rats. P3U and αD11 slices displayed a potentiation of 91.44 ± 54.32 % and 96.90 ± 57.82 % (mean ± STD) respectively, 1 hr after HFS and 41.58 ± 23.72 % and 15.61 ± 5.41 % (mean ± STD) respectively 20 min after the last set of LFS.

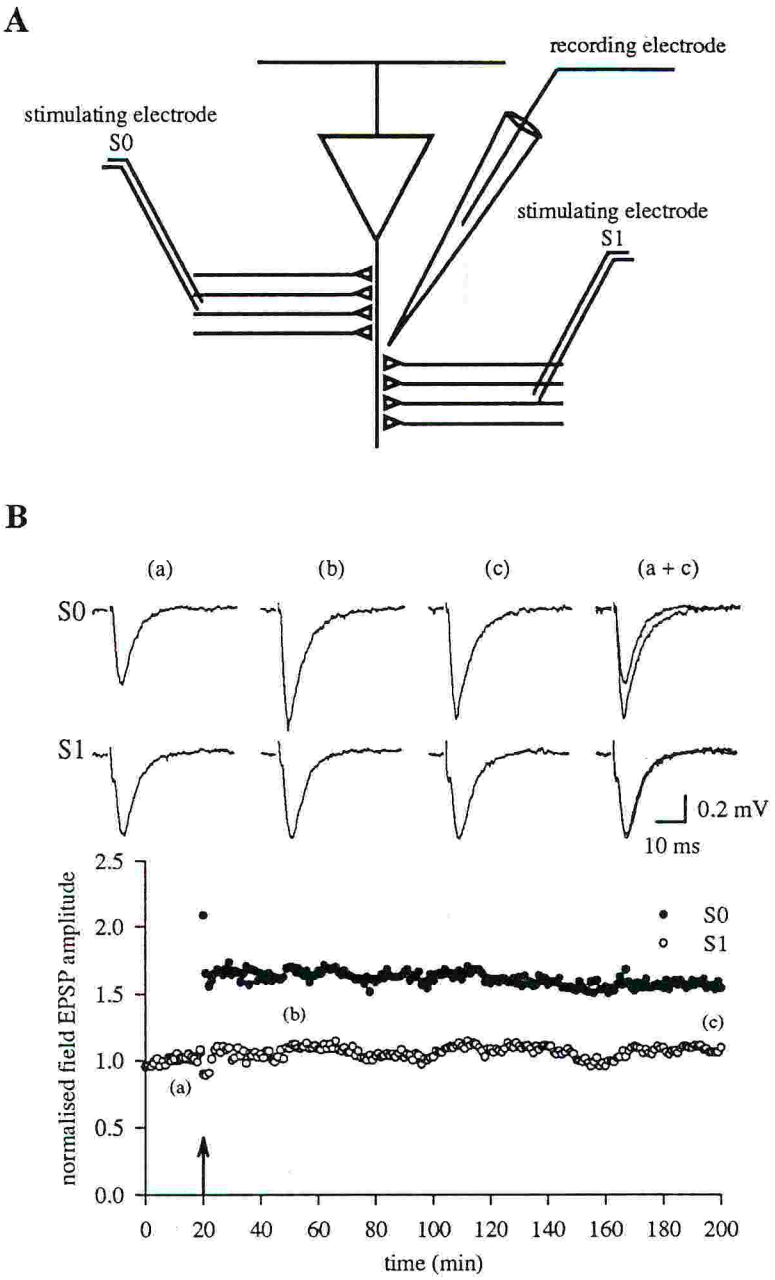


Fig.4.6A Schematic drawing for fEPSP recordings from two converging inputs. Two different fbers bundles (S0 and S1) synapsing on the same population of postsynaptic neurons were alternatively stimulated at 0.03 Hz, but only one of the two pathways received HFS train.

4.6B In αD11 slices no fading of potentiation could be observed after 3 hours from induction of LTP. A train of HFS delivered on S0 at the time indicated by the arrow, induced an input specific LTP of about 50% (filled circle) which persisted for after 3 hours of recording. Insets show the average of 4 responses taken at the time indicated by the letters on the graph.

4.2.2 Primed homosynaptic LTD is inhibited in αD11 rats

LTD in the CA1 region of the hippocampus has been implicated as one of the underlying mechanisms of spatial learning (Abeliovich et al., 1993; Abeliovich et al., 1993a; Mayford et al., 1995; Bach et al., 1995).

In hippocampal slices from behaviourally characterised P3U and αD11 rats, 1 hour after induction of CA1 LTP, LTD was produced in the same synaptic pathway by low frequency stimulation.

2 sets of low frequency stimulations, consisting of 900 pulses at 1 Hz, spaced by 30 minutes interval, elicited depotentiation in both αD11 and P3U rats. We have carried out field potential recordings in 12 P3U slices and 15 αD11 slices. Final analysis was accomplished on a lower number of slices, because in some slices we were not able, for technical reasons, to complete the long experimental protocol in a satisfactory way.

The results show that total depotentiation was diminished in αD11 slices (**fig. 4.5 A,B**), with respect to P3U slices. To quantify this effect we have determined the probability distributions of LTD slope for 7 αD11 an 10 P3U slices. As shown in **fig. 4.5 C (right)** the probability distribution for the LTD slope obtained from αD11 slices is significantly shifted to smaller values, as shown from statistical analysis, using the Kolmogorov-Smirnov non parametric test.

Thus, we conclude that in αD11 rats impaired in the radial maze learning test, CA1 LTP was normal and did not differ from that obtained in control P3U slices, while, on the other hand LTD in the same synaptic input after LTP was induced, is defective.

A direct implication of these results is that metaplasticity following LTP induction is altered in αD11 rats. This correlates with the learning impairment observed, but the mechanism underlying this correlation remains to be established.

4.3 *Discussion*

The data show that LTP with normal characteristics in both early and late phases is induced in the CA1 field of hippocampal slices from αD11 treated adult rats. Both the early and the late phases of LTP induced in αD11 rats were normal. The fact that a dramatic impairment in a spatial memory related behavioural task did not correlate to an impairment in CA1 LTP was not so surprising, in light of a growing body of recent work. We have started to turn our attention towards synaptic plasticity at the mossy fibre-CA3 synapses of αD11 rats. However, from the literature it appears that ablation of protein kinase A by gene targeting, produces a selective defect in mossy fibre LTP and the elimination of mossy fibre LTP, but does not affect spatial learning (Huang et al., 1995). On the other hand, in knockout mice deficient in mGluR2, LTD induced by low frequency stimulation was almost fully abolished, but this hippocampal LTD does not seem to be required for spatial learning (Yokoi et al., 1996). Thus neither LTP nor LTD at the mossy-fibre-CA3 synapses appears to be required for spatial learning.

My results suggest that the impairment in spatial memory found in αD11 rats correlates to an impairment of an effectively primed LTD. The use of the method of cell implantation in adult brain allows us to exclude the possibility that the spatial memory deficit is a function of a developmental effect, a criticism always difficult to answer in knock-out studies. In fact, physiological levels of NGF are present during neuronal development. To strengthen the correlation between the impairment in spatial memory and inhibition of primed LTD it will be interesting to analyse primed LTD in the αD11 rats which recovered learning performance after anti-NGF monoclonal antibodies were no longer detectable in the CSF.

In order to identify the time scale of requirement for NGF in synaptic plasticity the reversal of primed LTD inhibition could be obtained by treating of slices from αD11 rats with NGF. It has recently been shown for another neurotrophin, BDNF, that treatment of slices from BDNF knock out mice with recombinant BDNF reverses the deficit in LTP, suggesting an acute role for BDNF in LTP at the Schaffer

collateral -CA1 synapse (Patterson et al., 1996). If the action of NGF on synaptic plasticity is on a longer time scale and the deficit in primed LTD of αD11 rats reflects modifications with a longer time span, reversal may be achieved by implanting cells producing NGF in αD11 rats.

The interpretation that the spatial memory impairment of αD11 rats is caused by defectively primed LTD is limited however, by the fact that the electrophysiological characterisation was done only in vitro. The effect on LTP and LTD in the intact animals was not determined in the context of this thesis. Also, it is formally possible that the memory deficit arises from functional changes in other regions of the brain., since NGF receptors are also expressed in neocortex, striatum and BF. Future experiments will allow us to address this issue.

The impairment in spatial learning of αD11 rats resembles that observed in aged rats, thus providing a model of an adult animal with age-related impaired cognitive function, but it remains to be seen whether primed LTD is impaired in aged rats.

The synaptic mechanisms that underlie learning and memory in the mammalian brain are not well characterised. Nevertheless, most models of learning and memory invoke some form of use-dependent alteration in synaptic strength like LTP. However the definitive experiment to link LTP and learning has yet to be done. More recently LFS-induced LTD, a well-established model of synaptic plasticity, was proposed as the mechanism of hippocampal based learning. On the other hand, recognition of the presence of metaplasticity might yield new insights into the question of how information is stored in the nervous system (Bear, 1995). The analysis of transgenic mice expressing autonomous CaMKII provides intriguing speculations on the involvement of metaplasticity in memory. In these mice the entire frequency-response curve is shifted systematically to favour LTD (Mayford et al., 1995). Thus, at moderate frequencies of stimulation, LTP is lost in the transgenics and LTD is enhanced. The mutant mice showed a profound deficit in a task which requires them to learn to navigate to a specific location using only spatial cues (Bach et al., 1995). The spatial memory deficit does not correlate with a change

in HFS-induced LTP in CA1, which is normal in the mutants. The authors point out that this memory deficit might be a result of the observed deficit in LTP produced by 5Hz stimulation. However, it is plausible that the spatial memory deficit is a consequence of shifting θ_m ltd/p, which in theory would disrupt memory formation. It is within this framework that we could attempt to explain our results. There is the possibility that in αD11 rats blocking of NGF, interfering with synaptic functions and/or biochemical processes, can leave an enduring trace that affects the subsequent induction of synaptic plasticity. The cellular and molecular basis of this enduring trace is an interesting question for future experiments. This trace could interfere with spatial learning in a radial maze task. In order to asses this hypothesis we should perform other electrophysiological recording at different stimulation frequencies, to analyse the modification threshold (θ_m) at which the sign of the synaptic response reverts from LTD to LTP. We may or may not find differing θ_m values for αD11 and P3U implanted rats.

As Bear (Bear, 1995) sustains "when it comes to making the connection between synaptic plasticity and memory, we are still much like the blind man and the elephant". However, I would like follow the spur of C.A. Barnes who has recently invited all to "further heights of creativity thus saving us from what otherwise appears to be a continued technological and conceptual struggle with only slight glimmers of light at the end of the tunnel" (Barnes et al., 1995).

4.4 *Material and Methods*

The methodological part of the behavioural experiments, performed by Nicoletta Berardi and R.M. Costagliola, is not described, as these do not form part of the thesis.

Determination of αD11 antibody levels from the CSF was obtained by an ELISA, as described in chapter 2.

4.4.1 Electrophysiological recording from hippocampal slices

Electrophysiological experiments were performed on hippocampal slices obtained from 3 months old Long Evans rats. The standard method for preparing and maintaining the slices was as follows: the brain was quickly removed from the skull and the hippocampi were dissected free. Transverse 400-450 μm thick slices were cut with a tissue chopper and incubated at 33-34 ^{o}C in oxygenated artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl, 126; KCl, 3.5; NaH2PO4, 1.2; MgCl2, 1.3; CaCl2, 2; NaHCO3, 25; glucose, 11. The ACSF was equilibrated with 95% O2 and 5% CO2 (pH 7.3). Following a recovery period (1-3 h) one slice was transferred to a recording chamber in which it was continuously superfused at 33-34^{o}C with oxygenated ACSF at a rate of 3 ml/min. Schaffer collaterals-commisural fibres were stimulated by bipolar tungsten electrodes. Extracellular field potentials were recorded positioned in field CA1 with 2 M NaCl-filled microelectrodes (resistance 2-5 MΩ). Tetanus to evoke LTP consisted of 2 trains of 100Hz stimulation, each lasting 1 s at an interval of 10 s. LTD protocol was 900 pulses of 1 Hz stimulation as described (Dudek and Bear, 1992). If not otherwise stated, results are presented as mean +/- SD.

4.4.2 Probability distribution of slopes

Potentiation and depotentiation were evaluated as percentages of increase and decrease of the slope of EPSP responses. For statistical analysis, the data were expressed as cumulative probability distributions and comparison between two

experimental situations was performed using the Kolmogorov-Smirnov non parametric test. To obtain the cumulative probability distribution plot, the data set, $x_1, x_2, x_i, .. x_N$ of EPSP slopes was ranked into ascending order and each point was plotted as x_i on the x-axis and its relative frequency, i/N, on the y-axis.

Chapter 5

Expression of anti-NGF antibodies in the CNS of transgenic mice

5.1 Introduction

5.1.1 The neuroantibody approach as a complementary model to gene knock-out

Of the methods introduced so far to study the mouse nervous system, gene knock out by homologous recombination has proved extremely successful in the generation of mice carrying predesigned mutations in the germline (Capecchi, 1989). However, because the mutation, most commonly a null mutation, is introduced in the germline of the mutant animals, it will exert its effects from the onset of animal development. Studies published to date have described the generation of knock-out mice for NGF and its receptors p75 and TrkA. Both the NGF and TrkA knockouts have resulted in rather predictable, albeit dramatic phenotypes (Smeyne et al., 1994; Crowley et al., 1994), and, by and large, have confirmed some known facts about the role of NGF in supporting the survival of its target neurons. The animals develop sores and evidence of self mutilation and animals of both genotypes die within the first month of life. Both knockouts show dramatically depleted population of DRG and trigeminal neurons by birth, and both have virtually absent sympathetic ganglia by 10 days of age. The function of p75 have also recently been investigated by gene targeting (Lee et al., 1992; Lee et al., 1994; Davies et al., 1993). p75 (-/-) animals survive to adulthood, but develop evidence of loss of subpopulations of sensory neurons. All this work has provided more details on the function of NGF in the PNS, but the challenging question on the physiological function of NGF in the CNS remains open. Altought gene knockout is a valuable approach, for many applications it is important that the inactivation of a particular gene occurs in a conditional or partial manner (e.g. in a predefined cell lineage or tissue, or at a certain stage of development), and in particular in the adult organism. This would allow one to

overcome problems posed by the fact that null mutations in the germline are often lethal, and would also allow a more precise and unambiguous analysis of the impact of a mutation. While conditional gene targeting now seems promising, through the use of the Cre-loxP recombination system (Gu et al., 1993; Kuhn et al., 1995), the application of this method to the nervous system has yet to be proven, as is the question whether the recombinase enzyme may be active in postmitotic neurons.

Studies performed so far to investigate the response(s) of BF cholinergic neurons to endogenous NGF showed the need for methods alternative to gene knock-out. Lesion experiments in vivo, and in vitro cell cultures suggested that BF cholinergic neurons may require NGF for survival. Other experiments, in which the supply or subtraction of NGF in vivo was performed, revealed that NGF could regulate the phenotype of these neurons, for instance by modulation of ChAT. The forebrain cholinergic neurons of NGF -/- mice were not changed in number but seem to be stained more lightly for choline acetyl transferase than wt mice (**fig.5.1**). However, these mice did not show the interesting differences described in chapter 2, where a critical period in the sensitivity of BF cholinergic neurons to NGF deprivation was revealed by implanting hybridoma cells into the ventricles of rat brains at different post-natal times. It is noteworthy that the loss of functional NGF at P2 produced a marked decrease of ChAT enzyme in BF neurons at postnatal day P9 in rats implanted with αD11 hybridoma cells (chapter 2, fig.2.4). As shown in the previous chapters and from the collaborative experiments with the group in Pisa, the intracerebral grafting of αD11 hybridoma cells has shown to be a powerful method to study the function of endogenous NGF in the developing or adult CNS. However, it is a technical method subject to both advantages and disadvantages. In its favour, αD11 cells are not tumorigenic and the antibody penetrates to brain regions far from the site of cell localisation, at levels sufficiently high to block NGF function. However, both the number of cells and the level of antibody decreases over time (albeit over a time scale of weeks), therefore, only experiments in which transient loss of NGF function is required can be performed.

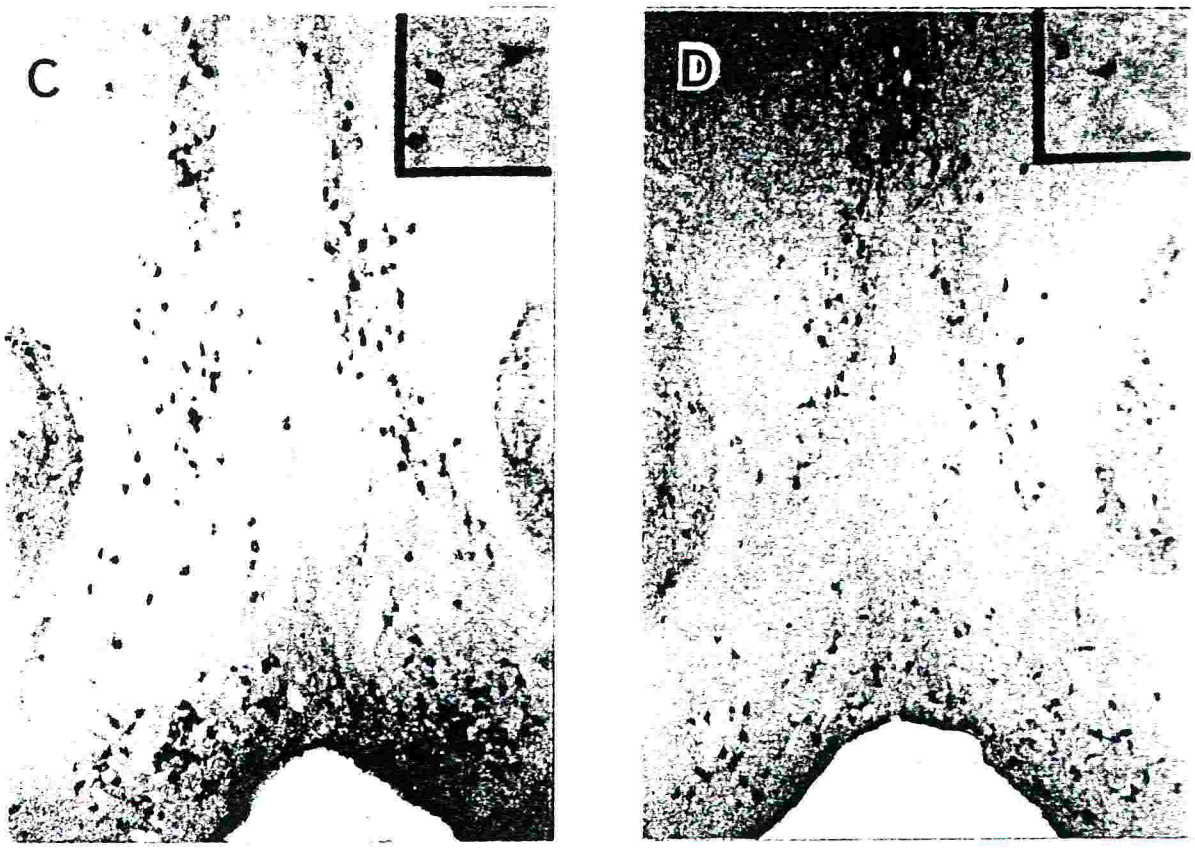


Fig.5.1 Basal forebrain cholinergic system in (-/-) mice lacking NGF. ChAT-immunoreactive neurons are apparent in the medial septum of 28-day-old (+/+) (C) and (-/-) (D) mice (from Crowley et al., 1994)

Moreover, the large interindividual and interexperimental variability requires a large number of samples for each experiment. This variability is due to the state of the cells in the injection buffer (cells are injected at a very high density), to their exact number and to the injection procedure per se. Finally, side effects due to surgical

manipulation, or to the need of treating injected animals with the immunosuppressant cyclosporine, should be considered in the interpretation of the results, even if, obviously, suitable controls are routinely performed. In order to perform functional and developmental studies in the central nervous system we have proposed and applied another strategy, utilising the expression of recombinant antibodies in the central nervous system, the so called neuroantibody approach (Cattaneo and Neuberger, 1987; Piccioli et al., 1991; Ruberti et al. 1993) (Introduction).

The successful application of the neuroantibody approach to interfere in vivo with the action of a target neuronal antigen such as the neuropeptide SP, recently provided by our group (Piccioli et al., 1995), confirms this approach as a complement to that of gene knock out by homologous recombination. One of the theoretical advantages of the neuroantibody approach with respect to the gene knock-out is represented by the theoretical possibility of achieving a localised spatiotemporal expression of the transgenic antibody. Another theoretical advantage of the neuroantibody method, over that of gene knock-out, is represented by the possibility of expressing the antibodies in transgenic rats, instead of mice, of note for those cases (such as for instance the visual system) in which much of the physiology has been studied in rats.

