In vivo function of NGF/TrkA signaling in the cholinergic neurons of the murine basal forebrain

Dissertation

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Vorgelegt von
Markus Müller

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Prüfungskommission:

Herr PD Dr. Rüdigher Klein (Vorsitz)
Herr Prof. Dr. Stefan Jentsch
Herr Prof. Dr. Benedikt Grothe
Herr Prof. Dr. Hans-Ulrich Koop

Umlauf:

Herr PD Dr. Mark Hübener
Herr Prof. Dr. George Boyan

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II ABBREVIATIONS

Aβ	Amyloid β
ac	anterior commissure
ACh	Acetycholine
AchE	Acetylcholine-Esterase
AD	Alzheimer's disease
ADAM	a disintegrin and a metalloprotease
APP	Amyloid precursor protein
BACE	β-site APP cleaving enzyme
BDNF	Brain-derived neurotrophic factor
bp	basepair
Ch	cholinergic cell group
ChAT	Choline acetyltransferase
CIPA	Congenital insensitivity to pain and anhidrosis
CNS	Central nervous system
C-ter	Carboxy-terminal
DB, hl	Diagonal band, horizontal limb
DB, vl	Diagonal band, vertical limb
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DRG	Dorsal root ganglium
E	Embryonic day
ec	External capsule
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signals related kinase

ES cells	Embryonic stem cells
FAD	Familial Alzheimer's disease
FGF	Fibroblast growth factor
GABA	Gamma aminobutyric acid
Gp	Globus pallidum
HRP	Horseradish peroxidase
IB	Immunoblotting
ic	Internal capsule
IHC	Immunohistochemistry
IRES	Internal ribosomal entry sequence
kDa	kilodalton
LTD	Long-term depression
LTP	Long-term potentiation
mAChR	muscarinic acetylcholine receptor
mRNA	messenger ribonucleic acid
nAChR	nicotinic acetylcholine receptor
NB	Nucleus basalis
Nes	Nestin
NGF	Nerve growth factor
NT-3	Neurotrophin-3
O.D.	Optical density
OD	Ocular dominance
P	Postnatal day
PCR	Polymerase chain reaction
PKC	Protein kinase C
PNS	Peripheral nervous system
PS	Presenilin
sAPP	soluble amyloid precursor protein
SCG	Superior cervical ganglion
St	Striatum
TNF	Tumor necrosis factor

Trk	Tropomyosin-related kinase
VACht	Vesicular ACh transporter
WT	Wild-type

III INTRODUCTION

After a first general part about neurotrophin signaling, this introduction will be focussed on NGF and its receptors TrkA and p75. Expression pattern and functions of these molecules will be described in details. In the third part the cholinergic system will be introduced and in particular the cholinergic neurons of the forebrain and their functions will be described extensively. Finally, the involvement of the cholinergic neurons and of the NGF/TrkA signaling in the pathological disorder of the Alzheimer's disease will be presented.

1 NEUROTROPHIN SIGNALING

The neurotrophins are a family of secreted proteins that potently regulate diverse neuronal responses (Bibel and Barde, 2000; Huang and Reichardt, 2001; Segal, 2003). Family members include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin 4/5(NT4/5). An additional neurotrophin has been found in fishes.

Neurotrophins bind two different classes of transmembrane receptor proteins, the tropomyosin-related kinase (Trk) family of tyrosine kinase receptors and the p75 receptor, a member of the tumor necrosis factor (TNF) receptor superfamily.

The neurotrophins bind the Trks receptor in a specific way. NGF is the preferred ligand for TrkA, BDNF and NT4/5 for TrkB, and NT3 for TrkC. NT3 is also able to bind TrkA and TrkB but only does so at much higher concentrations than do NGF and BDNF. Whereas the tyrosine kinase domains of the different receptors are highly related (80% amino acid identity), the extracellular domains are more divergent and confer specificity to the ligand-receptor interaction (see Figure 1).

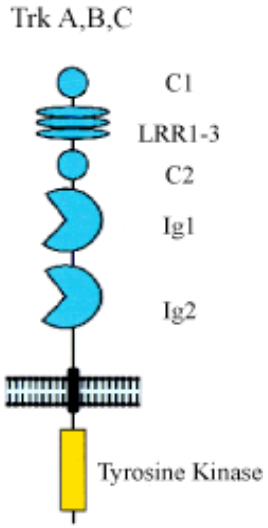

Figure 1 The Trk receptors.

The Trk receptors are transmembrane glycoproteins of ~140 kD. They are tyrosine kinases with an extracellular ligand-binding domain containing multiple repeats of leucine-rich motifs (LRR1-3), two cysteine clusters (C1, C2), two immunoglobulin-like domains (Ig1, Ig2), and a single transmembrane domain. Binding specificity of the Trk receptors is mostly determined by the second Ig-like domain, whereby each Trk receptor binds the corresponding ligand through a distinct specific sequence.

Neurotrophin binding to Trk receptors results in a series of events that characterize receptor tyrosine kinase signaling (Patapoutian and Reichardt, 2001). These include receptor dimerization and transphosphorylation of activation loop tyrosines leading to activation of kinase activity, followed by autophosphorylation of tyrosines outside of the activation loop. These autophosphorylation sites serve as binding sites for specific signaling proteins and adaptors such as PLCγ and Shc. Subsequent phosphorylation and activation of accessory proteins lead to the generation of a highly complex system of intracellular signaling pathways and results finally in a distinct cellular outcome. Each neurotrophin has numerous functions and the exact mechanisms by which the different functions are regulated are not yet completely understood.

1.1 Neurotrophins control cell survival

One of the most extensively studied properties of the neurotrophins is their ability to keep alive subpopulations of neurons in the peripheral nervous system (PNS) (Davies, 2003). In this regard, as the central concept of the neurotrophic factor hypothesis, targets of innervation were postulated to secrete limiting amounts of survival factors. Once a developing neuron has grown its process into its targets, it competes with other neurons for the limited supply of neurotrophic factors. Only some neurons are surviving, others are dying. This mechanism ensures a balance between the size of a target organ and the number of innervating neurons.

Animal models in which the expression of the different neurotrophins or their receptors is deleted (termed "knockout" animals) have evidenced clearly the essential role of neurotrophin signaling in ensuring the survival of peripheral neurons and correct innervation of the target organs.

NGF signaling through TrkA provides crucial neurotrophic support for the small and middle-sized sensory neurons (localized mainly in the trigeminal ganglia and in the dorsal root ganglia (DRG)) and almost all sympathetic neurons of the PNS (Smeyne et al., 1994). TrkB function is essential for the survival of about half of the sensory neurons of the trigeminal ganglia (Klein et al., 1993). In the nodose-petrosal ganglion that is responsible for the visceral sensory innervation almost all neurons are TrkB dependent. In the DRG, TrkB is responsible for the survival of the sensory neurons that innervate the mechano-receptors of the Merkel cells. In the ganglia of the vestibular organ, TrkB signaling upon BDNF binding is essential, while in the cochlear ganglia survival of a large part of the sensory neurons requires Trk receptor activation through NT-3 (Ernfors et al., 1995). TrkC controls together with TrkB the survival of the sensory neurons of the cochlear organ (Schimmang et al., 1997). Additionaly, in TrkC knockout mice, a subpopulation of the DRG neurons and the proprioceptive neurons that innervate the spindle organs in skeletal muscles are lost (Klein et al., 1994).

The phenotypes of the neurotrophin knockout mice in general are consistent with the phenotypes of the receptor knockouts and with what is known about the specificities of ligand interaction with the receptors. Thus, the phenotype of the NGF knockout is largely

identical with those of the TrkA receptor, indicating that NGF is the major ligand in vivo for TrkA (Crowley et al., 1994). Conversely, in the case of the NT-3 knockout phenotype, which is more severe than the one of the TrkC receptor, there is good evidence that in vivo NT-3 acts in some neuronal subpopoulations also as an important activator of TrkA and TrkB (Davies et al., 1995). Similarly, the phenotypes of the BDNF and of the NT-4/5 knockout mutants in the trigeminal and the nodose-petrosal ganglia are less severe than the TrkB knockout phenotype, suggesting that both ligands contribute in these neurons to the activation of TrkB. Interestingly, in the vestibular ganglia, the BDNF mutation appeared to have a more dramatic effect compared to the trkB knockout. BDNF has not been shown to cross-react with TrkA or TrkC receptors indicating that the differences between ligand and receptor knockouts may have a more general cause. One explanation might be the presence of non-catalytic isoforms encoded by the trkB and trkC genes. These isoforms are still expressed in the knockout mice and may mediate some sort of signal transduction that could partially prevent or delay cell death.

The neuronal losses in the PNS of the distinct neurotrophin and receptor knockout animals compared to the wild-type animals are summarized in Table 1.

	TrkA	NGF	TrkB	BDNF	NT-4/5	TrkC	NT-3
Sensory ganglia:							
Trigeminal	**70%**	**75%**	**60%**	**30%**	**NS**	**21%**	**60%**
Nodose-Petrosol	**ND**	**ND**	**90%**	**45%**	**40%**	**14%**	**30%**
Vestibular	**NS**	**ND**	**60%**	**85%**	**NS**	**15%**	**20%**
Cochlear	**NS**	**NS**	**15%**	**7%**	**ND**	**50%**	**85%**
Dorsal root	**70 - 90%**	**70%**	**30%**	**35%**	**NS**	**20%**	**60%**
Sympathetic ganglia:							
Superior cervical	**>95%**	**>95%**	**ND**	**ND**	**NS**	**NS**	**50%**

TABLE 1 : Neuronal losses in the PNS of neurotrophin and Trk-deficient mice
Neuronal losses are expressed as the percentage of neurons lost in the mutants compared with the wild-type animals. This table is modified from a table in Huang and Reichardt (2001)
NS: not significant; ND: not done

It is noteworthy that in some cases distinct neuronal subpopulations require more than one neurotrophic factor – receptor signaling pathway to survive. This fact is particularly evidenced by the generation of knockout mice, in which more than one neurotrophin or receptor is disrupted. While in some combinations of different knockouts, the effect on the distinct neuronal subpopulations were largely additive (e.g. trkA/trkC and trkB/trkC in the DRG), other combinations of mutant alleles such trkA/trkB (in the DRG) or trkB/trkC (in the vestibular organ) showed no significant increase in neuron loss over single trkA (in the DRG) or single trkB (in the vestibular ganglion) knockout mice (Minichiello et al., 1995). These data indicate that certain subpopulation of neurons require during development different Trk signaling pathways for survival. This requirement of two Trk receptors is most likely sequential and does not involve constant co-expression of both receptor pathways. In this regard, trigeminal sensory neurons have been shown to switch their neurotrophin specifity from BDNF/NT3 to NGF during embryonic development, presumably by changing the expression pattern of neurotrophin receptors. These kind of neurons would be vulnerable to both trkB and trkA mutations (Davies, 1997).

The survival role of the neurotrophins in the central nervous system (CNS) has been more difficult to study compared to the PNS. Nevertheless, there is clear evidence that at least during development neurotrophins provide essential survival signals also for cells of the CNS. TrkB and TrkC, which are expressed in a large amount of neuronal subpopulations in the CNS, ensure survival of distinct cells of the hippocampus and of the cerebellum (Alcantara et al., 1997; Minichiello and Klein, 1996). Interestingly, in this case TrkB and TrkC act in a redundant fashion; activation of either of the two receptors is sufficient for neuronal survival in the CNS during development. Later, it was shown that trkB-deficient mice exhibit also a small increase of apoptotic cells in the cortex, hippocampus and striatum during the first postnatal weeks (Alcantara et al., 1997). Recently, it was demonstrated that after development TrkB is no more necessary for the survival of CNS neurons but rather modulate complex bahavior (Minichiello et al., 2002; Minichiello et

al., 1999). The survival role of NGF/TrkA signaling for the TrkA-responsive cholinergic neurons of the basal forebrain will be discussed below in more details.

While neurotrophins in most of the cases promote cell survival, under some circumstances it may be possible that neurotrophins also invoke cell death. The neurotrophin receptor involved in the pro-apoptotic effects of neurotrophins appears to be the p75 receptor (Hempstead, 2002; Lee et al., 2001a). P75 binds all members of the neurotrophin family with approximately equal affinity. Neurotrophin binding to p75 triggers activation of signaling pathways distinct from these ones activated by the Trk receptors. In addition, p75 may interact directly with the Trk receptors and modulate their activity. In vivo, overexpression of p75 causes cell death in certain cell types and disruption of p75 may induce an increase of certain cell populations that express endogenously high levels of p75 (as the cholinergic neurons of the basal forebrain). In general, the effect of p75 activation on cell survival appears to depend crucially on the cellular context and on the level of expression of the other neurotrophin receptors and their ligands.

1.2 Neurotrophins control neuronal development and function

Neurotrophins have multiple effects on the functional properties of the neurons of the PNS and of the CNS beyond regulation of their survival.

There is a large body of evidence both in vitro and in vivo that neurotrophins regulate potently the growth of dendrites and axons in the PNS and the CNS. In vitro experiments using many different kind of neurons show that focal application of neurotrophins induces a rapid turning of neurite growth cones toward the neurotrophin source (Gundersen and Barrett, 1979; Ming et al., 1997). In vivo experiments show that overexpression of neurotrophins lead to an increase in the ingrowth of sympathetic and sensory neurons in different target organs (Albers et al., 1996; Hassankhani et al., 1995). Recently, an elegant in vivo study in mice demonstrated directly the chemo-attractant function of neurotrophins and the implication of this function in the elongation of peripheral mixed nerves (Tucker et al., 2001). Moreover, neurotrophin signaling plays a critical role in determining the phenotype of neurotrophin-responsive neurons. An early

illustration of this phenomenon has been provided by experiments in which antibodies to NGF were injected into young rats (Ritter et al., 1991). Reducing NGF levels led to a phenotypic switch of the sensory nerves that innervate the skin. The Aδ nerve fibers which innervate mechanoreceptors were replaced by D-hair-type nerve fibers which respond to low-threshold stimulation of hairs in the skin. This property of NGF signaling was further evidenced by an elegant mouse model in which the expression of TrkA was replaced by expression of TrkC (Moqrich et al., 2004). The phenotypical characteristics of sensory nerves in this mouse were changed importantly. Similar capacities in determining and maintaining neuronal phenotypes were demonstrated also for the other neurotrophins (Lewin, 1996). Finally, in the developing brain, neurotrophins are also involved in the proper timing of neuronal migration in the cortex (Medina et al., 2004).

Furthermore, neurotrophins modulate also the number of synapses and the efficiency of synaptic transmission. In vitro experiments demonstrated that neurotrophins regulate neuronal excitability and increase the release of neurotransmitter. One of the most intriguing functional properties of the neurotrophins is their involvement in neuroplasticity events (McAllister et al., 1999). Neuroplasticity is a life-long process that mediates the structural and functional reaction of dendrites, axons, and synapses to experience, learning, and injury. Characteristics of neuroplasticity can be found on molecular (e.g., protein phosphorylation states and insertion of receptors at synapses), synaptic (e.g., long-term-potentiation and –depression), cellular (e.g., number and shape of spines, dendrites, or axons) and system/circuit level (e.g., cortical representation/maps reorganization). Neurotrophins mediate various aspects of neuroplasticity. For example, neurotrophins are importantly involved in the activity-driven synaptic rearrangements of neurons from the lateral geniculate nucleus of the thalamus that result in formation of ocular dominance (OD) columns in the layer IV of the visual cortex. The role of neurotrophins in activity-dependent synaptic arrangement during cortical development has been tested mainly by using monocular deprivation during the critical period for OD development. Blocking the input from one eye leads to the shrinkage of the corresponding neuronal cell bodies and axons in the lateral geniculate nucleus, and the input from the nondeprived eye takes over. For instance, NT-4 infusion was shown to prevent many of the consequences of monocular deprivation (Gillespie et al., 2000).