Before undertaking attempts in this direction, I decided to undertake the supposedly more straightforward production of transgenic mice with the transcription of the recombinant αD11 antibody driven by the minimal promoter and enhancer of the cytomegalovirus (CMV) early gene (Schmidt et al., 1990; Furth et al. 1991), which should be active in all cells. This should provide a test case to verify the ability of transgenic αD11 to successfully inhibit NGF action in vivo under more refined situations. The first section of this chapter describes the cloning of the αD11 antibody, a necessary prerequisite for the neuroantibody approach, and show that the recombinant antibody expressed in non-lymphoid cells shares the same properties as the parental one. A simpler molecular form, such as single-chain

antibody has also been produced, which may be of future use for local delivery applications in the adult CNS, possibly mediated by ex-vivo or in vivo viral infection. In the second section the results concerning the generation and the study of transgenic mice that express recombinant anti-NGF antibodies are reported.

5.2 Results

5.2.1 Cloning of the αD11 antibody by RACE.

mAb αD11 was a difficult antibody to clone and the isolation of its variable regions required the improvement of the currently used techniques to isolate antibody V regions.

The PCR has been used extensively (also in our laboratory (Bradbury, et al., 1995a) to clone V region cDNA from hybridoma cells using degenerate mouse primers which anneal within the relatively conserved framework sequences FR1 and FR4 of V regions (Orlandi, et al., 1989). Amplification of αD11 by this method produced two bands for both light and heavy chains at the expected size of 330-360 bp. These were cloned and sequenced. The heavy chain, however, was found to have a frame shift in CD3 and so could not code for a functional immunoglobulin. The light chain appeared to be normal and was shown by immunofluorescence to indeed produce a light chain protein expressed in COS cells. In order to clone the required αD11 VH region we developed a modification of the RACE (Frohman et al., 1988) for the cloning of Ig variable regions which is independent of the V region sequence (**fig.5.2**). This method involves a first strand cDNA synthesis using a primer specific for the Ig isotype of the hybridoma annealing within the hinge region, addition of a polyA tail using terminal transferase, followed by amplification using one primer which anneals to the rat CH1 domain and another which anneals to the 5' polyA tail.

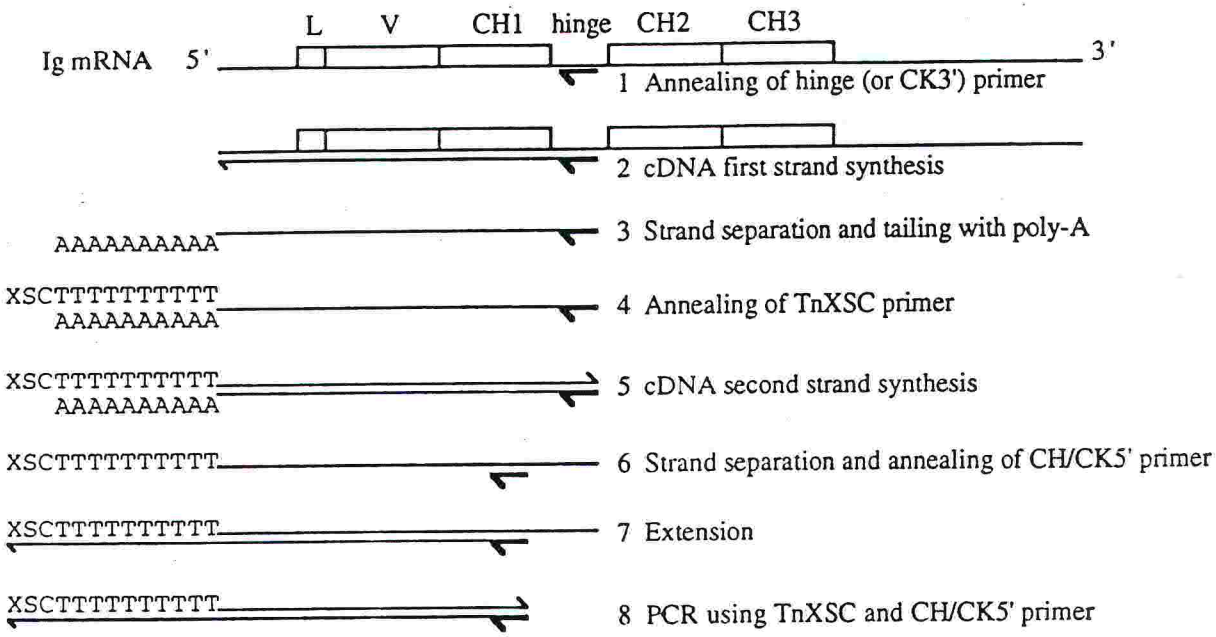


Fig. 5.2: The RACE technique as applied to the cloning of hybridoma V regions. Steps in the use of the RACE technique to clone hybridoma V regions are illustrated. The second strand cDNA synthesis, although indicated as a separate step in this figure (step 4, 5), occurs as the first cycle of the PCR. The sequences of the oligonucleotides used are given in the materials and methods.

This yielded a single band by agarose gel electrophoresis which, after cloning and sequencing, was shown to be a normal rat VH region with a constant region attached to it. Coexpression of this chain in COS cells with the light chain obtained earlier did not, however, reconstitute the anti-NGF activity of the parental

hybridoma, even though both chains produced immunoglobulin as shown by immunofluorescence of COS cell transfectants. For this reason we repeated the RACE procedure for the light chain, using a rat CK primers, and similarly obtained a single band on an agarose gel. This was found to contain a new light chain variable region, different from the previous one, as well as the parental hybridoma light chain (MOPC21), which appeared to be normal when sequenced. When the former was combined with the heavy chain obtained by RACE, it did finally reconstitute anti-NGF activity. A comparison of the αD11 V region sequences with those of the mouse consensus primers used for the first PCR attempts shows a number of differences, which may account for the failure to amplify them from cDNA (**fig.5.3**). It is interesting that attempts to amplify even the cloned αD11 DNA using the V region primers was only successful when the temperature of annealing was reduced to 37 C, a temperature at which amplification from hybridoma cDNA gives a very high background. The sequences obtained (**fig. 5.4**) differ from all other known antibody variable regions (Kabat, 1991) and that of the heavy chain (VH) appears to be most similar to that of the mouse IB VH family. The availability of the sequence of αD11 allowed to derive a three-dimensional model of its structure (Gonfloni, 1995), of interest for future studies aimed at engineering small molecular weight agonists/antagonists of NGF. The cloned variable regions were linked to human constant regions in order to facilitate the future detection of the recombinant antibodies against the background of mouse immunoglobulins, and inserted into expression vectors, to yield plasmids (pSVgpt-IgVHαD11, pSVhygro-IgVKαD11, pcDNAI-neo/VHαD11HuCγ and pcDNAI-neo/VkαD11HuCk) (**fig.5.5**) that direct the expression of the recombinant αD11 antibodies as rat/human chimaeric proteins. The two sets of vectors direct the synthesis of the recombinant antibody chains under the transcriptional control of the immunoglobulin promoter and of the cytomegalovirus early region promoter respectively (Boshart et al., 1985).

VH primers

```
5'
αD11          5' AG GTG CAG TTG AAG GAA TCA GG 3'
                        *   *     *
VH BACK       5' AG GTC CAG CTG CAG GAG TCT GG 3'
                    G A A         C     A


3'
αD11          5' GG GGT CAA GGA ACT TCA GTC ACT GTC TCC TCA 3'
                   *       *   * * *       *
VH FOR-2      3' CC CCG GTT CCC TGG TGC CAG TGG CAG AGG AGT 5'
```

VK Primers

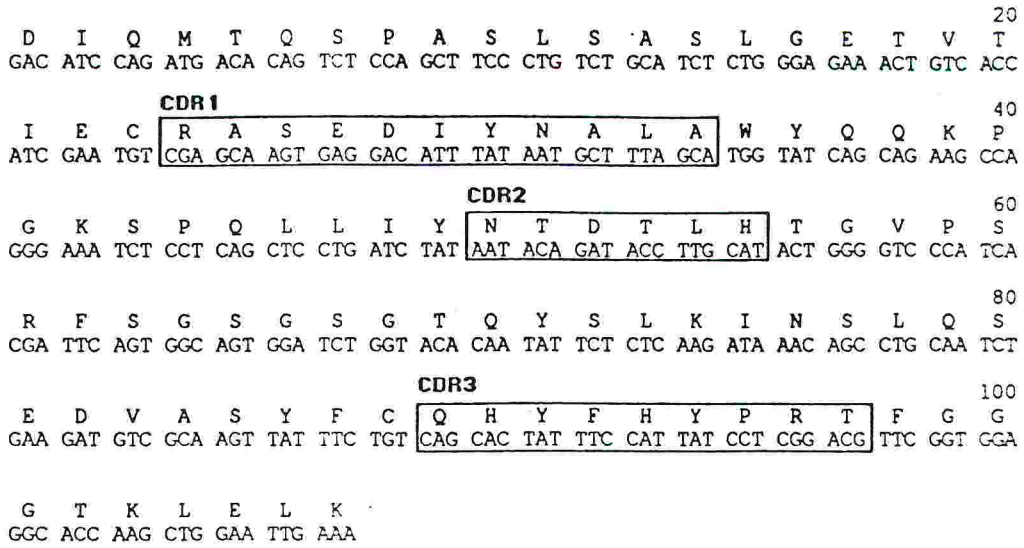
```
5'
αD11          5' GAC ATC CAG ATG ACA CAG TCT CCA 3'

                     *     *     *
VK BACK1      5' GAC ATT CAG CTG ACC CAG TCT CCA 3'
                    * *   * *   *
VK BACK2      5' GAC ATT GAG CTC ACC CAG TCT CCA 3'


3'
αD11          5' GGC ACC AAG CTG GAA TTG AAA CG 3'
                   *                 * * * *
VK FOR1       3' CCC TGG TTC GAC CTC TAG ATT GC  5'
                                      * *
MJK1FONX      3' CCG TGG TTC GAC CTT TAG TTT GCC 5'
                   *                  * *
MJK2FONX      3' CCC TGG TTC GAC CTT TAT TTT GCC 5'
                   *   *     *        * *
MJK4FONX      3' CCC TGT TTC AAC CTT TAT TTT GCC 5'
                   *                 * *
MJK5FONX      3' CCC TGG TTC GAC CTC GAC TTT GCC 5'
```

Fig. 5.3 Alignment of V region primers used to the authentic αD11 V region sequences. Primers used for PCR are indicated, and in the case of VK were used as follows: VK BACK1 and VKFOR1 together, or VK BACK2 and a mixture of MJK(1-4)FONX together. They are derived from (Orlandi et al., 1989) and (Winter et al., 1991). Mismatches between the primers used and the sequence of αD11 shown above are indicated by asterisks above the primers. VH BACK is a degenerate oligonucleotide, and the different bases in the same position are indicated.

VK



VH

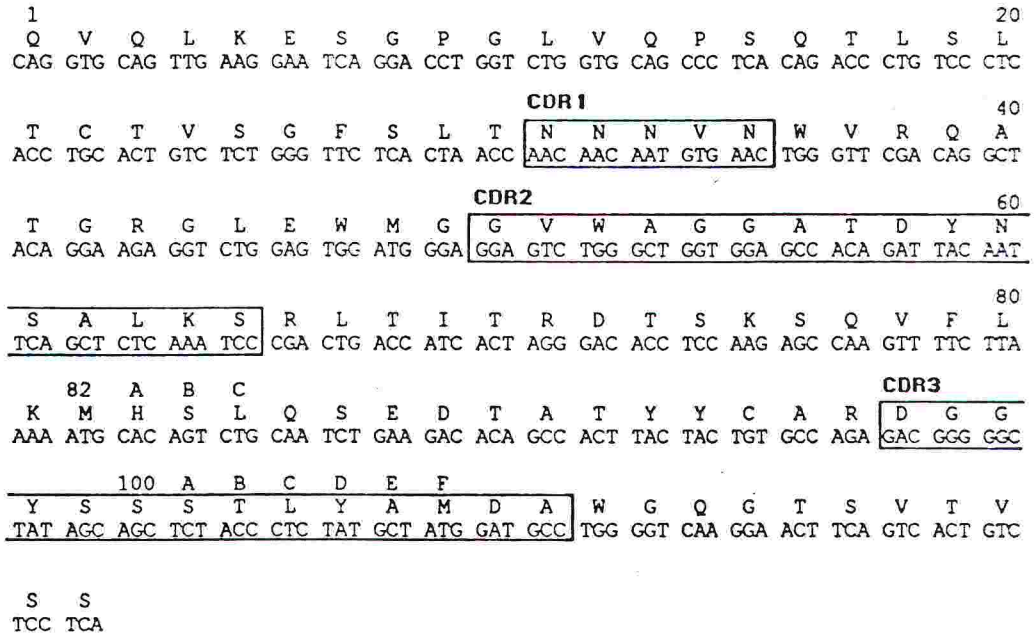


Fig.5.4 Nucleotide and deduced aminoacid sequence of αD11 light (VK) and heavy (VH) variable regions. The leader sequence for secretion is underlined, the complementary determining regions (CDRs) are boxed.

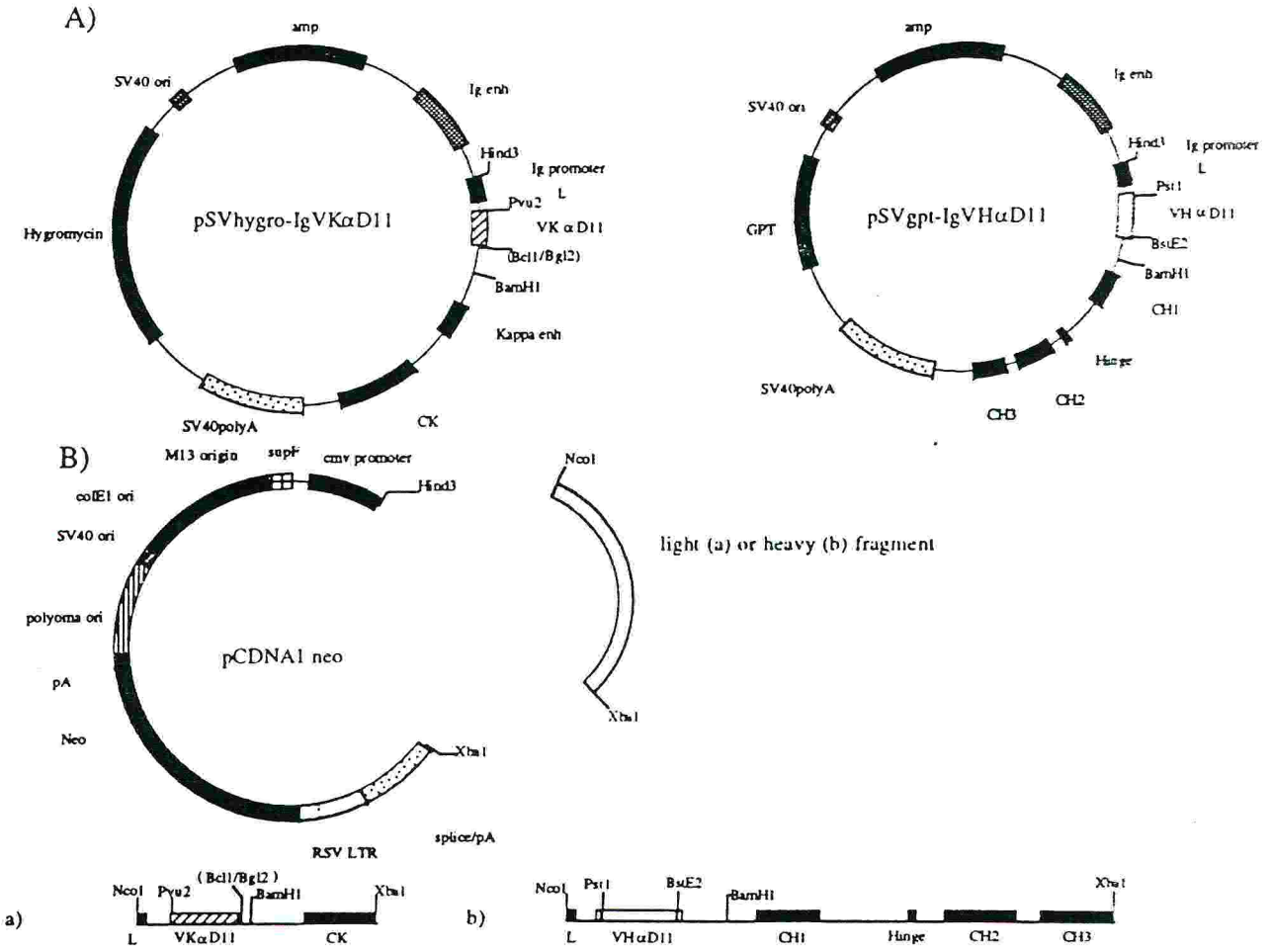
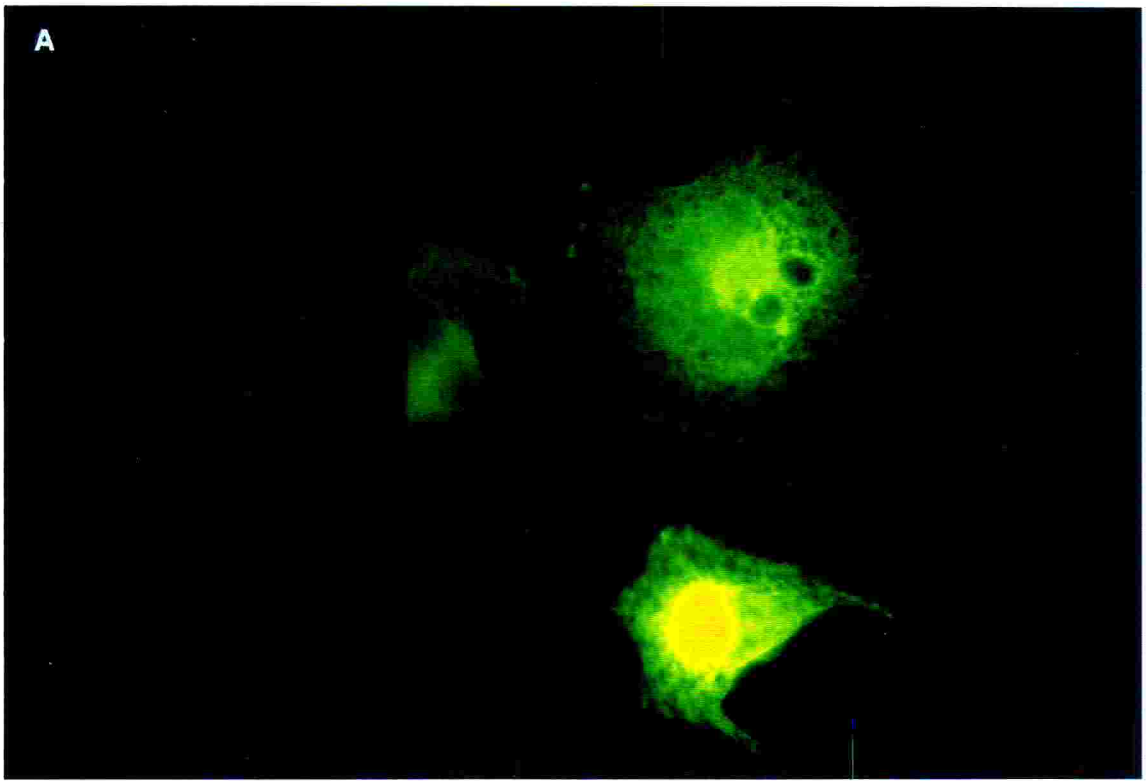


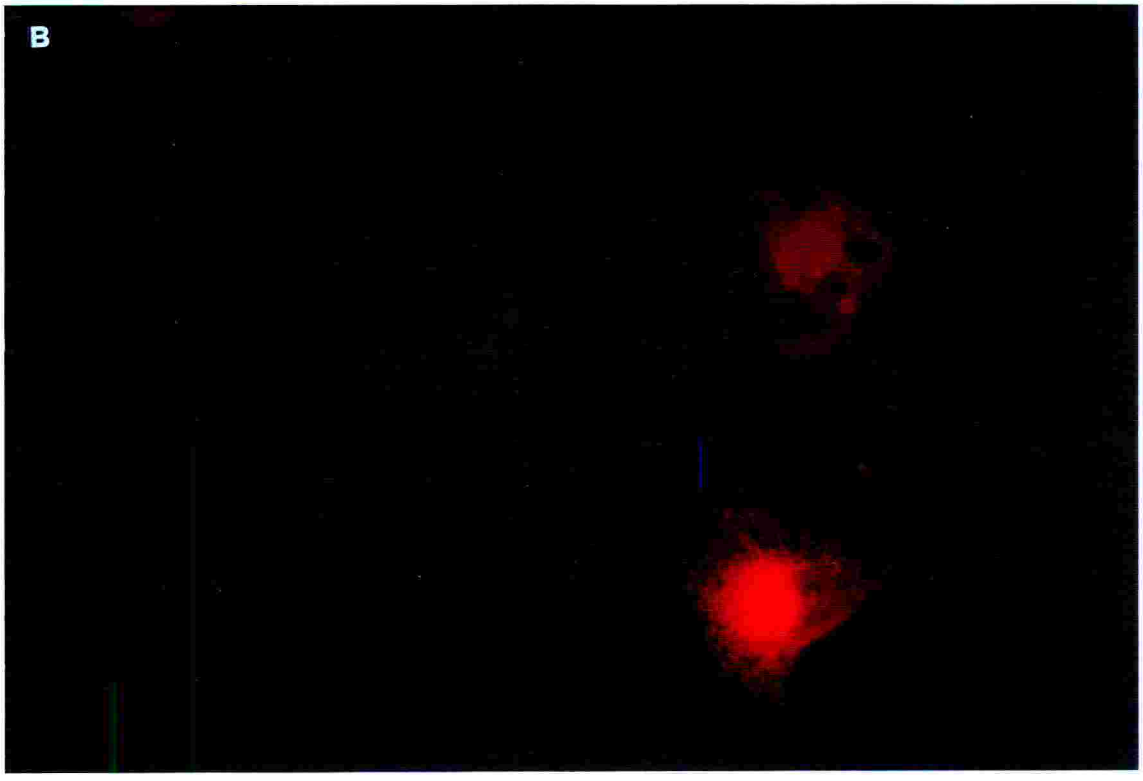
Fig.5.5 Structure of the plasmids for expression of recombinant αD11 antibodies. From top to bottom: pSVgpt-IgVHαD11, pSVhygro-IgVKαD11, pcDNAIneo-VHαD11HuCγ, pcDNAIneo-VKαD11HuCk.