Also, BDNF-mediated maturation of interneuronal cortical inhibition may be involved importantly in the regulation of visual cortical plasticity (Huang et al., 1999). Another important aspect of neuronal plasticity that is controlled by neurotrophin signaling are the long-term changes in synaptic activity. For instance, in vivo it was shown, that deletion of BDNF/TrkB signaling results in the inhibition of the formation of long-term potentiation of synaptic activity. The inhibition of long-term potentiation can be observed both in animals where BDNF is disrupted from early development on (Patterson et al., 1996) as well as in animals where TrkB is disrupted only after development (Minichiello et al., 1999). The impaired synaptic plasticity correlates with behavioral deficit in learning tasks in TrkB conditional mutant.

2 NGF AND ITS RECEPTOR TRKA

The neurotrophin NGF was discovered more than 50 years ago by Rita Levi-Montalcini, Stanley Cohen and Ernst Hamburger (Cowan, 2001). The ngf gene is located on the human chromosome 1 and in mice on the chromosome 3. The ngf gene contains 4 exons (Selby et al., 1987); only the last and largest exon 4 encodes for the NGF protein. Like the other neurotrophins, NGF is initially synthesized initially as an immature isoform (called proNGF) that contains a signal sequence and an amino terminal portion, which allows for correct protein folding. The mature NGF peptide is generated by protease digestion of proNGF and consists of a dimer of two 13 kDa polypeptides. Interestingly, recently it was shown that also proNGF is biologically active and binds preferentially the p75 receptor (Lee et al., 2001b).

TrkA was initially discovered in 1986 as an oncogenic fusion protein isolated from human colon carcinoma (Martin-Zanca et al., 1986). Genetic analysis revealed that in normal cells the proto-oncogene encoded for a single transmembrane-spanning polypeptide chain that make part of the receptor tyrosine kinase superfamily. Only 5 years after its discovery TrkA was identified to be the major receptor for NGF. As NGF, TrkA is located on the human chromosome 1 and in rodents on chromosome 3. The trkA gene contains 17 exons (Greco et al., 1996). The exons 1-8 encode for the extracellular domain of the receptor, while exons 13-17 encode for the kinase domain. Two isoforms of TrkA exist that differ in their extracellular domain through the inclusion of six

additional amino acids near the transmembrane domain of one of the variants. Inclusion of the insert appears to relax the specificity of TrkA activation; NT-3 mediated signaling is markedly enhanced through this receptor isoform (Clary and Reichardt, 1994).

2.1 Expression pattern of NGF and TrkA

2.1.1 Expression in the PNS

TrkA is expressed during development and in adulthood in sympathetic neurons and small-diameter peripheral sensory neurons that mediate nociception and thermoception.
The cell bodies of the sensory neurons of the PNS are localized in the dorsal root (DRG) and in the trigeminal ganglia. TrkA-positive sensory neurons are generated between embryonic day E11.5 and E13.5 (White et al., 1996).
All the sympathetic neurons are clustered in ganglia in the sympathetic chain alongside the spinal chord extending from the first thoracic spinal segment to the upper lumbar segments. One of the major and best-studied components of the sympathetic system is hereby the superior cervical ganglion (SCG) that contains primarily principal sympathetic neurons. TrkA expression in the SCG appears first at E13.5, becomes robust from E15.5 onward and remains high through adulthood (Fagan et al., 1996).
Most of the TrkA-positive neurons in the PNS express also p75 (Sobreviela et al., 1994). During development, p75 is expressed also in many motoneurons and in the myelin-producing Schwann cells of the PNS (Wheeler et al., 1998; Yan and Johnson, 1988).
NGF is produced during and often also after development in many non-neuronal target cells of sensory and sympathetic neurons (Wheeler and Bothwell, 1992; Yamamoto et al., 1996). These include targets in the skin (e.g. keratinocytes and melanocytes), vascular and other smooth muscle cells, and various endocrine tissues, such as testis and ovary, pituitary, thyroid and parathyroid, and exocrine salivary (e.g.submandibular) glands.

2.1.2 Expression in the CNS

TrkA and p75 expression in the CNS is found in the different cholinergic cell groups in the basal forebrain, which are discussed in more details below. Few groups of non-cholinergic neurons that express TrkA were described in the thalamus and other brain areas in the brainstem (Holtzman et al., 1995).

Glial cells are normally not expressing TrkA. Many non-neuronal, and in particularly oligodendrocytes, express the p75 receptor.

TrkA mRNA can be detected in the rat brain from the late embryonic development on. The mRNA levels then increase during the first postnatal days and reach adult levels towards the end of the third postnatal week (Li et al., 1995).

NGF production can be found in the CNS during development and throughout adult life. NGF-producing cells are most abundant in all the target areas of cholinergic innervation (Saporito and Carswell, 1995). Most such cells are neurons. In the hippocampus, pyramidal and dentate granule neurons express NGF, as do subpopulations of GABAergic interneurons (Rocamora et al., 1996). In the striatum, NGF is produced by GABAergic interneurons (Bizon et al., 1999). In the cortex, NGF is produced by neurons distributed throughout all the different layers except the layer IV, where only very few NGF-expressing neurons are found (Pitts and Miller, 2000).

NGF is produced occasionally also by astrocytes and microglia cells and this production can be importantly increased by inflammation or injury processes.

2.1.3 Expression in non-neuronal cells

TrkA is expressed in various non-neuronal tissue types (Shibayama and Koizumi, 1996; Wheeler et al., 1998) and many non-neuronal cell types were shown to be NGF-responsive (e.g. keratinocytes and myocytes). In particular, cells of the immune system were characterized in details in this regard. TrkA-positive cells in the immune system include mast cells, CD4+ T lymphocytes, B lymphocytes, monocytes, and macrophages. Also hematopoietic stem cells have been shown to express TrkA (Bracci-Laudiero et al., 2003). Many of these types of immune cells have also the capacity to express NGF.

2.2 Function of NGF/TrkA signaling

2.2.1 Knockout mice

The transgenic knockout mice, in which the ngf and the trkA genes were disrupted, were first described in 1994 (Crowley et al., 1994; Smeyne et al., 1994). Both knockout mice have a very similar phenotype, indicating that the major NGF functions are mediated in

vivo through TrkA. Both knockout mice show early lethality: Most of the mice die in the first 3 days of life and only very few mice get older than 4 weeks.

The first p75 knockout described in 1992 has a relatively mild phenotype and displays no premature lethality (Lee et al., 1992). More recently, it was shown that the p75 receptor is expressed also as a short isoform protein (lacking the exon 3 of the p75 gene), which cannot bind neurotrophins. This isoform is not deleted in the original p75 knockout. The complete p75 knockout shows a more severe phenotype and displays a perinatal lethality up to 40% (von Schack et al., 2001). This lethality is most likely due to a blood vessel-related phenotype, suggesting that p75 signaling has in vivo functions, which are not related to NGF and TrkA.

2.2.1.1 Function of NGF/TrkA signaling in the PNS

2.2.1.1.1 Sensory neurons

ngf and trkA knockout mice have a severe PNS phenotype. More or less 80% of the normal complement of DRG sensory neurons are lost. Roughly half of these are small- and medium sized neurons that express TrkA as well as calcitonine gene-related peptide in maturity. In addition, a group of nociceptive neurons that downregulate TrkA postnatally and upregulate glia cell line-derived neurotrophic factor receptor Ret also require NGF in embryonic life. DRG neurons in trkA and ngf knockout mice start to die around embryonic day E13.5 (White et al., 1996). The loss of the sensory neurons is the cause of the decreased responsiveness to pain in the knockout mice.

The complete p75 knockout mice show a reduction of DRG sensory neurons of about 50% (von Schack et al., 2001). It remains unclear whether this reduction is due to a direct pro-survival effect of p75 in sensory neurons or rather to secondary effects due to the lack of p75 in Schwann cells.

Because of the early death of sensory neurons in ngf and trkA knockout mice, it was difficult to analyze these mice in order to assess whether NGF/TrkA signaling in vivo is required also for the axon growth and differentiation of this neuronal subpopulation. This question was addressed in an elegant study by Patel et al. (Patel et al., 2000). In this study, ngf and trkA knockout mice were crossed with mice in which the expression of the proapoptotic bcl-2 homolog gene BAX is deleted. BAX is known to be essential for

apoptosis in neurons after withdrawal of neurotrophic support. Indeed, the sensory cell death in ngf and trkA knockout was efficiently rescued by eliminating the expression of BAX. In this animal model, it could be shown that NGF signaling is not required for the elaboration of proximal spinal collateral branches of dorsal root ganglia axons. In contrast, axonal outgrowth into distal nerves (e.g. the saphenous nerve in the hindlimb), peripheral sensory innervation and biochemical differentiation failed to develop in the double knockout mice. Thus, NGF/TrkA is not only crucial for the survival of sensory neurons in vivo, but also for the correct peripheral innervation of the target organs as the skin and for the acquisition of a functional biochemical phenotype.

2.2.1.1.2 In sympathetic neurons

Sympathetic ganglions are severely affected in ngf and trkA knockout mice. While the progenitor cells of sympathetic neurons develop normally, extensive cell death is present from embryonic day E17.5 on and develops progressively after birth (Fagan et al., 1996). By the end of the first postnatal week, sympathetic neurons in the SCG are virtually absent. Similarly to the studies done on the sensory neurons, it was shown that deletion of the BAX protein rescues most of the NGF-deficient sympathetic neurons from cell death. The rescued neurons formed initial axon extensions but failed often to elaborate correct target innervation (Glebova and Ginty, 2004). The severity of the deficits in target innervation varies thereby in the different target organs. For instance, while the sympathetic innervation of the heart was strongly impaired, innervation of the trachea was less affected. Thus, while in many target organs NGF signaling is absolutely required for terminal sympathetic innervation, in other organs target-derived neurotrophic support may be provided also by other molecules.

2.2.1.2 Function of NGF/TrkA signaling in the CNS

The original analysis of the ngf knockout mouse described no cholinergic cell loss and normal cholinergic innervation in the basal forebrain. In the case of the trkA knockout mice, the authors described a deficit in cholinergic innervation in the hippocampus and in the cortex but not in the striatum. Subsequently, the cholinergic phenotype in trkA-deficient mice was analyzed more in details (Fagan et al., 1997a). It was shown that in the few TrkA knockout mice, which survive until postnatal day P25, the cholinergic neurons in the medial septum were reduced by about 30% compared to wild-type mice. A

reduction of cholinergic neurons, although at lower extent, was found also in the striatum. In addition, it was demonstrated, that the cholinergic innervation in the hippocampus is markedly reduced in trkA knockout mice. Another study analyzed the phenotype of mice that are heterozygous for the ngf knockout mutation. These mice carrying only ngf allele are vital and healthy, but display a 75% - reduction of NGF expression in the hippocampus (Chen et al., 1997). This reduction resulted in adult mice in a 35%-reduction of cholinergic neurons in the medial septum and a reduction of cholinergic innervation of the hippocampus in these mice. The cholinergic impairment is accompanied by spatial memory deficits that were demonstrated by using the Morris water maze task. The deficits in cholinergic innervation and in memory performance could be rescued by infusion of NGF.

Interestingly, p75 function in the CNS seems to be related to cell death. While initial analyzes of the original incomplete p75 knockout gave controversial results (Peterson et al., 1997; Van der Zee et al., 1996), the study of the complete p75 knockout mice indicate a substantial 30% increase of the cholinergic neurons of the basal forebrain (Naumann et al., 2002). This effect may be caused either by a direct pro-apoptotic signaling of p75 in the basal forebrain cholinergic neurons or by a p75-mediated inhibition of pro-survival NGF signaling.

The study of the ngf and trkA knockout mice allowed the detailed analysis of the in vivo role of NGF/TrkA signaling in the neurons of the PNS. The question whether NGF/TrkA is required also for the survival and function of the cholinergic neurons in the CNS, however, was more difficult to address in the knockout mice. In fact, the results obtained from the very few trkA-deficient mice that survive the first week of life, are difficult to interpret in the light of the very poor health and development of these mice. In addition, due to the early lethality of the knockout mice, it was not possible to study the CNS function of NGF and TrkA in aged mice.

For this reason, a number of studies tried to inhibit NGF/TrkA signaling in a brain-specific way.

2.2.2 Inhibition studies

For instance, the injection of hybridoma cells that express a neutralizing antibody against TrkA in rat brains resulted in the reduction of cholinergic neurons in the medial septum by about 70% compared to control rats (Cattaneo et al., 1999). However, this effect was seen only when the injection was done in the first postnatal days and the cholinergic deficit was only transient and completely reversible. Three weeks after the injection no more reduction of the cholinergic neurons was observed. Similar results were found by another study in which an inhibiting antibody against NGF was used (Molnar et al., 1998). Recently it was shown that inhibition of NGF/TrkA signaling reduces the cholinergic phenotype of the forebrain also in adults rats (Debeir et al., 1999). This was demonstrated by injection of either an antibody against NGF or a small TrkA antagonist peptide. Similarly, reduction in cholinergic neurons in the medial septum of adult rats could be also induced by antisense oligonucleotides against trkA mRNA (Woolf et al., 2001). This treatment resulted in downregulation of TrkA expression and subsequently in reduction of cholinergic marker proteins and impaired performance in a memory consolidation-related learning task.

All these inhibition studies suggest a neurotrophic function of NGF/TrkA signaling in the cholinergic neurons of the forebrain, but the spatial and temporal extent of NGF signaling inhibition achieved by these studies is difficult to ascertain. Furthermore, injection of both hybridoma cells and antisense oligonucleotides is known to be often associated with unspecific side effects.

2.2.3 Transgenic anti-NGF mice

A temporal more consistent inhibition of NGF signaling after development is achieved in mice that express transgenically a neutralizing antibody (αD11) under the promoter of the human cytomegalovirus promoter (Ruberti et al., 2000). The ubiquitous expression of this antibody reach detectable levels only in adulthood and does therefore most likely not impair normal neuronal development. In adult mice, the extent of NGF inhibition varies in different tissue types and reaches about 50% in the brain. Analysis of the cholinergic basal forebrain showed no significant defect in the first two postnatal weeks. In adult mice, however, cholinergic neurons in the forebrain are reduced by about 55% and

cholinergic innervation of the cortex and the hippocampus was strongly affected. Behavioral analysis of the transgenic anti-NGF mice shows a reduced ability in spatial learning tasks (in a radial maze). The authors attributed this deficit to the cholinergic impairment in the forebrain. In parallel to the CNS phenotype, the adult transgenic anti-NGF mice display also a severe apoptotic damage of sensory and sympathetic neurons of the PNS. This is most likely explained by the absolute requirement of PNS neurons for NGF support also during adulthood.

Outside of the nervous system, the anti-NGF mice demonstrate a severe cell damage in the spleen and an inflammation-associated dystrophy-like muscle phenotype in the hind limbs (Capsoni et al., 2000a). It remains questionable, how far these disorders are caused directly by the lack of NGF signaling or whether there are rather induced by toxic effects of the forced transgenic antibody expression. For instance, in the case of the phenotype observed in the spleen, the rather low expression of TrkA in the spleen makes it difficult to explain the high degree of apoptosis directly by the inhibition of NGF (Lomen-Hoerth and Shooter, 1995). In summary, the studies of the transgenic anti-NGF mice suggest an important role of NGF/TrkA signaling in the basal forebrain during adulthood and aging (see also the part 4.4 of this introduction about the links between NGF and Alzheimer's disease). The results of these studies, however, should be interpreted carefully because they may be confounded by an immune response of the transgenic animal to the antibody expression. In addition, the strong PNS phenotype in adult anti-NGF mice may confound the analysis of the brain-specific functions of NGF/TrkA signaling. Finally, the inhibition of NGF signaling in these mice is not complete and the temporal and spatial extent of this inhibition is not precisely defined.