5.2.2 Expression of recombinant αD11 antibody in eukaryotic cells

The activity of plasmids pSVgpt-IgVHαD11, pSVhygro-IgVKαD11, pcDNAI neo-VHαD11HuCγ and pcDNAIneo-VkαD11HuCk was verified by transient transfection in COS cells, as well as by stable transfection in NSO myeloma cells. Transfected COS cells were assayed for expression of recombinant heavy and light chain by double indirect immunofluorescence with anti human isotypic antibodies (**fig.5.6**), confirming that many cells in the transfected population expressed both the heavy and the light chain at comparable levels. The specificity of the cloned antibody was verified by assessing the presence of anti NGF antibodies in the cell supernatants collected 72 hours after the transfection. This was done by ELISA, monitoring the binding to NGF in solid phase (**fig.5.7**). Bound recombinant antibodies were detected with anti-human constant region antibodies, while the parental ones with anti-rat Ig antibodies. The results obtained clearly demonstrate that transfected COS cells do secrete antibodies with anti NGF specificity. This was further confirmed by immunoprecipitation of 125I-NGF with recombinant αD11 and protein A-Sepharose (**fig.5.8**). It should be noted that while the parental mAb αD11 does not bind protein A, this property has been engineered in the recombinant one, which may be of interest for its future uses. COS cells were also transfected with the recombinant αD11 heavy chain alongside with plasmids directing the expression of light chains from unrelated antibodies. The antibodies secreted failed to bind to NGF (data not shown), thus showing that the original combination of both heavy and light chains is absolutely required to create the binding site for NGF.

Fig.5.6 Coexpression of heavy and light chains from αD11 in COS cells transfected with pSVgpt-IgVHαD11 and pSVhygro-IgVKαD11. 48 hours after transfection the cells were stained by double indirect immunofluorescence with FITC labelled anti human heavy chain (A) and with biotinylated anti human light chain (B) antibodies, followed by Texas Red Streptavidin. The same field is shown for the two fluorochromes used. Identical results were obtained by transfecting COS cells with the pcDNAIneo-VHαD11HuCγ and pcDNAIneo-VkαD11HuCk plasmids.


A


B

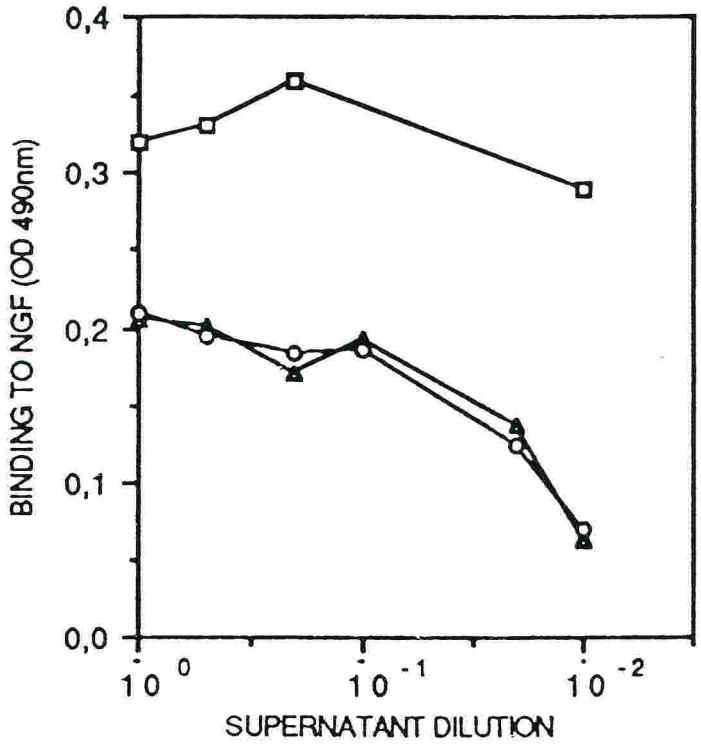


Fig. 5.7 NGF binding activity of recombinant and parental mAb αD11 determined by ELISA.

Cell supernatants from i) COS cells transiently transfected with pSVgpt-IgVHαD11 and pSVhygro-IgVKαD11 (filled triangles and squares, two independent transfections) and ii) parental αD11 hybridoma cells (empty squares) were incubated with NGF bound to solid phase. Binding is expressed as optical density values at 490 nm, after subtraction of the values obtained with control supernatants. Identical results were obtained by transfecting COS cells with the pcDNAIneo-VHαD11HuCγ and pcDNAIneo-VkαD11HuCk plasmids.

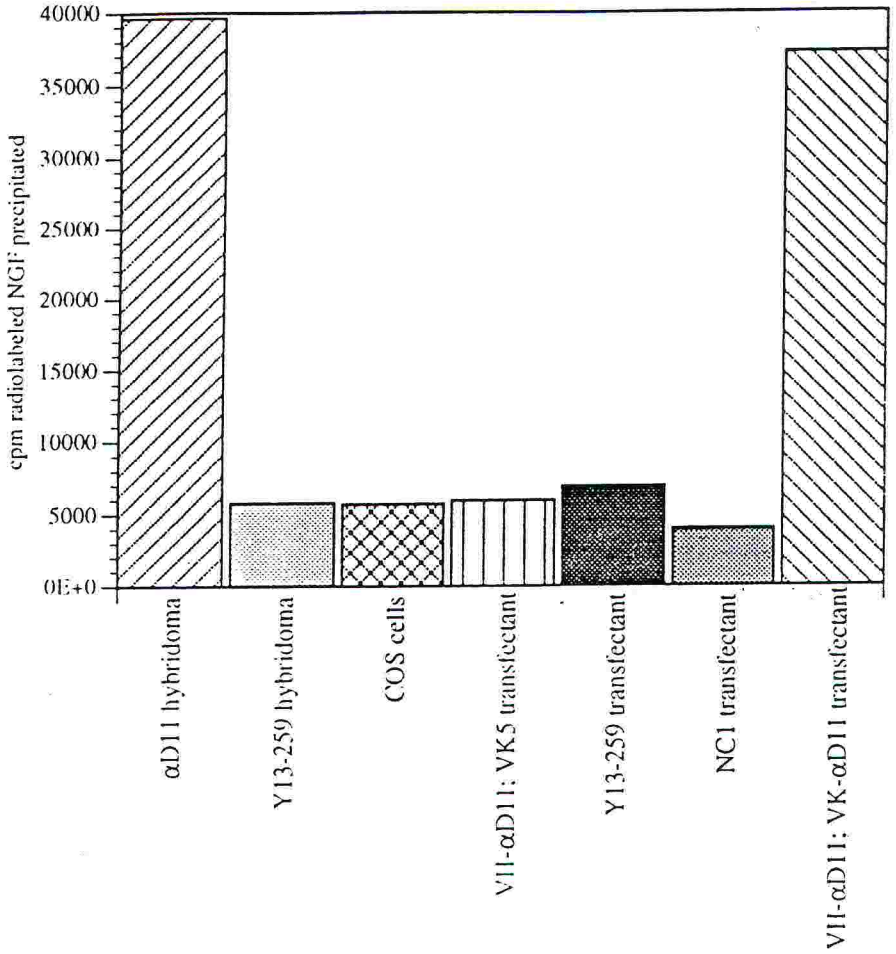


Fig. 5.8: Binding of recombinant αD11 antibodies to ^{125}I-NGF. Supernatants are as indicated below the columns. Y13/259 and NC1 are monoclonal antibodies we have cloned in the lab (Werge, et al., 1992; Piccioli, et al., 1991) which recognise p21ras and substance P respectively. VK5 is the first light chain cloned using V region primers which was functional, but did not show NGF binding activity.

5.2.3 Bioassay on PC12 cells

The parental MAb αD11 is very efficient at neutralising the biological activity of NGF, both in vitro and in vivo. In order to verify whether this property is retained by the recombinant antibody, PC12 cultures were challenged with 50 ng/ml NGF in the presence of supernatants from COS cells transiently transfected with pSVgpt-IgVHαD11 and pSVhygro-IgVKαD11. The results (**fig.5.9**) confirm that, also with respect to the property of neutralising NGF activity, the recombinant antibody retains the properties of the parental one.

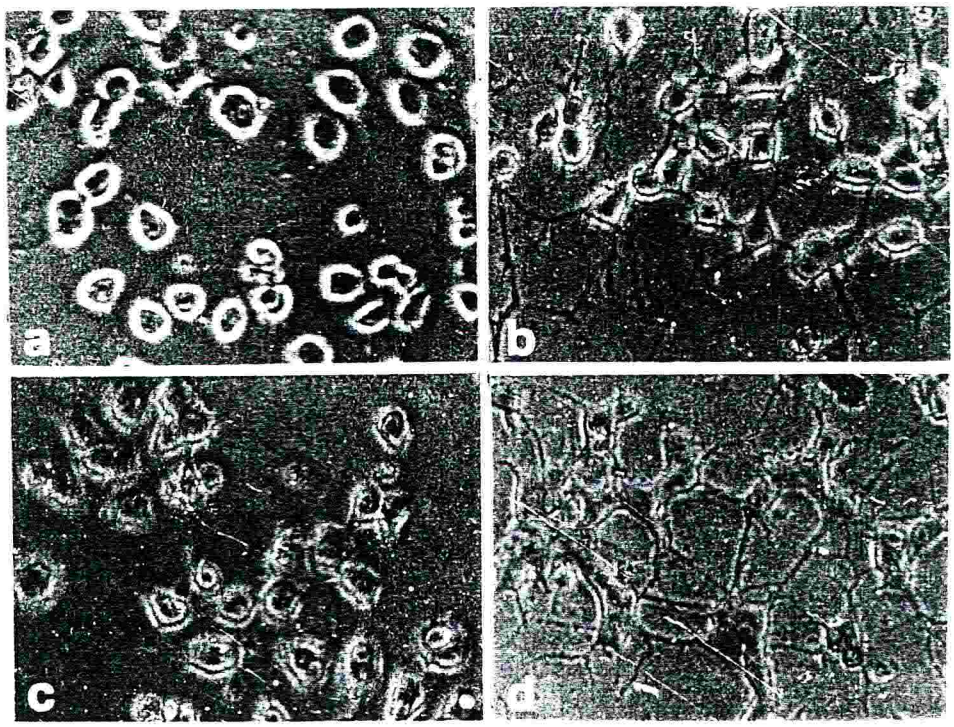


Fig.5.9 Inhibition of NGF biological activity in PC12 cells by recombinant αD11 mAbs.

Cell supernatants containing recombinant αD11 or NC1 mAbs were assayed for their ability to block the effect of NGF to stimulate neurite outgrowth in PC12 cells. PC12 cells were incubated for six days with [(B), (C) and (D)] or without (A) 50ng/ml of mouse NGF in the presence of control medium ((B), recombinant αD11 antibody (C) or recombinant NC1 (anti Substance P) antibody (D). Similar results were obtained by transfecting COS cells with the pcDNAIneo-VHαD11HuCγ and pcDNAIneo-VkαD11HuCk plasmids.

5.2.4 Expression of functional αD11 ScFv in bacteria and eukaryotic cells

The availability of the recombinant αD11 variable regions allowed us also to engineer a simpler form of the antibody (Single chain Fv fragment, or ScFv) which consists of the light and heavy variable regions joined by a linker peptide, linking the C-terminus of the VH chain to the N-terminus of the cognate VK chain (**fig.5.10**). An epitope of myc, linked at the C terminal end of the ScFv was used as a tag recognised by the monoclonal antibody 9E10. The heavy and light chains of the αD11 antibody have been subcloned in bacterial vectors for expression of secretory forms of ScFv molecules (**fig.5.11**). The αD11 ScFv purified from periplasmic space was able to bind NGF as shown by immunoprecipitation with NGF and protein A-Sepharose (**fig.5.12**). The biological activity of αD11 ScFv have been tested on PC12 cells primed for 1 week with 50 ng/ml of NGF. As shown in fig.**5.13**, αD11 ScFv inhibits the extension of neurites from primed PC12. Moreover bioassay on DRG neurons at embryonic stage 9 showed that αD11 ScFv inhibits NGF biological activity and does not crossreact with the other neurotrophins confirming the specificity of the chimeric molecule (data not shown). This suggests, although it does not formally prove, that the αD11 ScFv maintains the same affinity as the parental antibody. Direct affinity measurements will be needed for this. The same results have been confirmed for αD11 ScFv molecules expressed as secretory fragments in mammalian cells (not shown).

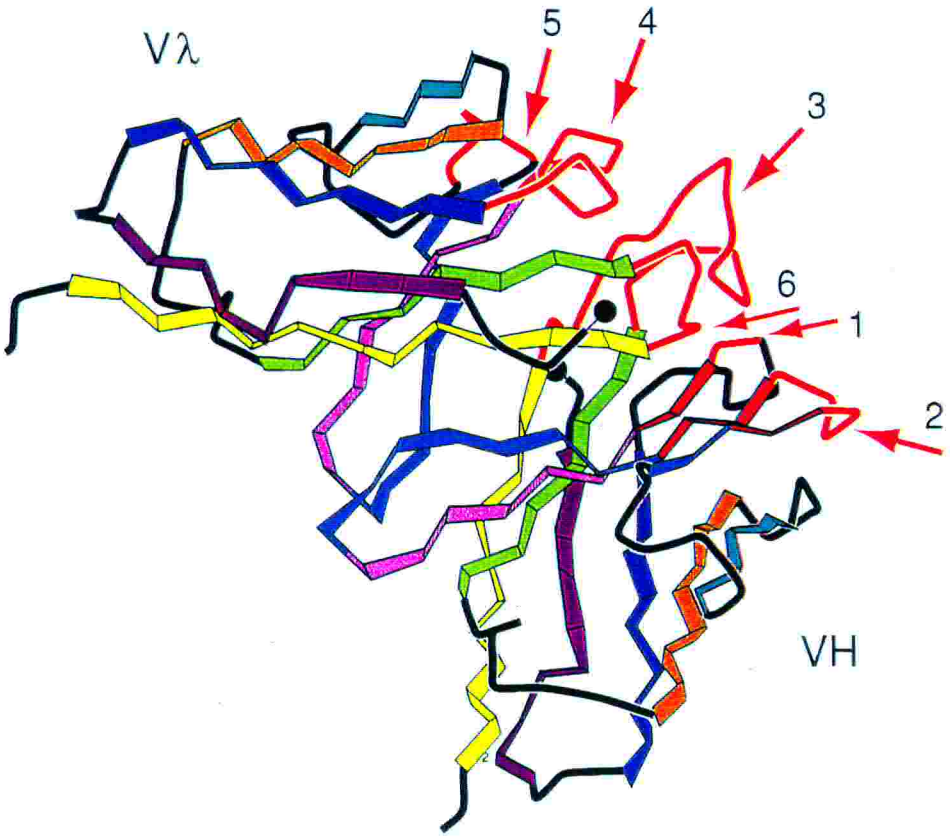


Fig.5.10 Schematic representation of a ScFv molecule. The hypervariable regions (VH, 1-3; VL, 4-6) are in red. (Reproduction from Winter and Milstein 1991).

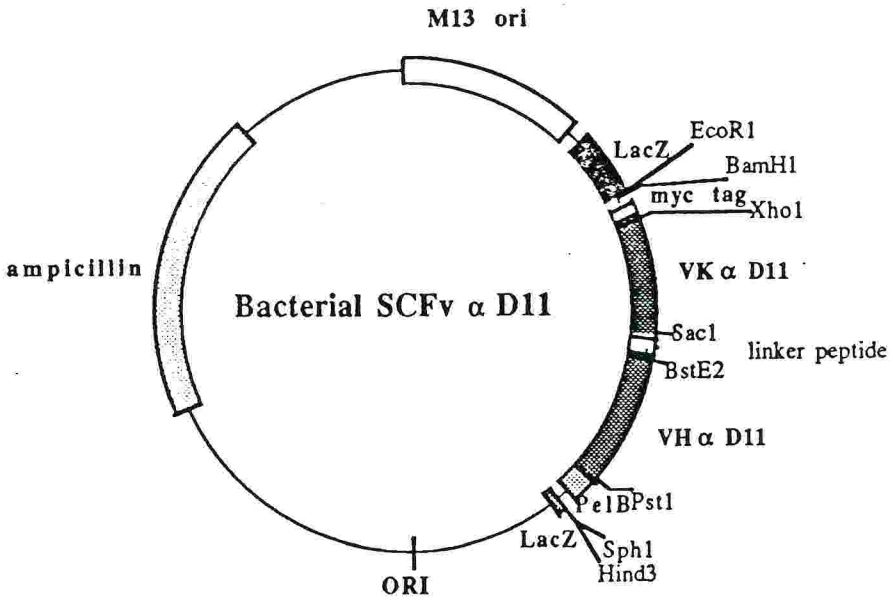


Fig. 5.11 Vector for expression of anti-NGF ScFv in E. coli

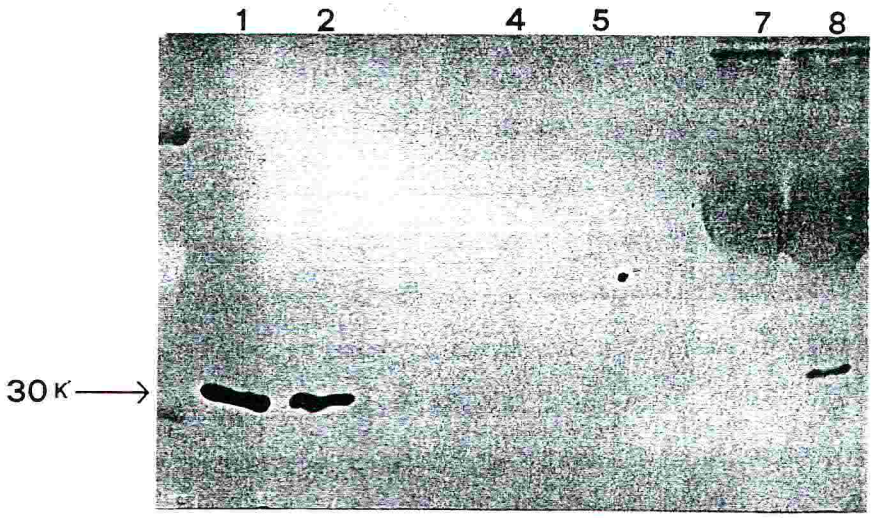


Fig. 5.12 Antigen binding activity of αD11 ScFv.

Periplasmic fractions from E. coli cells secreting ScFv from the αD11 antibody (lanes 1 and 2) or from a non relevant (anti-ras) antibody (lane 4 and 5) were chromatographed on NGF-Sepharose columns. Bound material was analysed by immunoblotting with mAb 9E10 directed against the myc-tag. Lanes 7 and 8: 1/10 of unbound chromatographed periplasmic fraction from anti-NGF and anti-ras ScFv respectively.