2.2.4 Neuromodulatory function of NGF/TrkA signaling

2.2.4.1 NGF and hyperalgesia

Beyond its role as survival and differentiation factor for sensory neurons, NGF modulates also the activity of mature nociceptive sensory neurons (Shu and Mendell, 1999). In fact, NGF stimulation of these neurons leads to hypersensitivity to nociceptive stimuli. This form of hyperalgesia can be observed both in animals and in patients where NGF was given in clinical trial for peripheral neuropathies. Blockade of NGF signaling by

inhibiting antibodies in animal models of skin injury and inflammation prevents development of hyperalgesia (McMahon et al., 1995). This function of NGF includes induction of mast cell degranulation and regulation of pain-related neuropeptides.

2.2.4.2 NGF and synaptic plasticity

NGF is able to modulate various aspects of synaptic activity. For instance, it is well known that NGF stimulation can increase neuronal excitability by regulating the expression of ion channels. Furthermore, NGF stimulation of culture of embryonic cholinergic forebrain neurons induces also a prolonged release of the neurotransmitter acetylcholine (Auld et al., 2001). Conversely, in sympathetic neurons of the heart that form synapses with cardiomyocytes NGF enhances synaptic activity (Lockhart et al., 1997). In the visual cortex, NGF was shown to be involved in the formation of ocular dominance columns in the layer IV. For example, in some cases exogenous supply of NGF is able to prevent the physiological effects induced by monocular deprivation. In this regard, it was also shown that NGF inhibits efficiently formation of long-term potentiation (LTP) in the visual cortex (Pesavento et al., 2000). Blockade of NGF rescues the formation of LTP. This effect is likely to be mediated by muscarinic acetylcholine receptors.

2.2.5 NGF/TrkA function in immune cells

A large body of in vitro experiments demonstrated that NGF regulates many aspects of immune cell functions (Vega et al., 2003). The most intriguing data concern the effects of NGF on B-cell and mast cell function. NGF is capable to regulate immunoglobulin production and serves as survival factor for memory B cells (Torcia et al., 1996). In vivo, it was shown that TrkA-deficient mice have major defects in the thymus development (Garcia-Suarez et al., 2000) and transgenic anti-NGF mice display massive cell death in the spleen (Ruberti et al., 2000). Recently, however, the in vivo function of NGF in immune cells were revalidated by the analysis of a mouse model, where TrkA is deleted from all cell types except from neuronal cells (Coppola et al., 2004). The mice lacking TrkA in non-neuronal tissues are viable and appear grossly normal. In contrast to the TrkA knockout mice and to the transgenic anti-NGF mice, these mice display no

abnormalities in organs related to the immune system and all major immune system cell population are present normally. Only a subtle but significant increase of certain immunoglobulin classes and accumulation of a distinct B cell subpopulation was observed.

2.2.6 TrkA mutations in humans

TrkA mutations have been identified as the cause of a human syndrome, Congenital Insensitivity to Pain and Anhidrosis (CIPA), also known as hereditary sensory and autonomic neuropathy IV(Indo et al., 1996). There are three main clinical symptoms of this disorder. The first feature of CIPA is defects in thermoregulation, with a lack of sweating and episodes of hyperpyrexia, which frequently are the cause of death. The defective thermoregulation reflects a lack of sympathetic neurons. The second symptom of CIPA is an extreme insensitivity to pain, leading to injuries and self-mutilation. This defect reflects absence of the small nociceptive sensory neurons. The third clinical feature is mental retardation. This may indicate that NGF/TrkA signaling in humans is needed not only for nociceptive and sympathetic neurons but also for higher cognitive functions.

3 CHOLINERGIC NEURONS

3.1 Cholinergic neurotransmission

One of the main neurotransmitters in the mammalian nervous system is acetylcholine (ACh). Neurons that use the neurotransmitter ACh are called cholinergic neurons. ACh is used as neurotransmitter at all neuromuscular junctions, at some synapses in the autonomic nervous system and finally at a variety of synaptic sites within the central nervous system. In the autonomic nervous system all the synapses in the ganglions and all the synapses of the post-ganglionic parasympathetic nerves are cholinergic, while the post-ganglionic nerves of the sympathetic system use the neurotransmitter norepinephrine.

In all cholinergic neurons ACh is synthesized from acetyl coenzyme A and choline, in a reaction catalyzed by choline acetyltransferase (ChAT) (Figure 2) (Sarter and Parikh,

2005). The presence of ChAT in a neuron is a very strong indication that ACh is used as one of its transmitters. The synthesized ACh molecules are packaged into synaptic vesicle by a vesicular ACh transporter (VACht).

In contrast to most other small-molecule neurotransmitters, the postsynaptic action of ACh at many cholinergic synapses are not terminated by reuptake but by a powerful hydrolytic enzyme, acetylcholinesterase (AChE). This enzyme is concentrated in the synaptic cleft, ensuring a rapid decrease in ACh concentration after its release from the presynaptic terminal. AChE has a very high catalytic activity and hydrolyzes ACh into acetate and choline. Cholinergic nerve terminals typically contain a high-affinity Na+-choline transporter that takes up the choline produced by ACh hydrolysis.

The post-synaptic receptors that bind ACh can be divided largely in two groups. The group of nicotinic ACh receptor (nAChR) is so named because the CNS stimulant nicotine also binds to these receptors. Nicotinic receptors are ligand-gated ion channel receptors. A second class of ACh receptors is activated by muscarine, a poisonous mushroom alkaloid, and they are referred to as muscarinic ACh receptors. Muscarinic acetylcholine receptors (mAChR) are G-protein coupled receptors and are the most abundant ACh receptors in the brain. Several subtypes of mACh receptors are known (Caulfield, 1993). Muscarinic receptors of the type M1 predominate in the hippocampus and cerebral cortex, whereas M2 receptors predominate in the cerebellum and brainstem and M4 receptors are most abundant in the striatum.