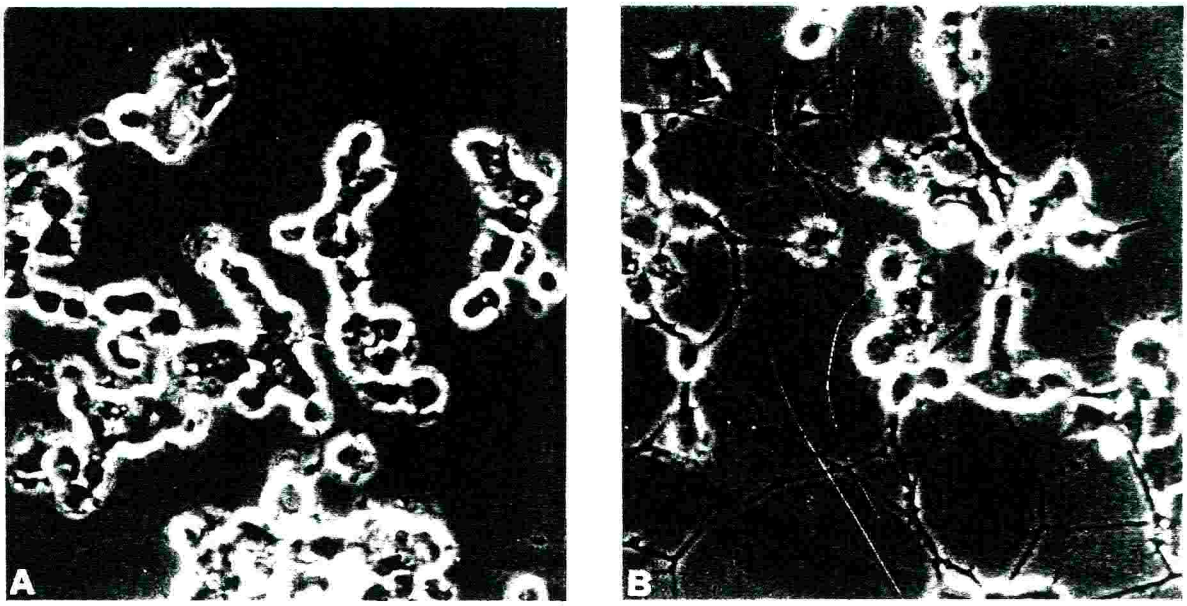


Fig.5.13 Inhibition of NGF biological activity in PC12 cells by recombinant αD11 ScFv

Periplasmic fractions containing recombinant αD11 or anti-phox ScFv were assayed for their ability to block the effect of NGF to stimulate neurite outgrowth in PC12 cells. PC12 cells, after priming for 7 days with 50 ng/ml of mouse NGF were incubated with 10 ng/ml of mouse NGF in the presence of recombinant αD11 ScFv (a) or recombinant anti-phox ScFv (b)

5.2.5 Production of lines of transgenic mice expressing recombinant αD11 antibody

As a first attempt towards the production of transgenic mice expressing anti-NGF antibodies, the linearized DNA from the separate plasmids for the heavy and light chains (**fig.5.14A**) was coinjected into fertilised mouse eggs. The injected DNA did not contain unwanted vector sequences, which have been removed by restriction of DNA.The expression of the transgenes was placed under the control of the Cytomegalovirus early gene promoter. 34 mice were born after egg implantation. Four mice had DNA for both chains integrated in the genome and two had the heavy chain DNA only. **Fig.5.14B** shows a dot blot for the heavy and light chains on DNA samples from the different founders, indicating that in some cases, more than one copy of the transgenes was integrated. In order to identify the genotype of αD11 transgenic mice in the course of the breeding a rapid screening method was established. Amplification of transgenic variable regions, without contamination of endogenous mouse immunoglobulins, was obtained by PCR from tail DNA using primers specific for αD11 variable regions (**fig.5.15**).

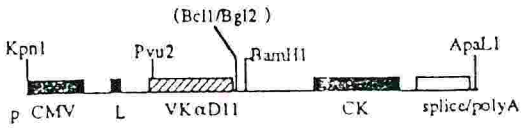
Transgenic αD11 antibodies are expressed in the founder mice. Three of the adult double transgenic founder mice express detectable levels of functional anti-NGF antibodies in the serum, as revealed by ELISA, with NGF coupled in solid phase (**fig.5.16**). Despite an intensive breeding program lasting over one year, we have been unable to obtain stable lines from these founder mice (2 males and one female), positive for the expression of αD11 antibody. The expressed antibodies in the founder mice appear to be effective in neutralizing the action of endogenous NGF, as assessed on a classical NGF target the Superior Cervical Ganglion (SCG) The SCG of one male founder mouse was analysed, showing a reduction of the overall ganglion size, as well as of the number of cells (data not shown).

Different possibilities could account for the inability of αD11 transgenic mice to yield offspring, as the transgenes could interfere with mating, behaviour, reproduction or survival of embryos (this could have been the case for the female

Fig.5.14 (**A**) DNA constructs used for the production of the transgenic mice. Light chain (a) and heavy chain (b) transgenes were used to generate transgenic mice by microinjection in fertilised 1-cell eggs. Linearized DNA was prepared by digestion with KpnI-ApaLI (light chain) and KpnI-XbaI (heavy chain) of pcDNAI-neo/VKαD11HuCk and pcDNAI-neo/VHαD11HuCγ respectively. Ck and CH1-CH3, human constant region domains of the light (k) and heavy (γ1) chains; VK and VH, light and heavy chain variable regions of the αD11 monoclonal antibody; CMV, immediate early gene promoter and enhancer (**B**) Slot blotting analysis of founders. DNA from tail biopsies was hybridised using the heavy (a) or light (b) chain constant regions as a probe. Three mice were double positive for the integration of the heavy and light chains in the genome. *Subsequent analysis showed that the 678 mouse was positive for the light chain too.

A

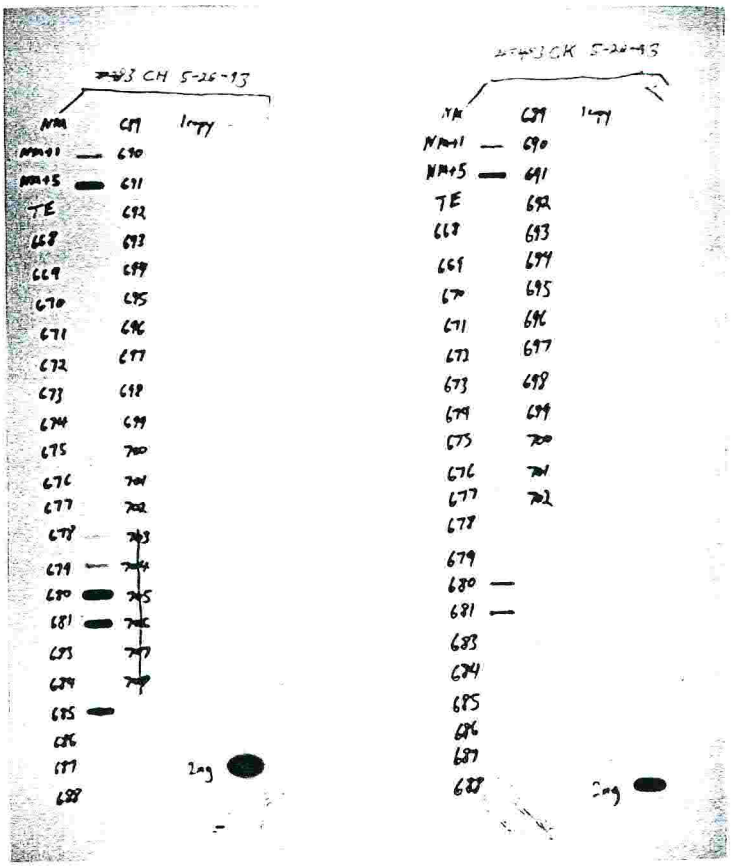
a)


(BclI/Bgl2)
Kpn1
Pvu2
BamH1
ApaL1
p CMV
L
VKαD11
CK
splice/polyA

b)


Kpn1
Pst1
BstE2
BamH1
Xba1
p CMV
L
VHαD11
CH1
Hinge
CH2
CH3
SV40 polyA

B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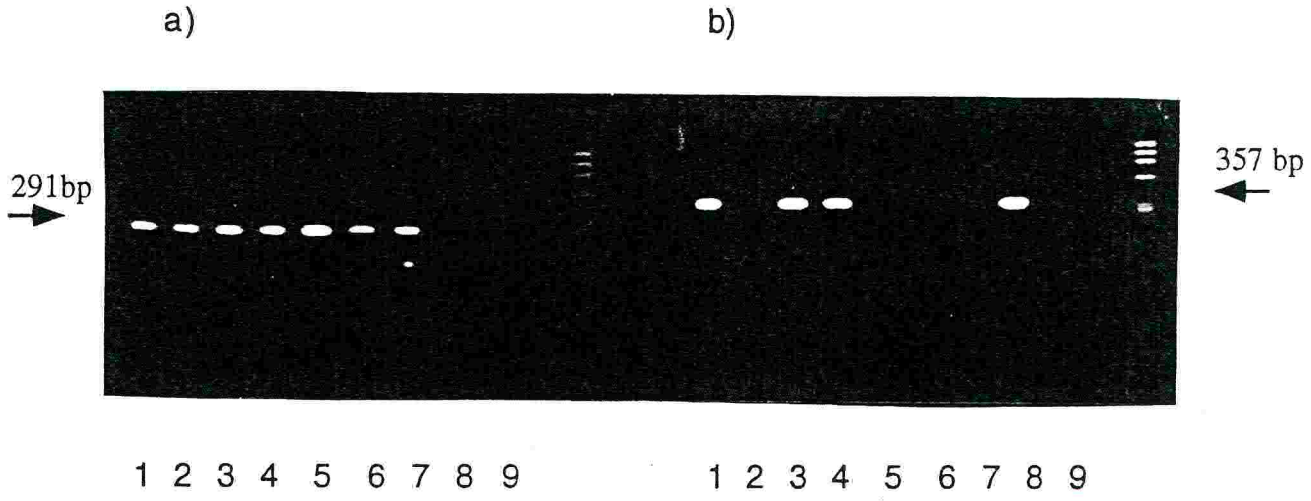


Fig. 5.15 Agarose gel analysis of PCR reaction products. PCR reaction products were analysed on a 1.5% agarose gel for the presence of VK (a; 291bp) or VH (b; 357bp) transgene sequences. PCR templates are: plasmid pcDNAI-neo/VKaD11HuCk (lane 1 (a)), plasmid pcDNAI-neo/VHaD11HuCγ (lane 1 (b)), tail DNA from eight individual littermates, including double transgenic (lanes 3,4) and single transgenic (lanes 2, 5-8), tail DNA from control mouse (lane 9). Size markers are HaeIII fragments of ΦX174.

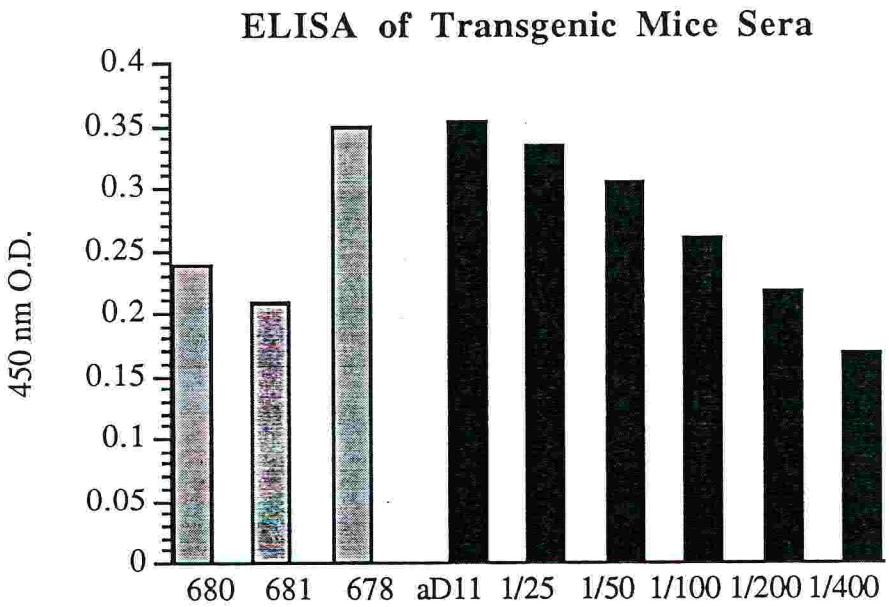


Fig.5.16 NGF binding activity in the serum of transgenic mice was compared to that one of the parental mAb αD11 by ELISA. Binding is expressed as optical density values at 450 nm, after subtraction of the values obtained with sera from control mice.

founder, which at some stage, became pregnant but did not give birth to a viable litter). It is noteworthy that the few TrkA knock-out mice which reach adulthood are likewise infertile (Klein, 1994).

Since both NGF and the LNGFR p75 are expressed in the male reproductive system (Ayer-LeLievre et al., 1988), one of the male founders was sacrificed and the testes analyzed in order to gain insight into the reasons for the observed infertility. The heavy chain of αD11 was found to be expressed in the seminifer tubules of testis, as shown in **fig.5.17.**

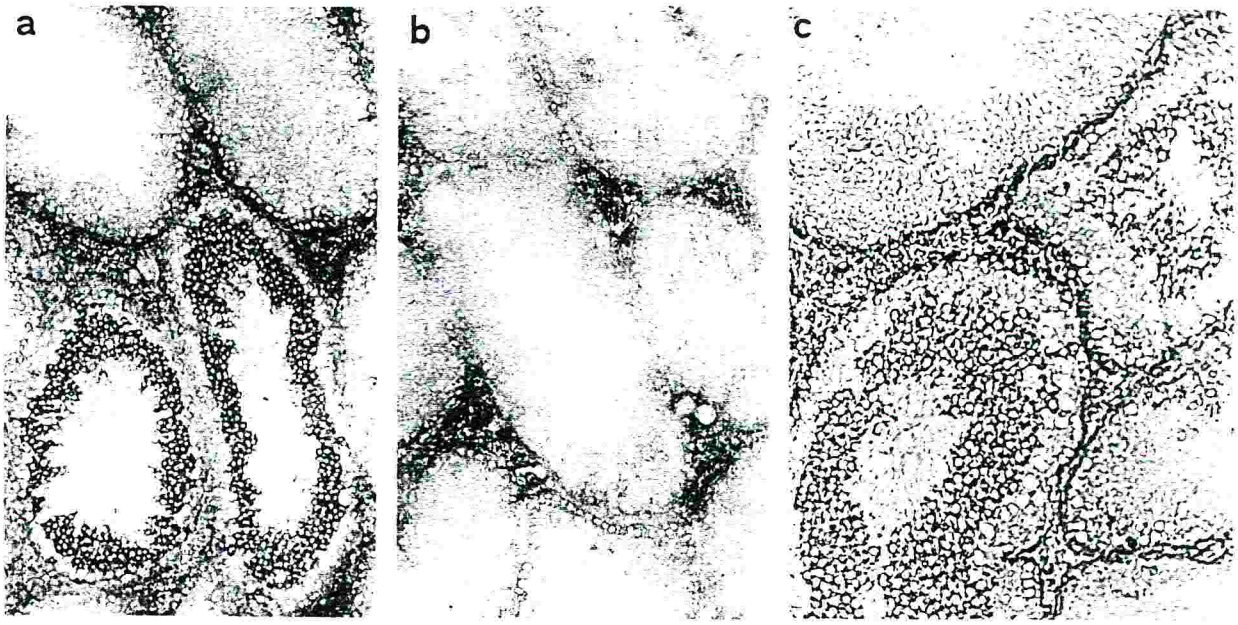


Fig.5.17 Immunoperoxidase micrographs of cryosections of transgenic mouse testis processed for the detection of anti-NGF antibodies. a. An intense specific immunoreactivity is evident in early postmeiotic germ cells present in a limited number of seminiferous tubules at specific stages of spermatogenesis. b. Control section, prepared with the biotinylated antibody omitted, showing endogenous peroxidase activity in the peritubular compartment and absence of specific staining in the seminiferous tubules. c. Phase contrast micrograph showing immunoreactivity for anti-NGF antibodies in the cytoplasm of round spermatids whereas elongating spermatids as well as meiotic germ cells appear negative.

However, anatomical inspection of semithin sections of transgenic mouse testis failed to show any abnormality in the developmental maturation of the germinal cells, which was shown to be identical to that in control mice, as shown in **fig.5.18**. Also the sperm motility in the transgenic mouse was found to be identical to that in control mice. It was therefore concluded that the male reproductive system was not impaired and cannot held responsible for the observed infertility.

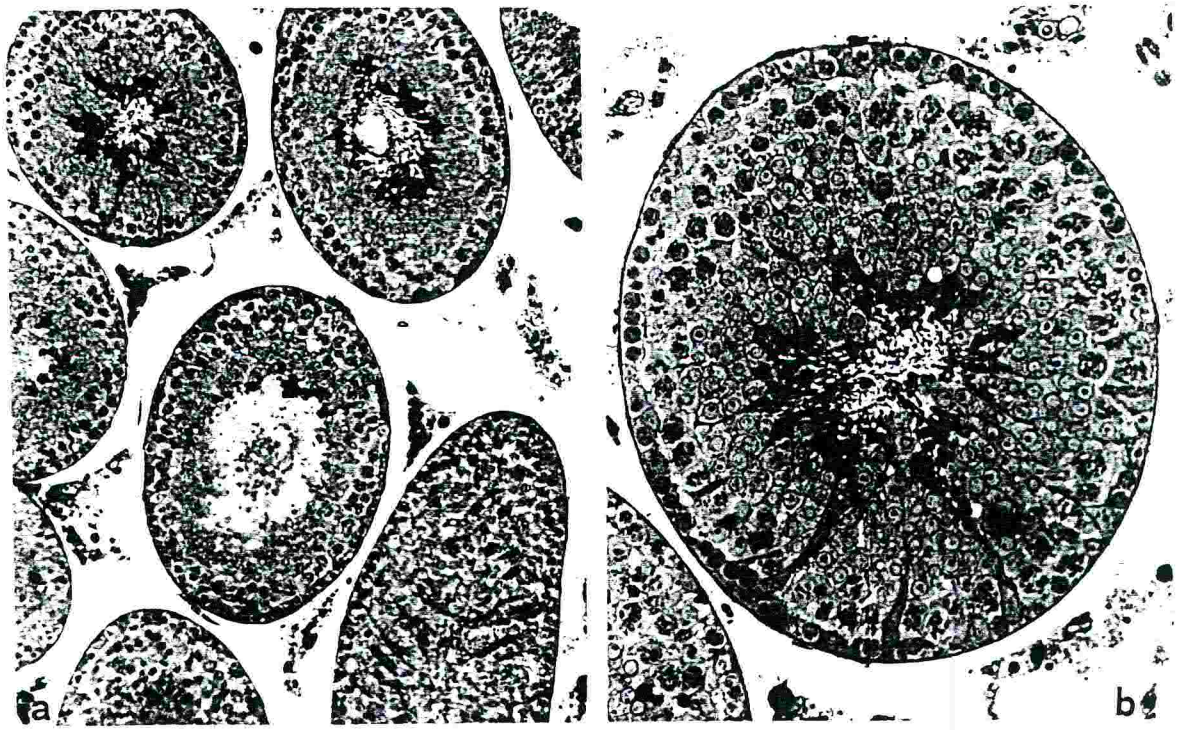


Fig.5.18 Semithin sections of transgenic mouse testis. a. Low-power view of seminiferous tubules which appear as typically seen in wild-type testis. b.High-power micrograph of a representative seminiferous tubule showing the structural integrity of the seminiferous epithelium. Several generations of germ cells at different stages of spermatogenesis are observable. Sertoli cells extend from the basal region to the tubular lumen surrounding bundles of maturing spermatids.

This first series of experiments showed that the expression of the αD11 antibody chains in founder mice did not allow us to establish a double transgenic line with both antibodies chains. Therefore we followed another strategy. As the single, isolated heavy or light chains of the αD11 antibody are unable to bind NGF in the absence of the other chain (see paragraph 5.2.2) we planned two separate microinjection experiments, to obtain two distinct lines of transgenic mice: one expressing the heavy chain only and another one expressing the light chain only. The breeding of these mice should produce transgenic mice which express functional anti-NGF antibodies (**fig.5.19**), provided the two antibody chains are co-expressed in the same cells, allowing their association within the ER and their secretion as a functional antibody. The advantage in this experiment derives from the possibility of obtaining stable lines of mice in which the expression of the transgene should not produce a phenotype (but see Discussion).

Therefore, a new round of distinct microinjections was performed, using (separately) the same linearized DNA of the previous experiment. Three founders were positive for the heavy chain by dot blot analysis (**fig.5.20A**). The expression of the transgene in different organs of adult mice was first verified at mRNA level, by RNAse protection analysis. Expression of the heavy chain mRNA was revealed in heart, brain, and kidney of adult mice. The lines generated from different founders show different overall levels of expression. We have selected for further study the line VH4645, which expresses the highest level of heavy chain mRNA (**fig.5.20B**). In this line the expression of heavy chain mRNA was also shown to be present in the spleen of adult mice.

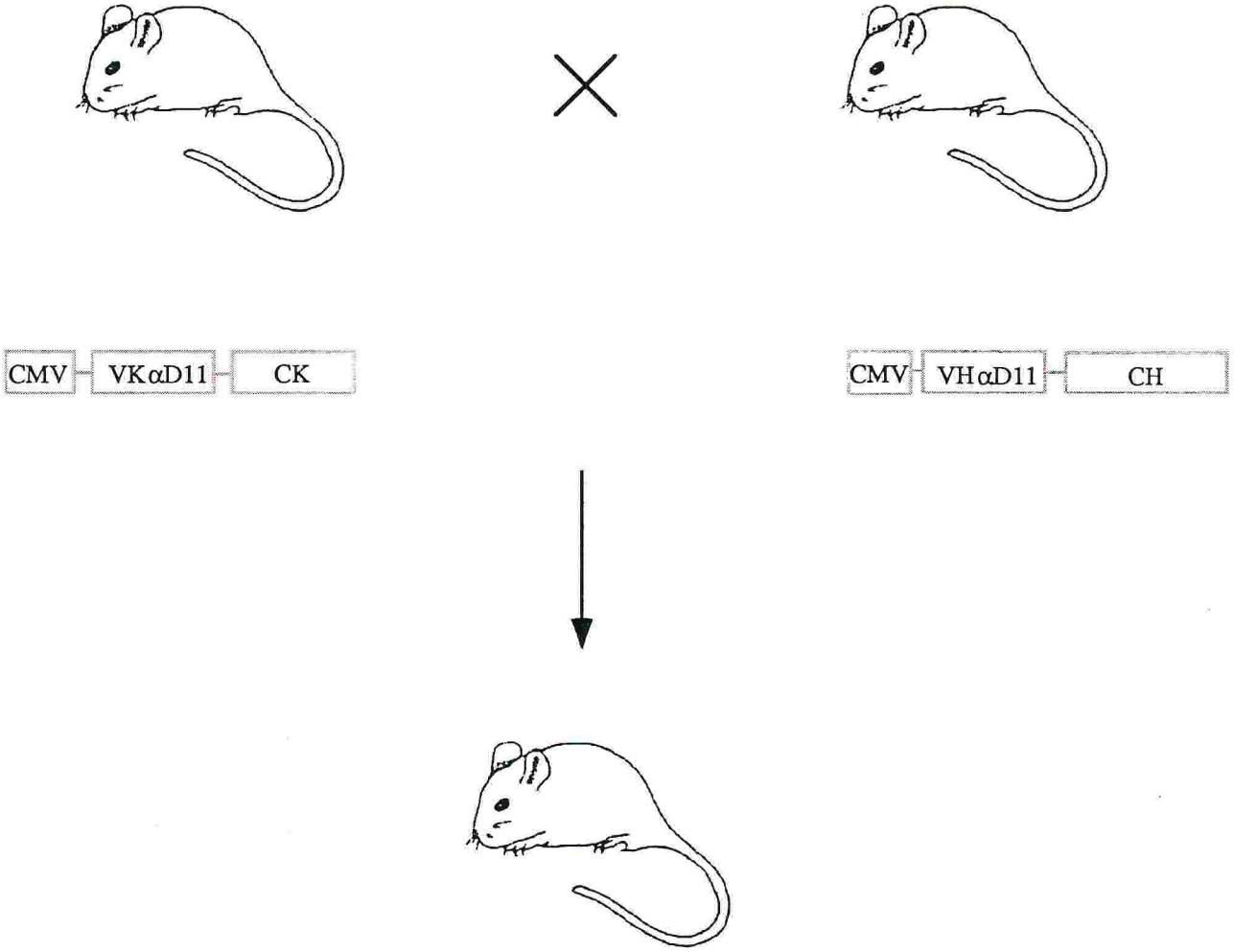


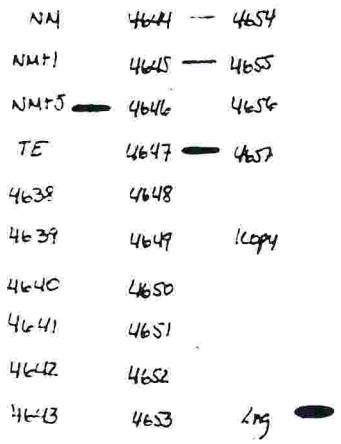
Fig.5.19 Two separate microinjection experiments have been performed to obtain two distinct lines of transgenic mice: one expressing the heavy chain and another expressing the light chain. The breeding of these mice should produce transgenic mice which express functional anti-NGF antibodies

Fig.5.20 (**A**) Slot blot analysis of mice derived from the microinjection of αD11 heavy chain. DNA from tail biopsies was hybridised using the heavy chain constant region as a probe.

(**B**) Rnase protection analysis of the transgenic VH chain. Total RNA was extracted from adult kidney and brain of transgenic mice of the line VH4645 (lane 1-8) and line VH4647 (lanes 9-14). Total RNA extracted from brain of adult wild type mice (lane 15) and of a cell line transfected with the DNA for the recombinant mAb αD11 (lane 16) were used as negative and positive control respectively. Total RNA (20 μg) was hybridised to a 32P-labeled antisense RNA probe specific for the αD11VH gene. A 340 bp protected fragment corresponding to the transcript from the VH transgene is evident in the kidney and brain of the mice from line VH4645. Numbers on the left and right, in B, represent molecular size (in bp) as derived from a 32P labeled DNA molecular weight marker; VH RNA probe is also shown (lane 17)

- 942 9/28/94

A)


NM
NMT1
NMT5
TE
4638
4639
4640
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4650
4651
4652
4653
4654
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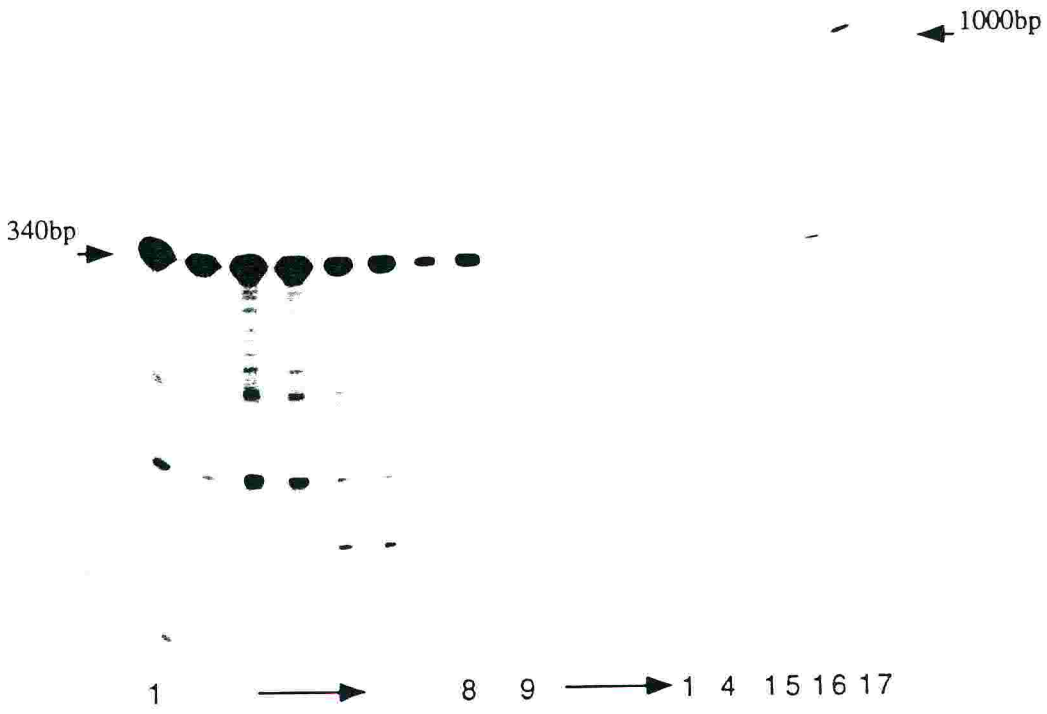
4656
4657
1 copy
2 mg

B)


1000bp
340bp
1
8
9
1
4
15
16
17

The spatial distribution of heavy chain protein in the CNS was studied by immuno-cytochemistry. The overall picture is that of an abundant staining of many discrete areas throughout the nervous system. An abundant expression can be seen in the piriform cortex and in the hippocampal region (**fig.5.21A**). A stronger density of positive cells is evident in the CA1, CA2 and CA3 regions; scattered neurons in the hilus are also labeled. The high level of transgene expression found in the hippocampus are particularly noteworthy in view of the high expression of NGF in this region. Other brain cortical areas also show good expression of heavy chain protein (**fig.5.21B**). High levels of heavy chain protein are found in the Purkinje cells of the cerebellum (**fig.5.21C**). As shown by inspection with higher magnification the staining is mainly neuronal and it is distributed throughout the cell extension, including its cellular processes and arborization. The expression in discrete regions and in group of cells of the CNS is consistent with data in the literature in which the expression pattern of the CMV promoter was studied (Schmidt et al., 1990).

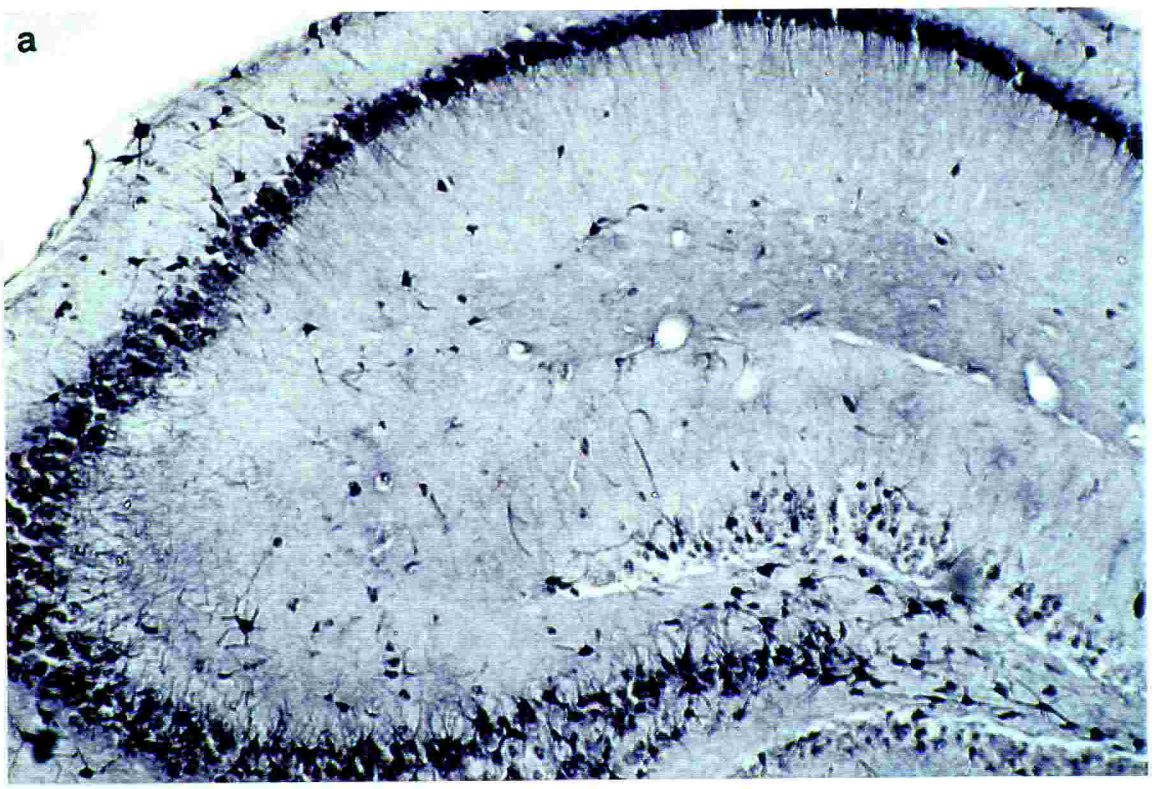
The microinjection of light chain DNA produced 4 transgenic mice, VK4014, VK4015, VK4019, VK4023 in which more than one copy of the transgene is integrated (**fig.5.22**). In order to obtain αD11 transgenic mice, two of these (VK4019 and VK4023) were bred with mice of the VH4645 line. Of the remaing transgenic lines, the VK founder, VK4015 mates and reproduces at a greatly reduced level compared to the others, and VK4014 did not transmit the transgene to its offspring.

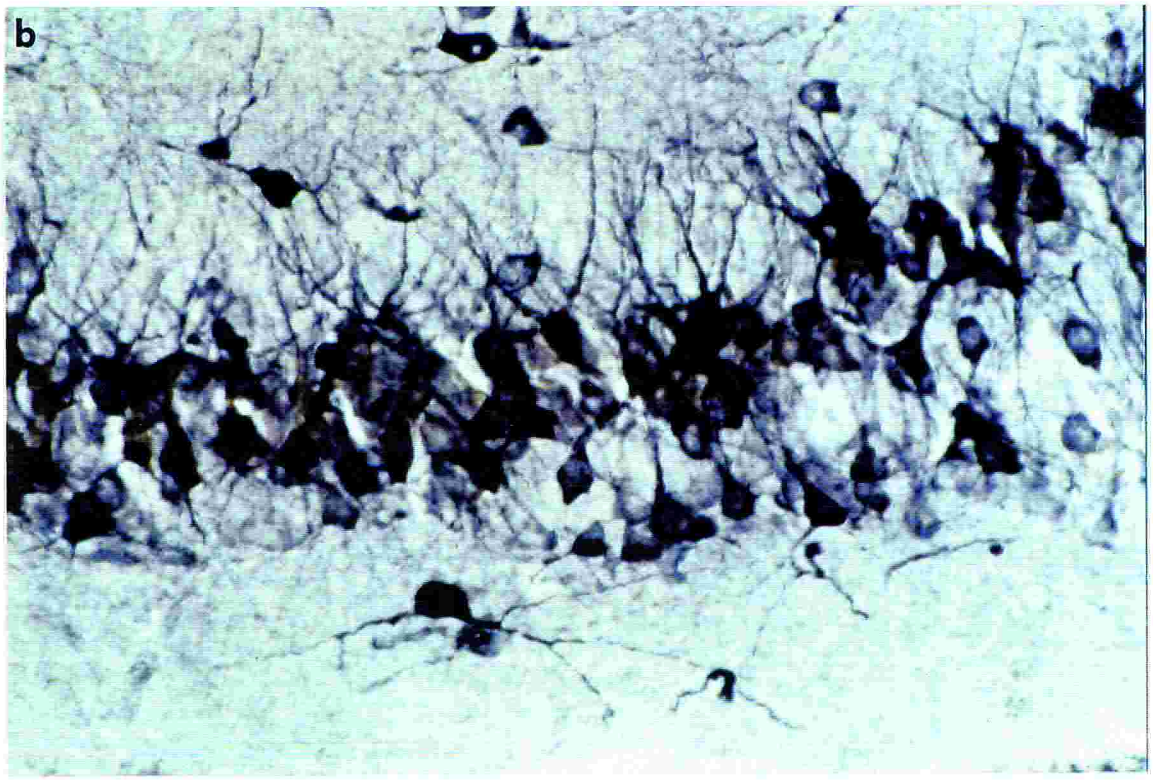
Two families of αD11 transgenic mice were derived: A(VK4019 x VH4645) and B(VK4023 x VH4645). The presence of functional anti-NGF antibody in the serum of adult mice was determined with an enzyme linked immunosorbent assay on solid phase-coupled NGF peptide.

Fig 5.21 Ectopic expression of recombinant anti-NGF heavy chain in the CNS of transgenic mice.

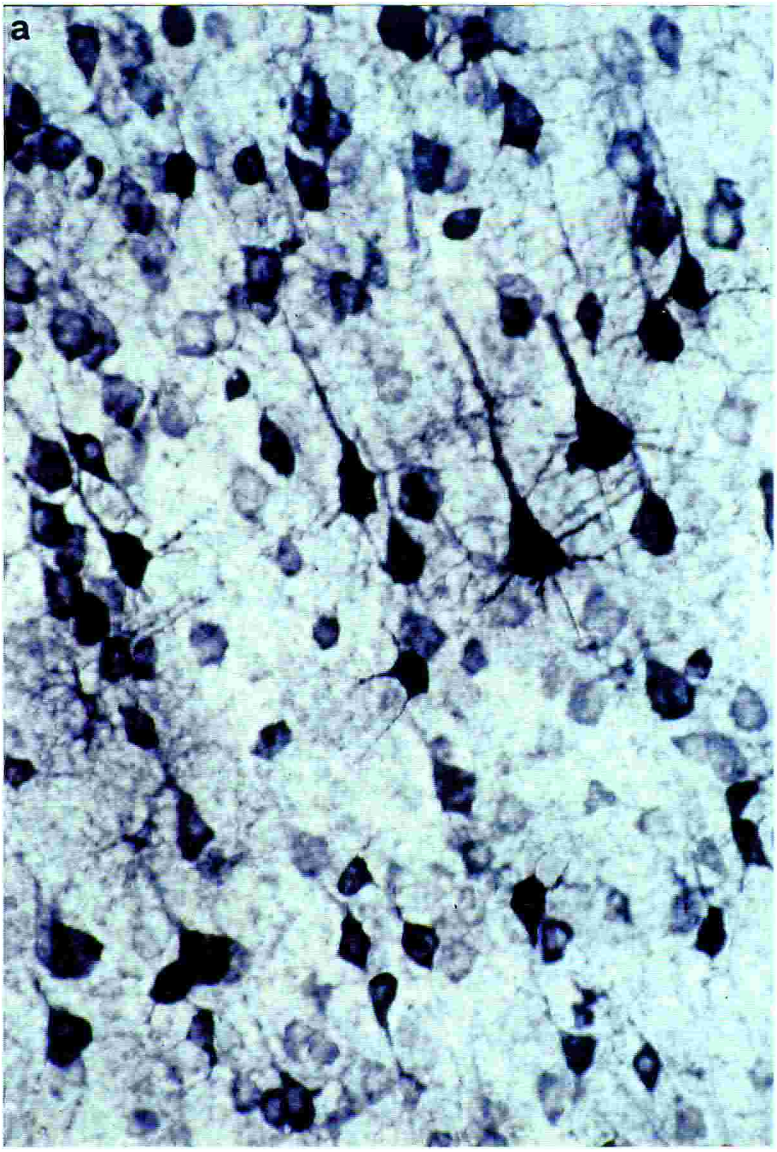
(A) 30 μm coronal sections of the brain from a transgenic mouse of the line VH4645 sacrificed at 2 months. Expression of the heavy chain can be seen in the hippocampus (a). A neuron-specific staining can be observed in (b), which shows at higher magnification, pyramidal cells of the hippocampus. **(B)** High level of transgene expression was found also in the frontal cortex (b). In (a) pyramidal neurons of the frontal cortex are shown at higher magnification. **(C)** Purkinje cells of the cerebellum are strongly stained.