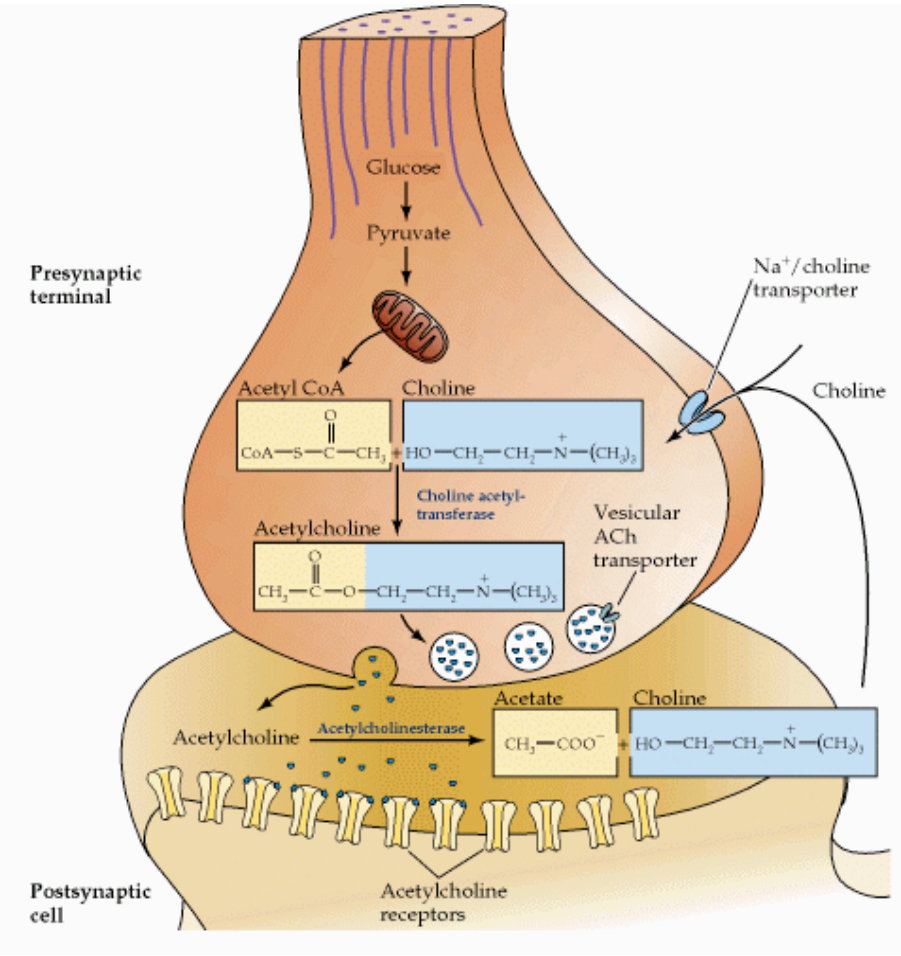

FIGURE 2: Acetylcholine metabolism in cholinergic nerve terminals
From the book "Neuroscience", 2nd edition. Purves, D.; Augustine, G.J.; Fitzpatrick, D.; Katz, L.C..; LaMantia, A.-S.; McNamara, J.O.; Williams, S.M., Sunderland (MA): Sinauer Associates, Inc. 2001.

3.2 Cholinergic neurons in the brain

All the cholinergic neurons in the brain are interneurons. The cholinergic interneurons can be largely divided in two different classes: While the cholinergic interneurons of the striatum form local circuits and their relatively short axons remain confined to the striatum itself, the large majority of the cholinergic neurons in the brain are projection interneurons, which send their axon in different areas of the brain. The cholinergic projection neurons in the brain can be divided in 6 different groups (Ch1-Ch6 after the

nomenclature of Mesulam (Mesulam et al., 1983) (see also Figure 3 and 4). Most of these cholinergic cell groups do not respect traditional nuclear boundaries, and their constituent cholinergic cells are intermixed with other noncholinergic neurons. The first 4 groups Ch1-Ch4 are located in the basal forebrain, while Ch5 and Ch6 are situated in the upper brainstem. The different groups of neurons can be distinguished not only by their anatomical localization but also by the different target areas that they innervate. In addition, they have distinct molecular characteristics. All of them express typical molecular markers of cholinergic neurons like ChAT, AChE and VaChT, but only the cholinergic neurons of the basal forebrain (including the striatal neurons) express TrkA. While most of the cholinergic projection neurons of the basal forebrain express also the neurotrophin receptor p75, the neurons in the striatum are p75-negative (Gibbs and Pfaff, 1994). In general, the molecular expression patterns of the cholinergic neurons in the brain appear rather heterogeneous. For example, a certain subpopulation of the cholinergic neurons expresses the neuropeptide galanin (Miller et al., 1998) and some neurons co-express the differentiation factors Islet-1 and bone morphogenetic protein-9 (Lopez-Coviella et al., 2000; Wang and Liu, 2001).

The genetic and developmental mechanisms that control the early formation of forebrain cholinergic neurons are just beginning to be elucidated. The vast majority of forebrain cholinergic neurons derive from a region of the subcortical telencephalon. This region contains different progenitor zones, including the medial ganglionic eminence, anterior entopeduncular area and preoptic area. It has been proposed that these progenitor domains contribute projection neurons to the globus pallidus, ventral pallidum, nucleus of the diagonal band, and parts of the septum and amygdala (Schambra et al., 1989). In addition, a substantial fraction of striatal interneurons originates from these progenitor zones and migrate tangentially to reach their final destinations (Marin et al., 2000). Knockout mouse models have demonstrated that the expression of the homeobox genes Nkx 2.1 and Lhx8 during early development is essential for correct development of the cholinergic neurons in the brain (Mori et al., 2004; Zhao et al., 2003).

In the rat forebrain, first weak ChAT mRNA was detected at the late stage of embryonic development, but adult ChAT mRNA levels are reached only at the end of the second postnatal week (Bender et al., 1996). Similarly, in the rat spinal chord, northern blot

analysis has shown that ChAT mRNA is expressed only at relatively low levels at the moment of birth (Lonnerberg et al., 1995). Analysis of the ChAT protein expression by immunostainings showed that in the medial septum of the rat brain ChAT-positive neurons can be detected only after birth (Bender et al., 1996). In mice, some studies have found a weak immunoreactivity in the forebrain around the end of the second prenatal week, while other authors report a consistent ChAT expression only after birth (Schambra et al., 1989; Villalobos et al., 2001). Generally, ChAT protein expression in the brain of mice seems to reach adult level at the end of the second postnatal week.

3.2.1 Cholinergic neurons in the medial septum (Ch1)

The Ch1 group of cholinergic neurons is located in the nucleus of the medial septum. Together with the neurons of the group Ch2, they provide the major cholinergic innervation of the hippocampus. The cholinergic projections to the hippocampus form synapses in the hippocampus onto pyramidal cells, dentate granule cells, and inhibitory interneurons. In the nucleus of the medial septum there is also a large subpopulation of GABAergic interneurons that express the calcium-binding protein parvalbumin. Like the cholinergic neurons also the GABAergic interneurons of the medial septum innervate the hippocampus. The GABAergic projections synapse onto GABAergic hippocampal interneurons, which in turn synapse onto pyramidal cells (Freund and Antal, 1988). Both the cholinergic and the GABAergic neurons send their projections to the hippocampus via the fimbria-fornix structure.

3.2.2 Cholinergic neurons in the diagonal band (Ch2+Ch3)

The neurons of the diagonal band (also called diagonal band of Broca) can be divided into two groups: the ones that make part of the nucleus of the horizontal limb of the diagonal band (DB, hl – Ch3) and the neurons of the nucleus of the vertical limb of the diagonal band (DB, vl – Ch2). The latter ones provide together with the neurons of the Ch1 group the cholinergic innervation of the hippocampus. The neurons of the Ch3 project intensively to the olfactory bulbs.

3.2.3 Cholinergic neurons in the nucleus basalis (Ch4)

In human, the largest group of cholinergic neurons in the basal forebrain is constituted by the cholinergic neurons of the nucleus basalis (also called nucleus basalis of Meynert)

(Mufson et al., 2003). In mice the basal nucleus has less homogenous boundaries than in humans: The cholinergic cells of the basal nucleus straddle the border between the globus pallidus, the substantia innominata and the internal capsule, invading all these structures. The cholinergic cells of the nucleus basalis are responsible for the major part of the cholinergic innervation of the entire cerebral cortex and of the amygdala.
In the cortex the most abundant ACh receptor is the muscarinic receptor of the type M1. The major function of the muscarinic receptors in the cortex is to modulate the polarization state of GABAergic neurons, so that these neurons become more susceptible to other incoming excitatory inputs (Kawaguchi, 1997). This is why ACh is also known as excitatory neurotransmitter in the cortex.

3.2.4 Cholinergic neurons in the striatum

The cholinergic interneurons in the striatum (which consist of the four components nucleus caudate, putamen, olfactory tubercle and nucleus accumbens) are large spiny neurons that make up about 2% of the striatal neurons (Zhou et al., 2002). The axons of the striatal cholinergic interneurons remain confined to the striatum and innervate predominantly the medium spiny neurons. Both muscarinic and nicotinic cholinergic receptors are found in the striatum. Muscarinic receptors on glutamatergic terminals are thought to inhibit release of the excitatory transmitter, acting as a modulator of glutamatergic stimulation of striatal neurons, whereas nicotinic receptor activation enhances transmitter release.