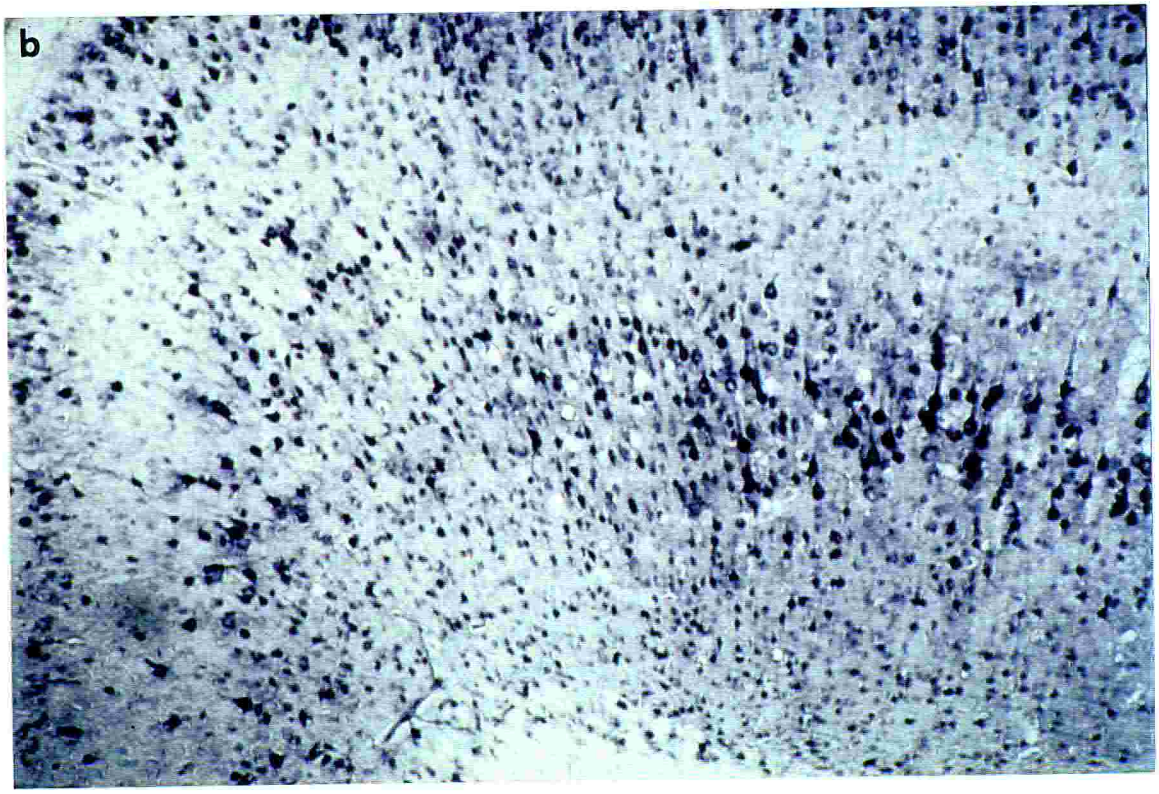
A



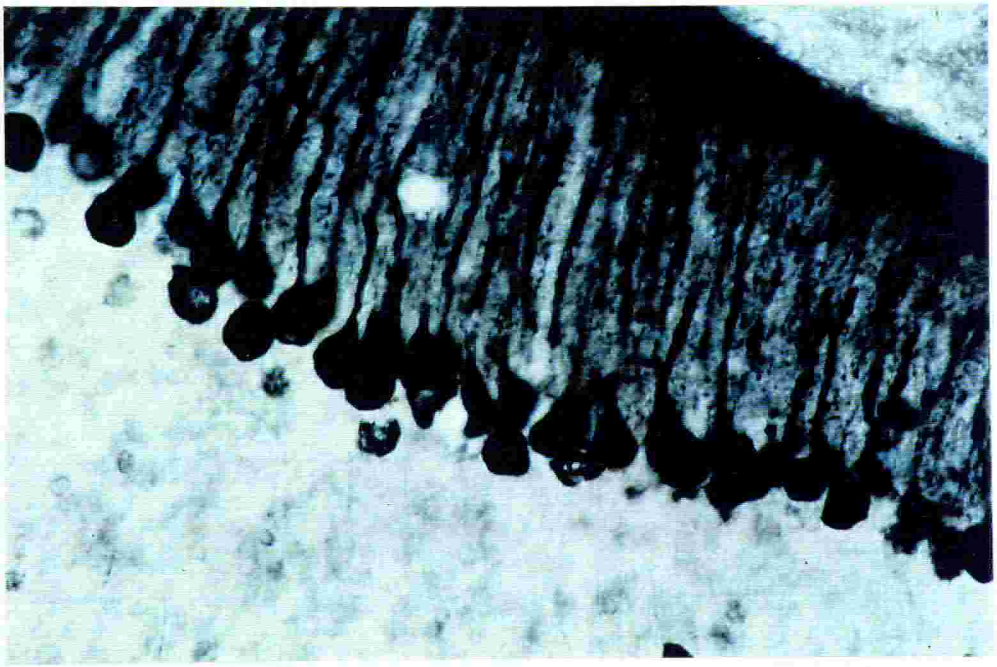


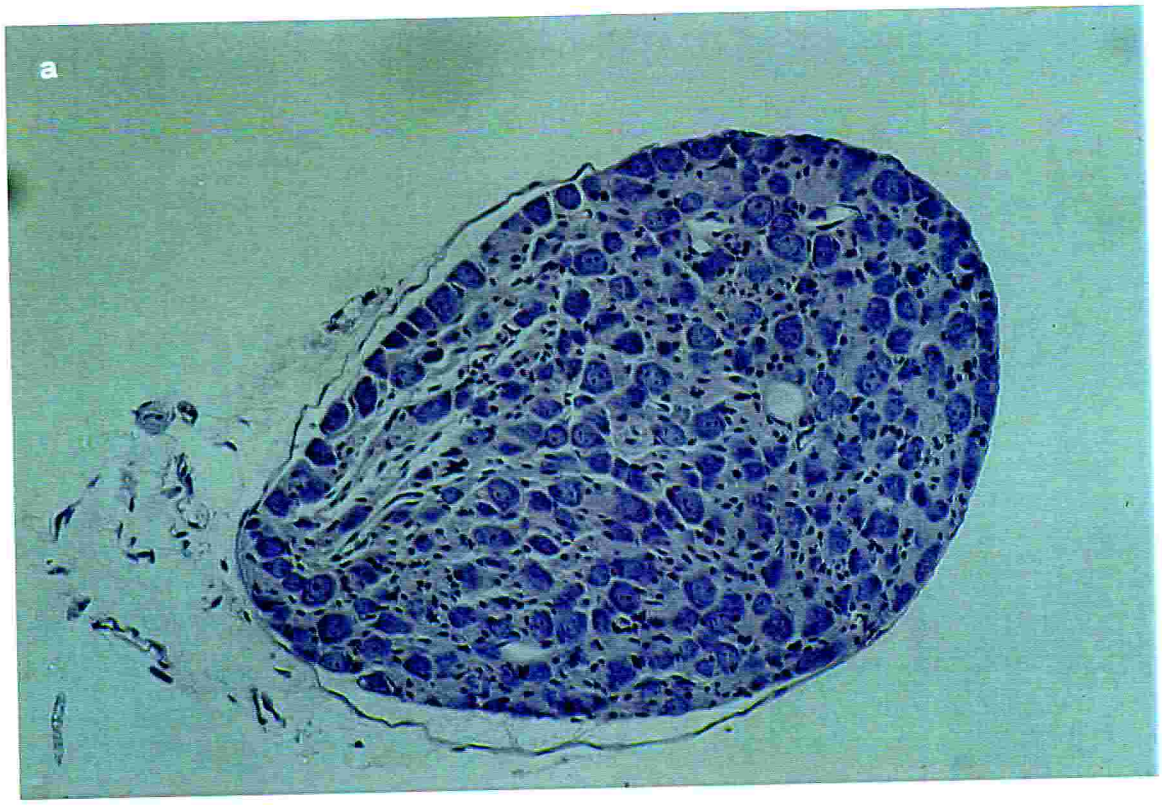
B

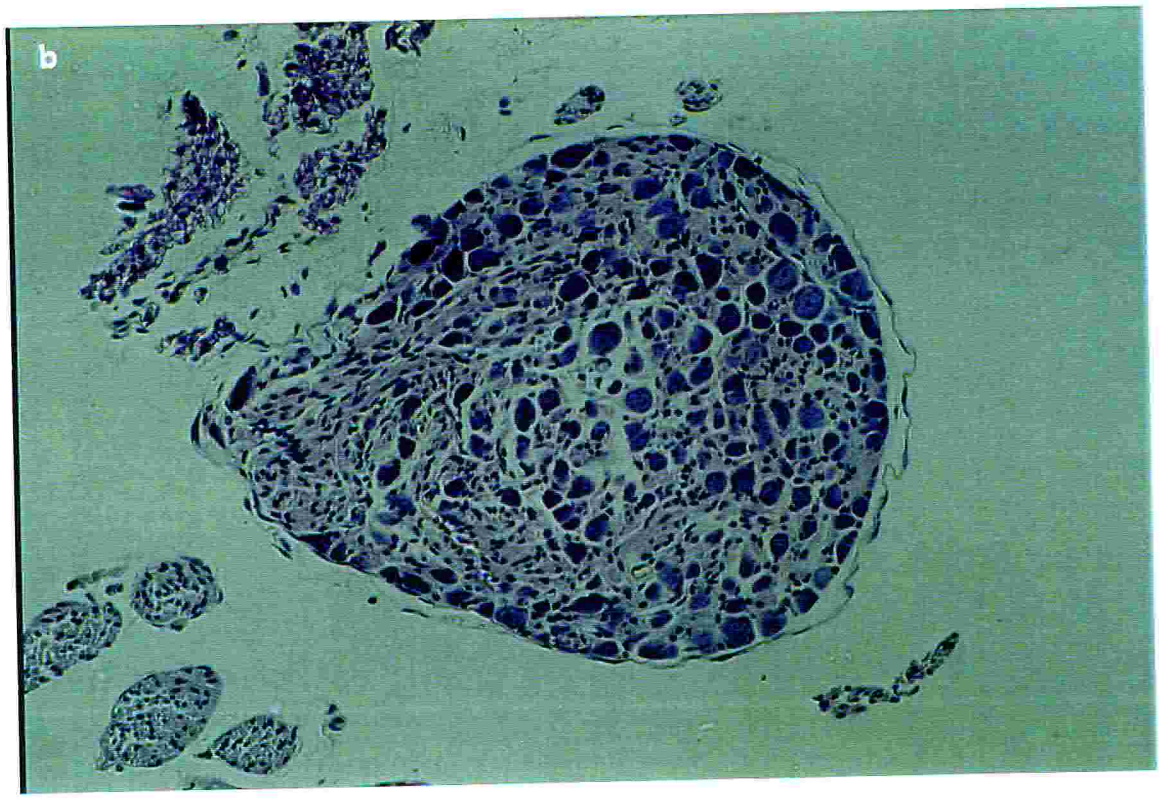




C




a


b

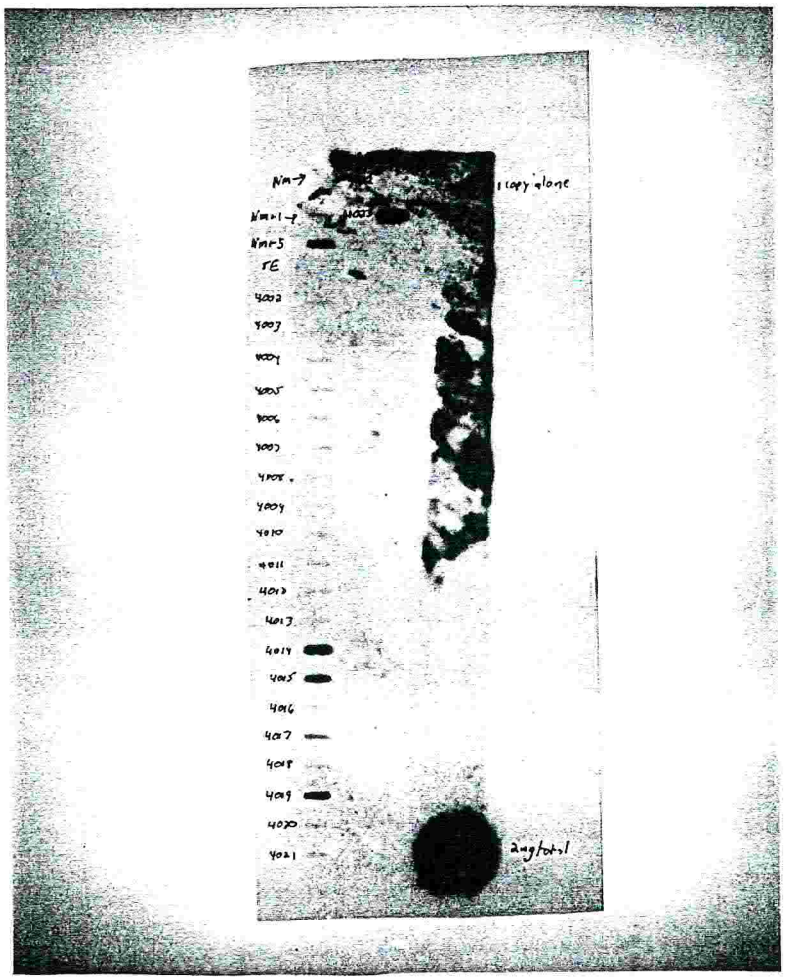


Fig.5.22 Slot blot analysis of mice derived from the microinjection of αD11 light chain. DNA from tail biopsies was hybridised using the light chain constant region as a probe.

The amount of αD11 antibody found in adult mice of family A was in the range of 5-10 ng/ml, wheras it was 50 ng/ml for family B. Two main conclusions can be drawn from this experiment: first, NGF-binding antibodies can indeed be detected in the serum, demonstrating assembly (and therefore coexpression) of the antibody chains, and second, higher levels of antibody are present in the serum of family B with respect to family A.

To understand the reasons for this variability, we performed RNAse protection analysis on different organs of single transgenic mice deriving from VK4019 and VK4023 respectively. The protected fragment for the light chain mRNA from VK4019 mice was barely detectable in all the organs tested, while that from VK4023 was readily detected. The difference of mRNA expression of the light chain between the VK4023 and the VK4019 lines (**fig.5.23**) is consistent with the different levels of αD11 antibody in the serum of transgenic mice A and B. Light chain mRNA expression from line 4023 was shown in brain, kidney, heart and spleen, similar to the pattern of heavy chain mRNA expression.

For technical (and rather unfortunate!) reasons, mice of family A were obtained before those of family B and for this sole reason the studies described below refer to the former. Mice from the family B (high expressors) are only now becoming available in sufficiently large numbers to perform functional studies.

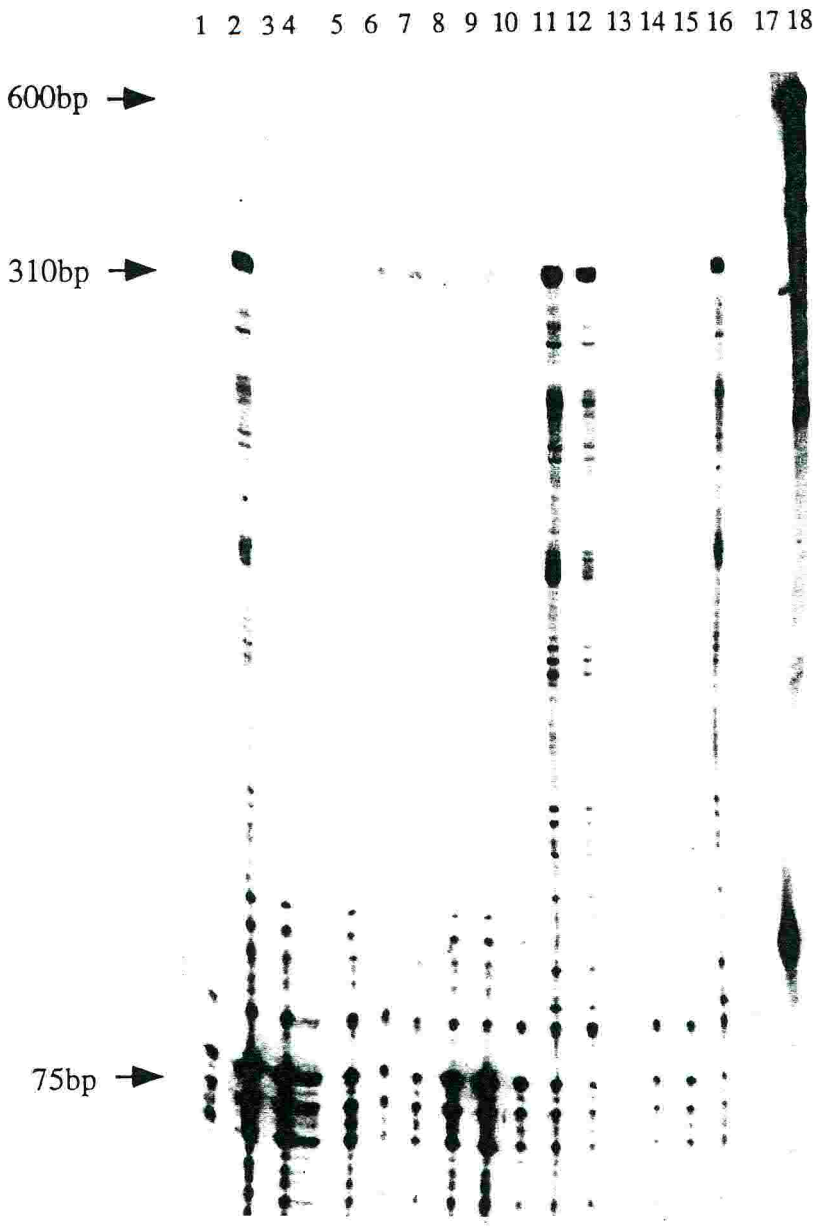


Fig 5.23 Rnase protection analysis of the transgenic VK chain. Total RNA was extracted from adult brain, kidney and heart of transgenic mice of the line VK4023 (lanes 1,2; 6,7; 11,12) and line VH4019 (lanes 3,4; 8,9; 13,14). Total RNA extracted from brain, kidney, heart of adult wilde type mice (lanes 5, 10, 15) and of a cell line transfected with the DNA for the recombinant mAb αD11 (lane 16) were used as negative and positive control respectively. Total RNA (20 μg) was hybridised to a 32P-labeled antisense RNA probe specific for the αD11VK gene. A 310 bp protected fragment corresponding to the transcript from the VK transgene is evident in the brain, kidney and heart of the mice from line VH4645. Numbers on the left represent molecular size (in bp) as derived from a 32P labeled DNA molecular weight marker; β actin and VK RNA probes are also shown (lane 17 and 18)

5.2.6 Analysis of some classical targets of NGF in transgenic mice of family A

We first focused our attention on the analysis of classical targets of NGF in the peripheral nervous system. As anticipated from previous studies, employing anti-NGF antibodies, and more recently from NGF and TrkA knock out mice, survival of small peptidergic DRG neurons, which are thought to mediate pain and thermal receptive functions, depend on the presence of NGF during prenatal life. Several lines of evidence suggest that NGF also acts outside the embryonic period to regulate the function of nociceptors in neonatal life and in adulthood, for example in regulating the synthesis of CGRP neuropeptide, a typical neurotrasmitter of small DRG neurons, as well as the phenotypic expression of nociceptive afferents, without any effects on neuronal survival (Lewin and Mendell, 1993).

In five αD11 transgenic mice, from family A, we tested acute nociception by tail immersion and hot plate tests. These functional tests were performed on adult mice (3 months old) whose circulating anti-NGF antibodies were individually determined, and found to be in the range of 5-10ng/ml. The reaction times in the hot plate test and the tail withdrawal latencies did not significantly differ in anti-NGF transgenic mice and in age-matched controls (**fig.5.24**). The mice were sacrificed in order to evaluate the CGRP levels by radioimmunoassay. Sciatic nerve CGRP level in transgenic mice were identical to those in controls.

Considering the rather low level of anti-NGF antibodies in family A (5-10ng/ml), a possible explanation for the lack of inhibition in the acute nociceptive behavioural tests is that the amount of αD11 antibody is insufficient to compete for the binding of NGF to its receptors, TrkA and p75. In fact TrkA knock out mice develop sores and evidence of self mutilation, and die within the first month of life, and p75 (-/-) animals, even if they survive to adulthood, develop nonetheless evidence of loss of subpopulations of sensory neurons. An alternative or additional explanation for this negative result may be related to a temporally inadequate/inappropriate expression of the transgenic antibodies (see below).