3.2.5 Cholinergic neurons in the brainstem (Ch5+Ch6)

The brainstem cholinergic neurons are located in the region of the pedunculopontine tegmental nucleus and laterodorsal pontine tegmentum. They innervate principally the thalamus. A very small number of the brainstem neurons provide also cholinergic innervation of the hippocampus, olfactory bulb and neocortex (Mesulam et al., 1983). The neurons in the brainstem are the only cholinergic neurons in the brain that do not express TrkA.

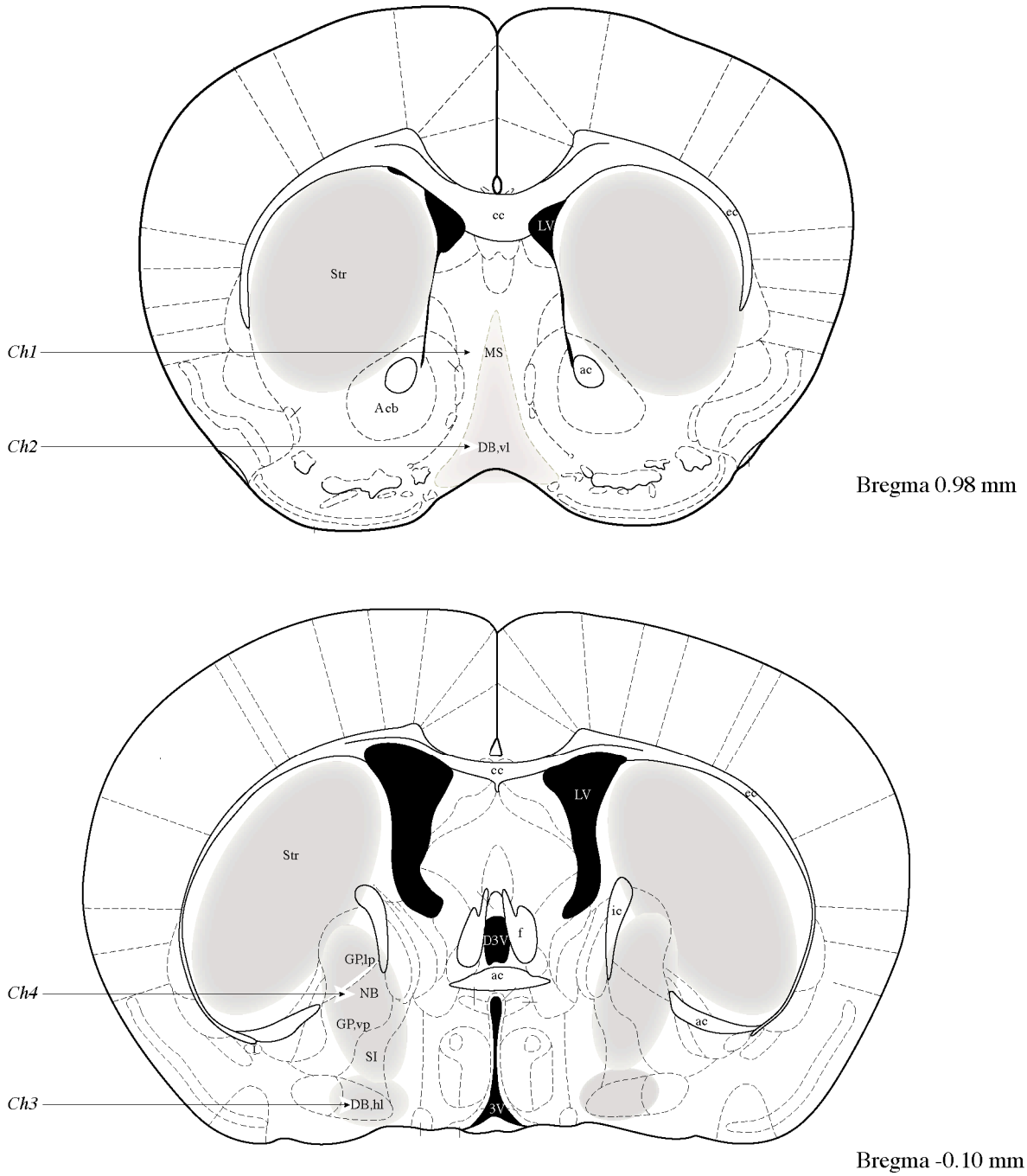

FIGURE 3: Anatomical localization of the cholinergic cell groups in the basal forebrain
Schematic representation of coronal sections from the rostral part (upper figure) and from the caudal part (lower figure) of the mouse basal forebrain. Precise coronal plane of the sections is indicated by the Bregma coordinates. The cholinergic cell groups are highlighted in grey and the *Ch* nomenclature (after Mesulam) is indicated.
Modified from "The Mouse Brain in Stereotaxic Coordinates", Second edition, Paxinos, G. and Franklin K.; Academic Press

MS - Medial septum; Acb - Nucleus accumbens; DB,vl - Diagonal band, vertical limb; DB,hl - Diagonal band, horizontal limb; NB - Nucleus basalis; Str - Striatum; GP,vp - Globus pallidum, ventral part; GP,lp - Globus pallidum, lateral part; SI - Substantia innominata; cc - Corpus callosum; ac - Anterior commissure; ic - Internal capsule; ec - External capsule; f - Fornix ; LV - Lateral ventricle; 3V - Third ventricle; D3V - Dorsal third ventricle

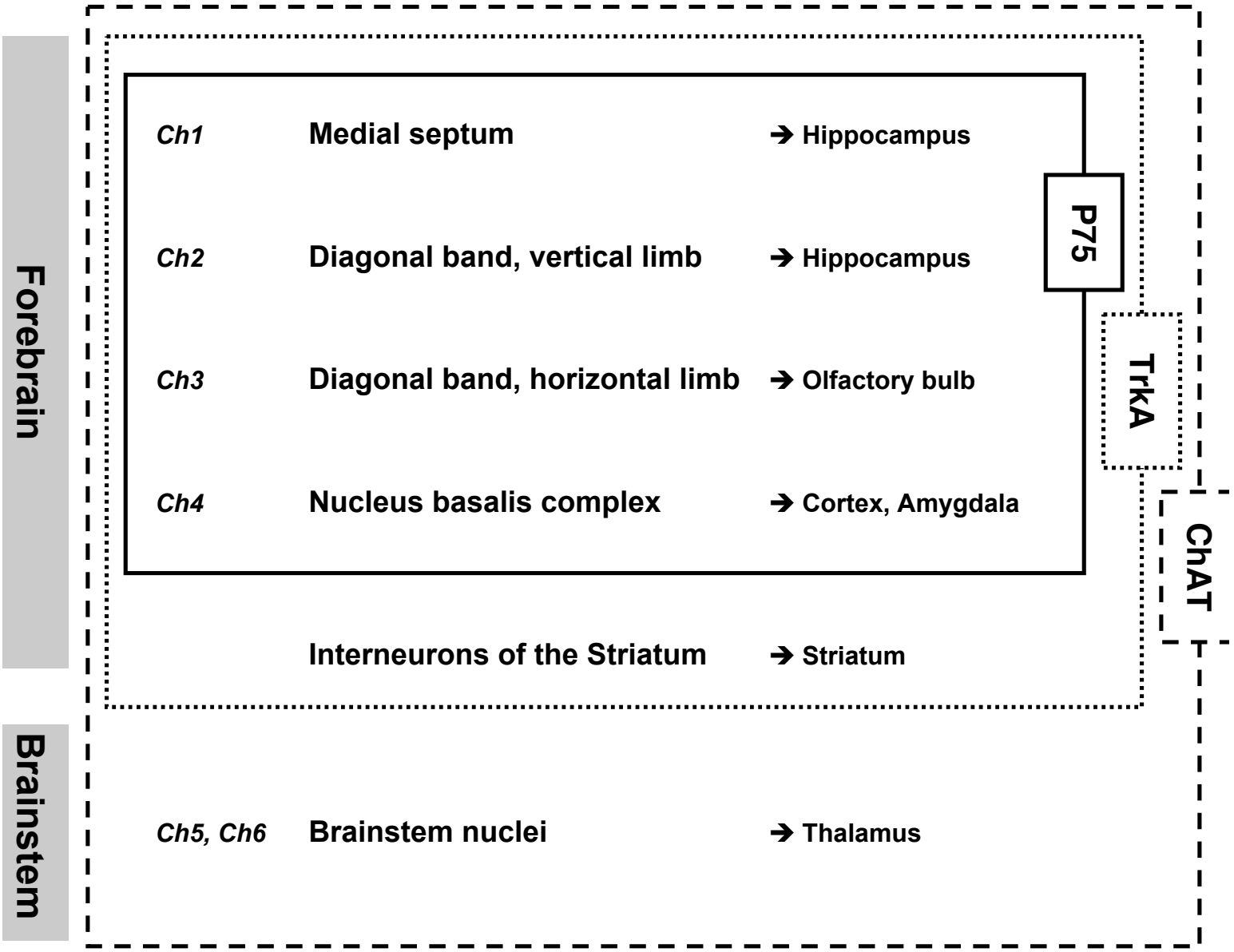

FIGURE 4: Schematic representation of the cholinergic cell subpopulations in the brain
For each neuronal cell group, main target areas of innervartion and expression of some major marker proteins are indicated.