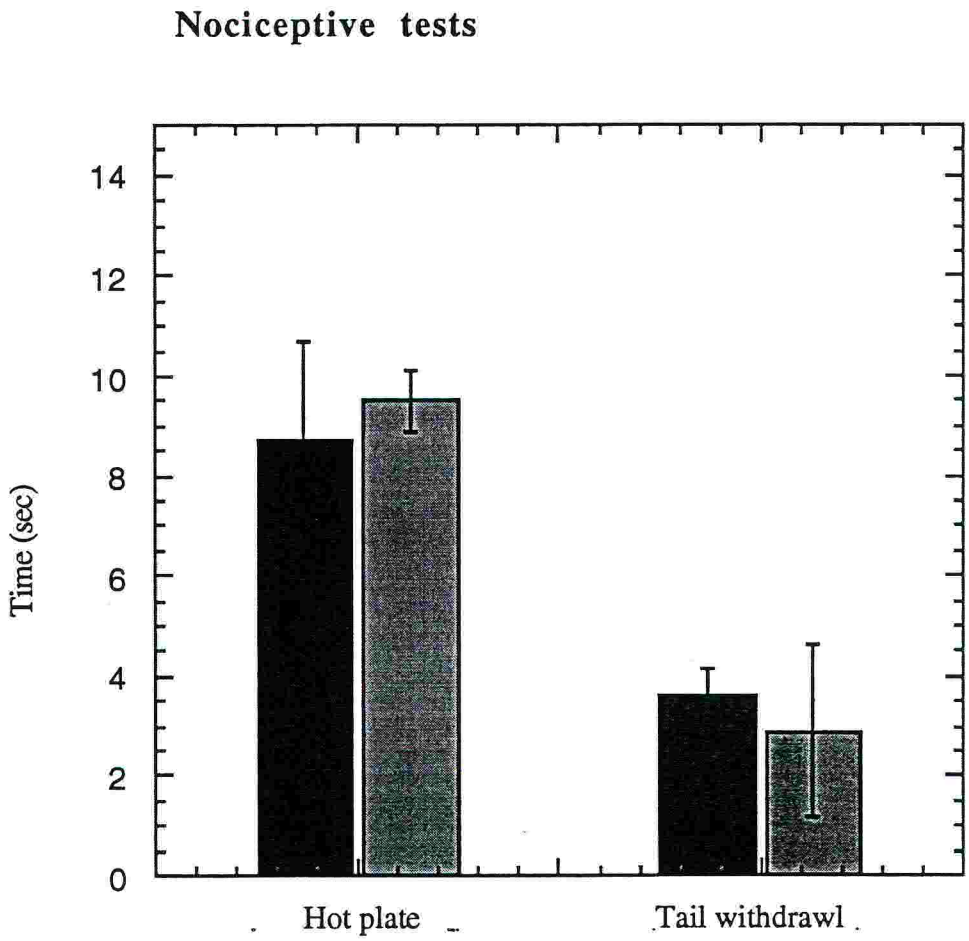


Fig.5.24 Functional tests.

Thermonociception was evaluated by the reaction time in the hot plate test and by the tail withdrawal latency from 50 C water (gray bars, family A [n=5] black bars control [n=5]

Many sympathetic ganglion neurons go through a period of NGF dependence at a much later stage than do DRG neurons. Sympathetic ganglia cells, in particular, undergo a period of massive neuronal death in the first few days of postnatal life. However, NGF deprivation during adult life (Ruit et al., 1990) also leads to a reduction in the number of Superior Cervical Ganglion (SCG) cells. Therefore, adult αD11 transgenic mice were sacrificed to perform anatomical analysis of SCG. The SCG of αD11 transgenic mice was found to be visibly smaller than those of w.t. mice. Quantitative analysis of a prototypical case, revealing a 30% decrease in the number of ganglion neurons present in αD11 transgenic mice, compared to control mice, is shown in fig. **5.25**.

Thus even with these low antibody levels in this particular transgenic family, an effect on SCG cells can be conclusively demonstrated.

Fig.5.25 Superior cervical ganglia (SCG) (a) Coronal section through the center of a SCG of wilde type mouse. (b) Coronal section through the center of SCG of family A mouse. The loss of neurons in the transgenic mice results in a reduction in the total area of the ganglion.

5.2.7 Is the expression of the recombinant αD11 antibody temporally regulated?

As stated above, an alternative and complementary explanation for the lack of effects of transgenic anti-NGF antibodies on the nociceptive system and the incomplete reduction of SCG neurons could be due to the temporal pattern of antibody expression.

No systematic comparison of CMV promoter activity during mouse pre and post natal development is available from data in the literature. Moreover, chromosomal integration could affect the expression pattern of the transgene. In order to verify these possibilities, the expression of the transgenes in different organs of mice, at different ages, was first analyzed at the mRNA level by RNAse protection analysis. Expression of heavy chain mRNA was confirmed in the brain, heart and kidney of transgenic mice both at P1 and at 3 months of age, but the expression is lower at postnatal day 1 than at 3 months (**fig.5.26**). Strong differences are evident in some tissues, in particular, the levels of mRNA for heavy chain from the hearts of adult mice were significantly higher than those of mRNA from neonatal mice. A quantitative analysis (**5.26B**), performed by normalising the mRNA levels with a house keeping mRNA (for β-actin), showed that the levels of mRNA for the heavy chain, in the heart of adult mice are six times higher than at day P1. Likewise, differences, between P1 and P90, are present in the levels of heavy chain mRNA from brain and kidney.

Using the same type of analysis, the expression of the light chain mRNA from VK4023 mice appears to parallel that of heavy chain mRNA (**fig.5.27A,B**),being almost four-fold higher in the adult than in neonatal mice. This pattern of expression i.e. low expression around birth and high expression in the adult, is very convenient for a creation of an anti-NGF transgenic model in which the role of NGF in the adult nervous system could be studied after normal development has occured.

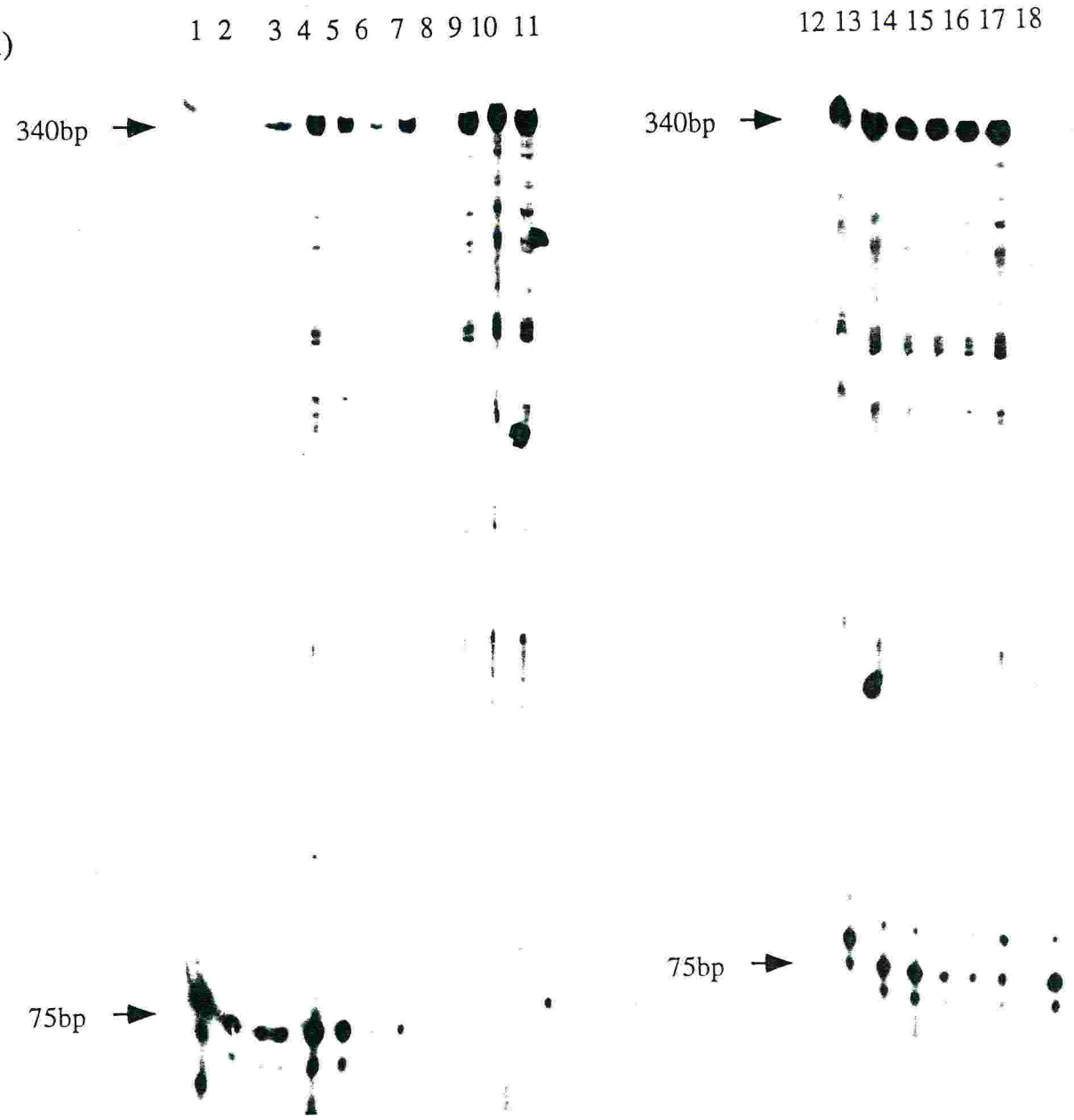
A time course of mRNA transgene expression during the first postnatal weeks will allow us to define precisely at which postnatal time the levels present in adult mice are achieved. A time course of αD11 antibody expression in the serum will allow us

to establish the direct relevance of the mRNA developmental regulation, in terms of its effects on the levels of antibody protein achieved. Also, data on the embryonic expression of the antibody mRNA/protein expression will be important.

Fig.5.26 (A) Rnase protection analysis of the transgenic VH chain at different postnatal ages : P1 and 3 months old mice of the line VH4645. Total RNA was extracted from brain, heart and kidney of P1 transgenic mice (lanes 1-2, 6-8, 12-14) and adult transgenic mice (lanes 3-5, 9-11, 15-17). Total RNA (20 μg) was hybridised to a 32P-labeled antisense RNA probe specific for the αD11VH gene in presence of a 32P -labeled antisense RNA probe specific for βActin. Total RNA extracted from kidney of adult wilde type mice (lane18) was used as negative control. Numbers on the left represent molecular size (in bp) as derived from a 32P labeled DNA molecular weight marker.

(**B**) The level of VH αD11 expression from heart at different postnatal ages was evaluated by phosphorimaging analysis. The amount of the 340 bp protected fragment corresponding to the transcript from the VH transgene was normalized respect to the 75bp protected band specific for the endogenous βActin gene. The graph shows mean counts ± S.E..

A)


1 2 3 4 5 6 7 8 9 10 11
12 13 14 15 16 17 18
340bp
340bp
75bp
75bp

B)

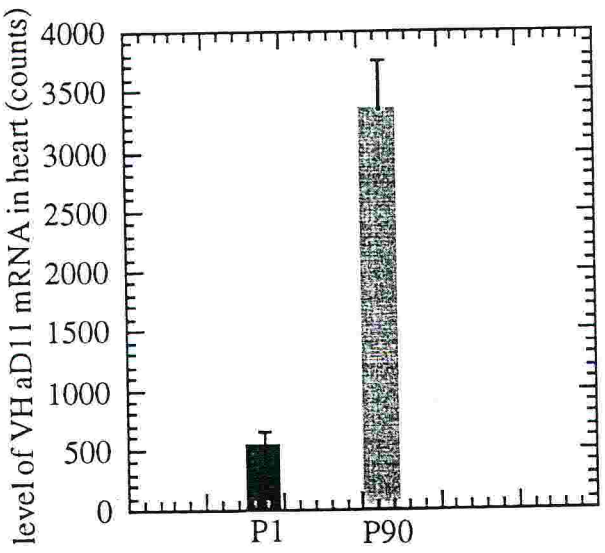
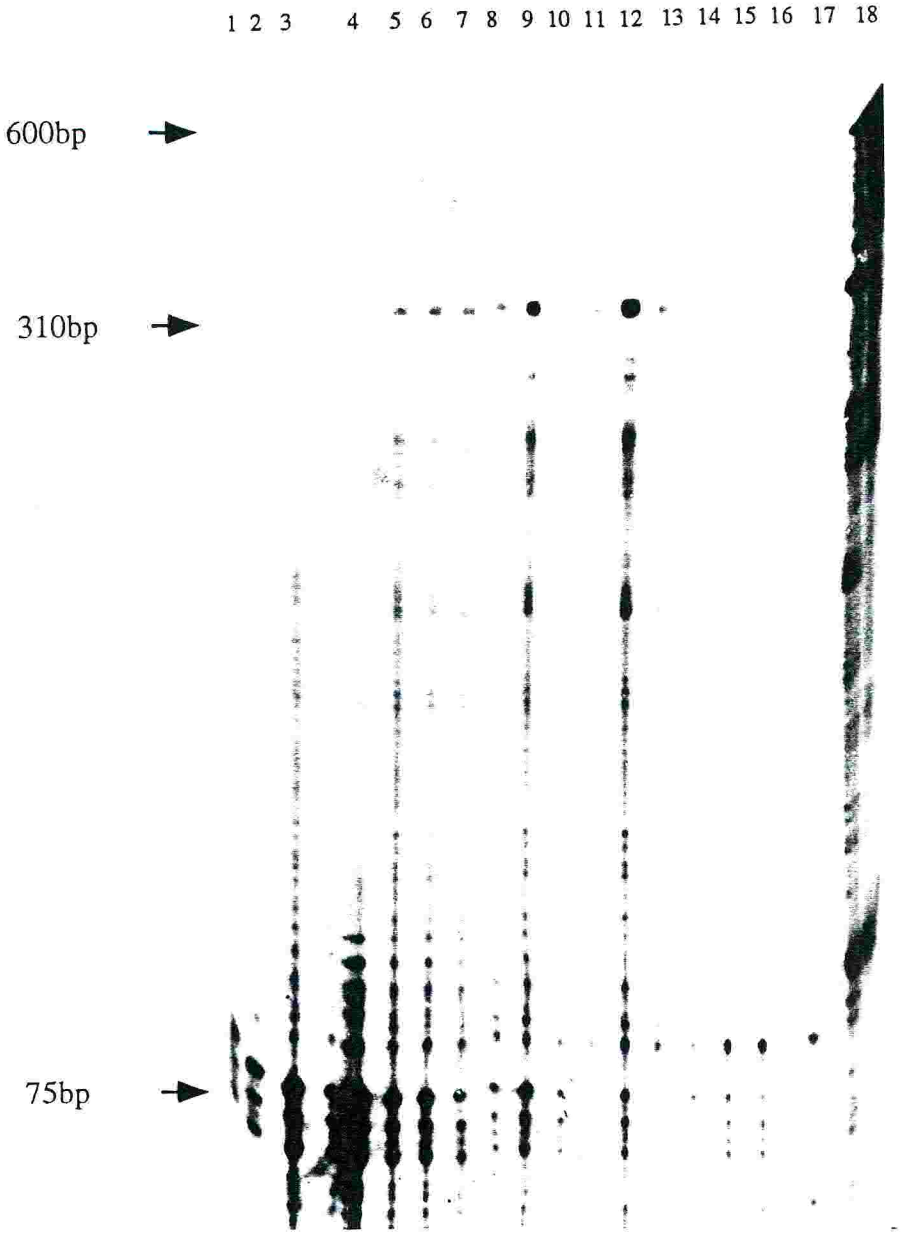

level of VH aD11 mRNA in heart (counts)
4000
3500
3000
2500
2000
1500
1000
500
0
P1
P90

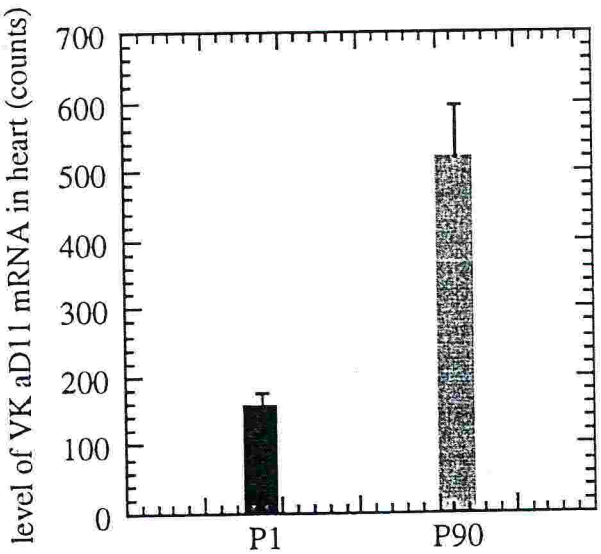
Fig.5.27(A) Rnase protection analysis of the transgenic VK chain at different postnatal ages : P1 and 3 months old mice of the line VK 4023 Total RNA was extracted from brain, kidney and heart of P1 transgenic mice (lanes 3-4, 8-10, 14-16) and adult transgenic mice (lanes 1-2, 5-7, 11-13). Total RNA (20 μg) was hybridised to a 32P-labeled antisense RNA probe specific for the αD11VK gene in presence of a 32P -labeled antisense RNA probe specific for βActin mouse. Total RNA extracted from kidney of adult wild type mice (lane17) was used as negative control. Numbers on the left represent molecular size (in bp) as derived from a 32P labeled DNA molecular weight marker.

(B) The level of VK αD11 expression from heart at different postnatal ages was evaluated by phosphorimaging analysis. The amount of the 310 bp protected fragment corresponding to the transcript from the VK transgene was normalised with respect to the 75bp protected band specific for the endogenous βActin gene. The graph shows mean counts ± S.E..

A)


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
600bp
310bp
75bp

B)


level of VK aD11 mRNA in heart (counts)
700
600
500
400
300
200
100
0
P1
P90

In the light of these results, the negative data on the nociceptive tests can be interpreted as being due to the antibodies not having been present at concentrations sufficiently high enough, during prenatal development, to induce cell death. On the other hand, the tests performed were not suitable to study the more subtle, sensory-modality related, actions of NGF on sensory neurons in adulthood. Direct experiments in this direction are being planned. Also, a comparison with forthcoming results obtained with the family B will be instructive.

5.2.8 The immune response of αD11 transgenic mice could be altered.

There is some evidence that NGF may play important roles outside the nervous system and particular interest has been directed to the immune system. Early studies had demonstrated the presence of NGF high affinity receptors in thymocytes (Cattaneo, 1985) and recently expression of the TrkA protooncogene has been shown in cells of the immune system such as monocytes (Ehrhard et al., 1993) or T lymphocytes (Ehrhard et al., 1993a). Moreover, elevated plasma levels of NGF have been observed in patients with autoimmune disease (Dicou et al., 1993; Bracci-Laudiero et al., 1993). Very recently, Torcia et al. (1996) have shown that NGF is synthesized and released under basal conditions by normal human B lymphocytes, which also constitutively express both p75NGFR and p140Trk-A receptors, suggesting that NGF could be involved in a B lymphocyte autocrine loop. A single administration of neutralizing anti-NGF antibodies in vivo caused strong reduction in the titer of specific IgG in mice immunized with tetanus toxoid, nitrophenol or arsonate, and reduced numbers of surface IgG or IgA B lymphocytes. The authors suggest that NGF could be an autocrine survival factor for memory B lymphocytes. As shown above, αD11 transgenic mice express anti-NGF antibody in the serum. Moreover, the presence of heavy and light chains mRNA in the spleen suggests that the local levels of αD11 protein could be higher than those in the serum. Following the recent results by Torcia et al., we are undertaking experiments to analyse the immune response in αD11 transgenic mice, in order to study whether also in our

mouse model (in which circulating anti-NGF antibodies are present in a continuous fashion) the activity of memory B lymphocytes is affected. In a preliminary experiment, we have used the two antigens Nitrophenol-BSA and Oxazolone, whose immune rsponse is very well characterized in the mouse. Thus, two group of three αD11, or VK 4019, transgenic mice were immunized with the relevant antigen. After the second immunization with the respective antigen the serum concentrations of antigen-specific IgM and IgG will be determined by Elisa.

5.3 Discussion

The creation of a stable line of αD11 transgenic mice was a long and knotty work, but, in the end, the results obtained lay the grounds for an experimental model that may provide interesting results in the near future.

The first experiment discussed above with founder mice which express both heavy and light chains of the αD11 mAb leave us with some "suspicions" about the requirement of endogenous NGF for the normal physiology of the male reproductive system and possible lethal effects of NGF deprivation during prenatal life due to blockade of both maternal and/or fetal NGF.

To achieve our aim — the production of transgenic mice which express functional αD11 antibody — the creation of transgenic mice which expressed either heavy or light chains of the αD11 antibody was an adequate solution. This allowed us to obtain continuous stable lines of founders in which the expression of the transgene should not produce any phenotype. The validity of this assumption (lack of anti-NGF activity in the transgenic mice for the individual chains) may be challenged by arguing that since the expression of the transgenes is under the control of the CMV promoter, which is also active in B lymphocytes, it is in principle possible that αD11heavy or light chains may assemble with endogenous mouse immunoglobulin chains in B lymphocytes, thereby recostituting an anti-NGF antibody. However, this is an unlikely event, since we have previously demonstrated that heavy or light

chains of the αD11 antibody, assembled by cotransfection with some irrelevant antibody chains are unable to bind NGF (see Results, Expression of recombinant αD11 antibody in eucaryotic cells). The possibility of the odd antibody chain from the mouse repertoire yielding a functional anti NGF binding site in combination with one of the two αD11 antibody chains is a rare, but possible event, that, at most, would provide a very low level of circulating anti NGF antibody. A direct test for the presence, in the single transgenic mice, of (hybrid) anti NGF antibodies, has shown that no significant anti-NGF activity is found in the serum of the single transgenics. Obviously, we cannot exclude the remote possibility that assembly of the αD11 heavy or light chain with mouse immunoglubulin chains may produce antibody molecules recognising other epitopes of endogenous mouse antigens. In the lack of any evidence of the contrary, we shall consider the single transgenic mice as "normal mice".

Finally, αD11 transgenic mice have been otained by mating transgenic mice expressing individual heavy and light chain of the αD11 antibody. It was shown that : i) that functional αD11 antibodies can indeed be detected thereby proving that the two chains are coexpressed in the same set of mouse cells where they can assembled and be secreted and ii) the αD11 transgenic antibodies are indeed able to compete with some of the known biological actions of NGF (superior cervical ganglion neurons), iii) the transgenic antibodies are expressed in a temporally regulated manner, with a pattern of regulation that is very convenient for a novel anti-NGF transgenic model, i.e. very low expression around birth, with the expression reaching the peak in the adult. In fact, this is also compatible with the result that in αD11 transgenic mice the number of SCG neurons, a population of neuronal cells typically depending from NGF at somewhat later stages of postnatal life, was reduced, while DRG neurons,which depend on NGF during prenatal life, were unaffected, (on the basis of the negative results on simple nociception tests). These results are consistent with higher expression of mRNA transgenes in adult mice and lower expression in neonatal mice.

If further more detailed analysis will confirm this developmental regulation of antibody/protein expression, the αD11 transgenic mice should provide a useful model for studying the role of NGF in adult mice, after a normal development has occured. Unfortunately, the experiments just summarized were (as previously described) carried out with the low expressing mice of family A (because of availability reasons!). Experiments with family B (high expressors) are forthcoming, and the results eagerly awaited.

Mating of the αD11 transgenic mice to p75 -/- knock-out mice may increase some, but not all, of the phenotypic effects eventually observed. The lines of transgenic mice expressing individual heavy or light chains of the αD11 antibody, driven from the CMV promoter, could be further exploited to obtain a more refined spatial and/or temporal control of αD11 expression. This will be achieved by mating mice transgenic for one of the two chains to new transgenic lines expressing the cognate chain under the control of a tissue specific or an inducible promoter. This will provide a more restricted expression of functional antibody. Expression of one or both of the αD11 antibody chains driven by a promoter specific for expression in mammary glands (such as for instance the promoter of the goat beta-casein gene) will allow a very high expression of the recombinant antibody (in the order of 1 mg/ml) in the milk of lactating transgenic mice. This will allow to achieve a time controlled delivery of anti-NGF antibodies to neonate mice, through a time controlled exposure of mice pups to transgenic lactating mothers. Engineering an IgA version of the transgenic αD11 antibody may help in this oral route of antibody administration. It is noteworthy that lines of mice transgenic for the individual chains also allow to bypass problems that in double transgenic mice would arise from the maternal expression of recombinant antibody, that could produce a lethal phenotype for the embryos. An example of this was found in our laboratory for transgenic mice expressing anti SP antibodies (Piccioli and Cattaneo, manuscript in preparation). In conclusion, the mouse model derived should allow us

to address questions related to functions and roles of NGF in the adult nervous system, which cannot presently be addressed with other transgenic models.

Most of the experimental models to study brain insults (ischemia etc.) and the neuroprotective actions of NGF are in the rat. Also, most of the physiology of sensory systems (visual, olfactory, auditory) is better developed for rats than it is for the mouse. The availability of recombinant anti-NGF antibodies will allow us to develop neuroantibody-based models of transgenic rats, a species where the knock-out technology is not yet possible.

The availability of recombinant αD11 ScFv will allow affinity maturation of this antibody by the use of phage display technology, to further improve its performance in vivo. (Winter et al., 1994; Bradbury and Cattaneo, 1995b). The smaller size of the ScFv version of the recombinant αD11 antibody may facilitate its delivery across the blood-brain barrier, possibly by engineering its binding to transferrin receptors (Friden et al., 1993).

The transgenic approach, which sofar has been restricted to mice, can be complemented by viral mediated gene transfer on other species, by the expression of blocking anti-NGF ScFv.

5.4 *Material and Methods*

5.4.1 Plasmids.

M13-VHPCR1, M13-VKPCR1, pSV-gpt-Huγ1 and pSV-hyg-HuCk were kindly provided by Greg Winter (Medical Research Council, Cambridge, U. K.). Plasmid pcDNAIneo was from InVitrogen.

5.4.2 Cloning of αD11 variable regions and plasmid construction.

The cloning of αD11 variable regions was performed by a modification of the RACE method of Frohman et al. (Frohman et al., 1988) as outlined below. Cytoplasmic mRNA was prepared from $5x10^6$ αD11 hybridoma cells with standard techniques (Sambrook et al., 1989). cDNA was synthesized by incubating 1μg of poly(A) mRNA at 65°C for 5 min in DEPC treated water, this was quenched on ice and then added to a mixture containing 5μl 5xRT buffer, 1μl RNasin (Promega), 10 pmol primer (specific for rat γ2a or rat k chains, as appropriate), 250μM of each of the four deoxynucleotide triphosphates (dNTPs) and 10U of Moloney murine leukemia virus reverse transcriptase in a total volume of 25μl. The oligonucleotides used were 5'CTCATTCCTGTTGAAGCTCTTGAC3' (CKRat), complementary to the 3' region of rat Kappa constant region, and 5'ACAAGGATTGCATTCCC-TTGG3' (Rat γ2aHinge) complementary to the hinge region of rat γ2a constant region (the isotype of αD11). The reaction mixture was incubated at 42°C for 60 min and then at 52°C for 30 min. After inactivation at 95°C for 5' the reverse trascription mixture was diluted with 2 ml of 0.1 TE (1mM Tris/0.1mM EDTA pH7.4) and excess primer was removed using a Centricon 100 spin filter (20 min at 1000g, twice). The retained liquid was collected and concentrated to 10μl, and a polyA tail added to the 5' end of the cDNA by adding 4μl 5X Tailing buffer, 4μl dATP 1mM and 10U of Terminal deoxynucleotidyl transferase (Promega). This was incubated for 5 min at 37°C and then 5 min at 65°C. The volume of the cDNA reaction was adjusted to 500μl and 10μl were amplified with Vent polymerase as

follows: 1 precycle (5min 95°C ; 5 min 60°C; 40 min 72°C) and 40 cycles (1min 95°C; 1 min 60°C; 3 min 72°C). For amplification of the VH region, PCR was performed using one oligonucleotide, 5'CTC AAT TTT CTT GTC CAC CTT GGT GC 3'(MOCG124), internal to Rγ2aHinge (used for cDNA synthesis) and another, XSCTnTag, (5'GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT3'), which hybridizes to the poly(A) tail added to the 5' end of cDNA. For amplification of the VK we used the XSCTnTag primer and the oligonucletide CK Rat Bam (5'CAAGT-CCGGATCCCTAACACTCATTCCTGTTGAAGCTCTTGACGACGGG3')
which encompasses the sequence of the CK rat primer, and extends six bases beyond. The 3' oligonucleotide used for amplification of the VK DNA fragment contained a restriction site (BamHI) for cloning, whereas the VH DNA fragment was cloned using the PstI site found in the CH1 region. In both cases the 5' restriction sites (XhoI, SalI, ClaI) are provided by the XSCTntag oligonucleotide. The amplified DNA fragments were cloned into the Bluescript KS+ vector (Stratagene) and sequenced using the Sequenase DNA sequence kit (USB).

In order to incorporate the appropriate restriction sites required for cloning into the available expression vectors, the αD11 variable regions had to be reamplified as in Orlandi et al., (Orlandi et al., 1989) with V-region specific primers. Although performed on cloned material, the PCR had to be performed at the reduced annealing temperature of 37°C. The amplified bands were subcloned first into M13-VHPCR1 or M13-VKPCR1 (to yield M13-IgVHαD11 and M13-IgVKαD11), checked by sequencing and subsequently in pSV-gpt-Huγ1 or pSV-hyg-HuCk ,as decribed (Orlandi et al., 1989), to yield plasmids pSVgpt-IgVHαD11 and pSVhygro-IgVKαD11, which direct the expression of chimeric (αD11 variable regions attached to human constant regions) heavy and light chains under the transcriptional control of the immunoglobulin heavy chain promoter and enhancer.

Plasmids pcDNAI-neo/VHαD11 and pcDNAI-neo/VkαD11 were derived by subcloning the variable regions (from the NcoI site encompassing the initial ATG to a BamHI site in the V-C intron) into pCDNAI-neo. The Human γ1 and k constant

regions were amplified from pSV-gpt-Huγ1 and pSV-hyg-HuCK and subcloned into pcDNAI-neo/VHαD11 and pcDNAI-neo/VK αD11 respectively, to yield pcDNAI-neo/VHαD11HuCγ and pcDNAI-neo/VK αD11HuCK. These plasmids direct the expression of the recombinant antibody chains from the cytomegalovirus (CMV) early promoter and enhancer (Boshart et al., 1985).

5.4.3 Cells, transfections and immunofluorescence.

αD11 hybridoma (Cattaneo et al., 1988) and simian COS cells were cultured in Dulbecco's Modified Eagle's Medium with 10% Foetal Calf Serum (FCS). PC12 cells were grown in RPMI containing 10% Horse Serum, 5% FCS.

COS cells transfection by the DEAE-dextrane/chloroquine method and indirect immunofluorescence were performed as previously described (Biocca et al., 1990). 10^6 COS cells were plated onto polylysine-coated 35 mm Petri dishes, transfected with the relevant plasmid DNA and incubated with 1 ml of culture medium. Cells were analyzed by indirect immunofluorescence 48 hours after the transfection, while the supernatant was harvested 72 hours after the transfection (referred to as COS cell transfectants supernatant).

5.4.4 Assays for anti-NGF antibodies

2.5 S NGF was purified from the mouse submandibular glands according to the method of Bocchini and Angeletti (1969) and was kindly provided by D. Mercanti.

5.4.5 NGF bioassay on PC12 cells

PC12 cells (Greene and Tischler, 1976) were plated in 96-microwell collagen-coated plates at a concentration of 25000 cells/well and cultured in the presence of 50ng/ml NGF and of 100μl of relevant supernatant (from transfected COS cells or

from αD11 hybridoma cells). Supernatants were collected from COS cells 72 hours after the transfection. Growth of neurites was scored after four days.

5.4.6 ELISA

96-well plates were coated with 5μg/ml NGF in 0.1M Carbonate buffer pH 9.6. Binding to NGF of antibodies secreted from COS cell transfectants was revealed with biotinylated anti-human or anti-rat costant γ heavy-chain region (Cγ) antibodies, as appropriate, followed by streptavidin-peroxidase (Amity).

5.4.7 Immunoprecipitation of 125I-labelled NGF

NGF was labelled with 125I by the chloramine T method at a specific activity of 10^5 cpm/ng as described (Cattaneo et al., 1983). Aliquots of COS cell transfectants supernatants were incubated with 125I-NGF and antigen-antibody complexes were precipitated with Protein A-Sepharose (Pharmacia) (or with anti rat Ig-Sepharose in the case of MAb αD11 supernatants). In all these assays, controls included i) culture medium, ii) supernatants from non relevant hybridomas, iii) supernatants from COS cells transfected with plasmids directing the expression of non relevant recombinant antibodies.

5.4.8 Western Blot

SDS-gel electrophoresis was performed as described by Laemmli (1970). Proteins were transferred into a nitrocellulose membrane, in a semi-dry system (Tris-glicine-methanol). The membrane was incubated in PBS (0.1% Tween 20, 2% dry milk) for 1 hr with gentle agitation. After 5 washes in PBS (0.1% Tween 20), the membrane was incubated in a solution of 9E10 antibody (ascites, 1:2000 dilution) for 1 hr with gentle agitation. The 9E10 antibody is directed against the myc peptide tag. After 5 washes a labelled secondary antibody (peroxidase conjugated) has been

added and incubated for 1 hr. Then after 5 washes the membrane was transferred to the substrate solution (3-3'-Diaminobenzidine tetrahydrochloride tablet dissolved in PBS containing 3% hydrogen peroxide).

5.4.9 Tail DNA extraction .

For tail biopsies , mice were weaned at 18 to 22 days of age, at which time the distal 1 cm of the tail was escised with a sterile razor blade. Tail biopsies were placed in 1.5 ml microcentrifuge tubes and digested overnight at 55 C with 0.5 ml of tail digestion buffer [50 mM Tris-HCl, pH 8, 100 mM EDTA, 100 mM NaCl, 1% SDS, 300 µg Proteinase K (Boehringer-Mannheim)]. Each digest was extracted with 1 volume of Tris-buffered phenol/chloroform, pH 7.2. DNA was precipitated from the acqueous phase with two volumes of absolute ethanol. Pellets were then redissolved in 500ml sterile water.

5.4.10 Screening of transgenic mice by PCR analysis of DNA

The PCR cocktail was performed in 25 µl total volume containing approximately 100ng tail DNA. The PCR cocktail was made up to give the following concentration when added to the sample solution: 10 mM Tris-HCl pH 9, 1.5 mM MgCl2, 0.1% Triton X-100, and 0.2 mM dNTPs. Each 25 µl PCR sample contained 12.5 pmoles of both 5' and 3' PCR primers and 0.7 U Taq plymerase (Perkin Elmer, Norwal, CT). The conditions for temperature cycling were: 94 C for 5 min followed by 30 cycles of 94 C for 1 min., 65 C for 1 min. and 72 C for 1 min. Cycling was followed by a final extension step at 72 C for 10 min. and the reactions were then held at 4 C until removed for analysis. PCR products were resolved by 1.5% agarose gel electrophoresis.

For amplification of αD11 VH transgene PCR was performed using th following 5' and 3' primers: αD11 VH BACK, 5' CAG GTG CAG CTG CAG GAA TCA GGA CCT3' and αD11 VH FOR, 5' TGA GGA GAC GGT GAC CGA AGT TCC TTG ACC 3'. For amplification of αD11 VK transgene PCR was performed using the

following 5' and 3' primers: VK1 BACK, 5' GAC ATT CAG CTG ACC CAG TCT CCA 3', and CD3 VK αD11, 5' CGT CCG AGG ATA ATG GAA ATA GTG CTG 3'.

5.4.11 Slot blot

Briefly, 20 μg of genomic DNA was trasferred to nitrocellulose and hybridised with 32P labeled DNA fragment encompassing human constant regions as probes. For the heavy chain , a BamHI-XbaI fragment was purified from the vector pcDNAI-neo/VHαD11HuCγ, while for the light chain we used a BamHI-ApaLI fragment from plasmid pcDNAI-neo/VkαD11HuCk. Probes labeled with 32P to a high specific activity by random priming were used under high stringency hybridization (50% formamide, 6x SSC, 5x Denhardt's, 0.5% SDS, 0.2 mg/ml sonicated salmon sperm DNA at 42 C) and washing (0.2 x SSC, 0.1% SDS at 65 C) conditions.

5.4.12 RNA analysis

Anesthetised mice were sacrificed, and tissues were dissected, immediately frozen in liquid nitrogen, and stored at -70 C until use. Total RNA was isolated following the guanidine-isothiocyanate procedure (Chomcynzski and Sacchi, 1987) and analysed by RNase protection assay using the following probes: VH (PstI-BamHI; 555bp) from pcDNAI-neo/VHαD11HuCγ cloned in Bluescript KS (+); and VK (Sac-BamHI) from 3JFαD11.

PvuII fragments from these vectors (983 and 600 bp, respectively) were used as templates for RNA transcription with T7 polymerase in the presence of [32P]UTP (Amersham; 800 Ci/mmol). The size of the expected protected band is 340 bp for VH and 310 bp for VK.

The template for βActin mRNA transcription was a RsaI, 1700bp fragment in pGEM4Z (kindly provided by R. Possenti). Transcption with T7 polymerase produced a 110 bp probe. The size of the expected protected band is ~75 bp.

VH, VK and βActin antisense RNA probes were hybridised (or cohybridised) 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% acrylammide, 8M urea gel and autoradiographed.

5.4.13 Histological Methods

5.4.14 Testis analysis:

Immunoperoxidase procedure. Pieces of testes from transgenic mice were embedded in OCT compound (Tissue Tek, miles Elkhart, IN) and frozen in liquid nitrogen. Cryostat section (7 μm) were mounted on gelatin-coated slides, fixed in methanol at -20 C for 10 min., then transferred to phosphate-buffered saline (PBS). The sections were incubated for 15 min. in 0.3% H2O2 in absolute methanol to inhibit endogenous peroxidase, 60 min. in PBS containing 10% fetal calf serum (FCS) to block aspecific binding and finally incubated with biotinilated anti-human antibody (dilution 1:500 in PBS) for 1 hr at room temperature. The sections were then washed and incubated for 30 min. in Vectastain ABC kit reagent (Vector Laboratories, USA). Peroxidase activity was revealed by incubation for 7 min. in peroxidase substrate solution containing 0.05% diaminobenzidine tetrahydrocloride, 0.01% H2O2 and 0.25% nickel sulfate. The specificity of the reaction was checked on control sections prepared with biotinylated antibody omitted or cut from testis of wilde type mouse.

Light mcroscopy Small pieces of transgenic mouse testis were fixed by immersion in 2.5% glutarldehyde in 0.1% M cacodylate buffer for 2 h, then postfixed for 1 h in 1 % OsO4, followed by dehydratation in ethanol and embedding in EPON 812. The section were cut at the thickness of 1 μm, stained by flotation on warm 1% acqueous toluidine blu, studied and phographed with a Leitz Orthoplan microscope.

5.4.15 Brain analysis:

Immunocytochemistry Mice were deeply anesthetized (chloral hydrate 6ml/kg, 10% in saline solution) and transcardially perfused with saline solution followed by 4% paraformaldehyde in phosphate buffer 0.1M (PB). After cryprotection with 30% sucrose in PB 0.1M, coronal brain section were cut at 30 μm and incubated in 0.1% H2O2, 10% fetal calf serum (FCS) in PBS before being analysed as floating sections for the presence of transgenic antibody using the avidin-biotin-peroxidase Elite Standard Kit (Vector Laboratories). Biotinylated anti-human IgG (Amersham) at 1:500 (0.1% triton X-100, 10% FCS in PBS) and anti-human light chain chain (Amersham and/or Vector) at 1:50-1:100 were used as primary antibodies. Sections from age-matched control mice were also analysed in parallel under the same conditions, in addition to sections in which primary antibodies were omitted.

5.4.16 SCG analysis:

Mice were deeply anesthetized (chloral hydrate 6ml/kg, 10% in saline solution). SCG were dehydrated in ethanol, embedded in paraffin, serially sectioned at 8-10 μm and stained with hematoxolin and eosin method.

5.4.17 Functional and behavioral experiments

The CMV anti-NGF transgenic mice were used for the experiments at the age of 2-3 months. Family A mice have been tested for acute nociception. For the hot plate test, mice were placed on a 55°C hot aluminium plate 19 cm in diameter with a 20 cm high perspex cylinder. Licking of the hindpaws or jumping was used as endpoint. For the tail withdrawal test, mice were held in tubular restraining cages, and the terminal two-thirds of the tail were dipped into water at 50°C.

CONCLUSION

In order to study the role of endogenous NGF on neuronal populations of the CNS and on synaptic plasticity of the hippocampus, we have pursued two different approaches to make the CNS experimentally accessible to selective inhibition of NGF by the specific neutralising antibody αD11: 1)intracerebral grafting of αD11 hybridoma cells and 2)production of transgenic mice harbouring the recombinant antibody αD11. The following results have been obtained:

i) Injection of αD11 hybridoma cells can be used to effectively antagonise endogenous NGF, as demonstrated by the reduction of ChAT staining in BF cholinergic neurons, a population of cells known to be sensitive to NGF.

ii) The intraventricular implant of αD11 cells at different postnatal ages allowed us to demonstrate the existence of a critical period in the sensitivity of BF neurons to NGF deprivation.

iii) In the developing hippocampus, NGF deprivation during the first postnatal week, inhibits the induction of MF-LTD, at least as observed between postnatal days P9 to P12. Our experiments do not allow us to conclude whether the observed inhibition of LTD observed derives from a direct effect of NGF deprivation or to an indirect effect, secondary to a reduction of cholinergic input to the hippocampus.

iv) In adult αD11 implanted rats, separately shown to be severelly impaired in radial maze test, CA1 LTP was normal, while on the other hand, depotentiation of the same synaptic input is defective.

v) a transgenic mouse model has been created that will allow to study the effects of blocking anti NGF antibodies in adult mice, after normal development has occurred.

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