3.3 Function of cholinergic neurons

A large number of studies have demonstrated that training-induced learning processes and memory formation activates the cholinergic system in the brain. For instance, maze

training produces a long-lasting increase in hippocampal ChAT concentration (Park et al., 1992). Conversely, aged rats that display memory deficits often display correlated decreases in hippocampal ACh markers (Aubert et al., 1995). More recently, in vivo microdialysis has allowed analyzing changes in hippocampal extracellular ACh levels in rats during behavioral tests of leaning and memory. With few exceptions, the results of such studies show that hippocampal-dependent learning and memory is associated with an increase in hippocampal extracellular ACh levels (Chang and Gold, 2003). Moreover, it was shown by electroencephalographic analysis, that neurons of the nucleus basalis are activated during learning processes (Whalen et al., 1994).

These and many other studies indicate a positive correlation between the activation state of the cholinergic system and the cognitive functions of the brain. However, none of these evidences answer the question whether the cholinergic neurotransmission in the basal forebrain is necessary for memory and learning. This question was addressed more directly by two main approaches: first, pharmacological modulation of the ACh pathway, and second, direct lesions of the basal forebrain cholinergic neurons. In particular the immunotoxin 192 IgG- Saporin was used in many studies with rats as a relatively specific tool to lesion the cholinergic neurons of the basal forebrain. The immunotoxin binds with the p75 receptor expressed on the cholinergic neurons of the forebrain, and is then internalized into these neurons, which allows the cytotoxin saporin to kill the cells.

3.3.1 Memory

The amnestic properties of anticholinergic drugs such as scopolamine and atropine have long been known (Drachman and Leavitt, 1974). In animals, both systemic and local (intra-hippocampal) application of anticholinergic drugs consistently inhibit hippocampal-dependent memory acquisition. In this regard, both nicotinic and especially muscarinic antagonists were shown to be efficient in impairing memory (Levin, 2002; Ohno et al., 1994). Conversely, lesions of the fimbria-fornix structure, or lesions of the medial septum impair hippocampal-dependent learning and memory (Kelsey and Vargas, 1993). These kinds of lesions, however, are rather unspecific and necessarily result in loss of both cholinergic and non-cholinergic septohippocampal projections.

Surprisingly, findings from experiments using injections of the immunotoxin 192 IgG-Saporin into the medial septum or the nucleus basalis often fail to cause any impairment

in spatial learning in the water maze, spatial working memory in the radial arm maze, or contextual fear conditioning (Baxter et al., 1996; Frick et al., 2004; Kirby and Rawlins, 2003). With few exceptions, deficits in learning performance were observed only in studies where high concentrations of 192 IgG-Saporin were used and where the immunotoxin was applied in a rather unspecific fashion by intracerebroventricular injections. In these cases, however, it is difficult to link the results to a specific cholinergic lesion. In fact, it was shown that under these kind of circumstances the 192 IgG-Saporin damage also cells of the cerebrellum or other non-targeted cells. In addition, in rats with different kind of lesions, it was shown that only rats with both cholinergic and non-cholinergic displayed deficits in a spatial discrimination task. In contrast, rats with specific cholinergic lesions showed no learning deficits (Cahill and Baxter, 2001).

These controversial results taken together seem to indicate that cholinergic neurotransmission in the basal forebrain may be involved in some but is definitely not necessary for all memory functions (Everitt and Robbins, 1997; Parent and Baxter, 2004).

3.3.2 Attention

In contrast to the controversial issue of the involvement of the cholinergic basal forebrain system in memory functions, there is considerable agreement concerning the importance of this system to various aspects of attention (Baxter and Chiba, 1999). For instance, rats that had received a selective lesion of the nucleus basalis by injection of low doses of 192-IgG Saporin failed to maintain sustained visuospatial attention in a Five-Choice Serial Reaction Time Task. In this study, it was also shown, that particulary the cholinergic projection from the nucleus basalis complex into the cortex are important for sustaining attention; the projection from the medial septum into the hippocampus, on the other hand, seem to be less necessary for attentional functions (Lehmann et al., 2003). In another study, it was shown that the attention deficit in nucleus basalis-lesioned animals was accompanied by a decreased cortical ACh efflux (McGaughy et al., 2002). Similarly, rats with nucleus basalis lesions failed to increase attentional processing under conditions where expectancies regarding stimulus relationships were violated. In general, the impairments following selective damage of the nucleus basalis are best described not as an impairment of attention itself, but rather as an impairment in the ability to respond appropriately to demands placed on attention.

3.3.3 Neuroplasticity

Cholinergic neurons are also well known to modulate events related to neuroplasticity. In particular, the reorganization of adult motor and sensory cortical representations seem to depend crucially on correct cholinergic innervation of the cortex. In general, cortical resources are allocated in such a way that discrete subsets of cortical neurons selectively process information related to a given part of the body. The amount of cortical resource allocated to a given region of the body is thereby not fixed and can under a certain set of circumstances be altered. Removal of the cortical cholinergic input inhibits this remodeling and has therefore a dramatic impact on the regulation of information processing. For instance, electrical stimulation of the nucleus basalis paired with auditory cues results in reorganization of the primary auditory cortex, increasing the area of auditory cortex that respond preferentially to the paired stimulus (Kilgard and Merzenich, 1998). Rats that had received 192 IgG-Saporin lesions did not show any remapping of the auditory cortex, indicating that this effect is cholinergically mediated. Basal forebrain neurons also appear to be essential for reorganizing the somatosensory cortex in response to removal of whiskers (vibrissae). Indeed, removal of the cholinergic nucleus basalis neurons by application of 192 IgG-Saporin eliminated experience-dependent plasticity in the somatosensory cortex (Baskerville et al., 1997). Remarkably this latter function of cholinergic neurons can be enhanced by application of NGF to the cortex (Prakash et al., 2004). Recently, it was shown that the cholinergic neurons of the nucleus basalis are also essential for remapping of the motor cortex. Rats that had impaired nucleus basalis activity showed reduced reorganization of the motor cortex after training and this impairment was combined with defects in performing complex motor tasks like reaching the footpad with the forelimb (Conner et al., 2003).

Another form of neuroplasticity, synaptic plasticity, can be also modulated by cholinergic neurotransmission. For instance, it was shown that activation of acetylcholine receptors induces long-term depression (LTD) of synaptic activity in the visual cortex and in the perirhinal cortex (Kirkwood et al., 1999; Massey et al., 2001). In both areas the cholinergic effect on the synaptic plasticity is transmitted most likely through the M1 muscarinic receptor. Recently, it was shown that in the perirhinal cortex the muscarinic antagonist scopolamine disrupts efficiently the production of LTD of synaptic plasticity.

This disruption was accompanied by an impaired performance in a visual recognition memory task. Thus, the acetylcholine-induced reduction of activity in perirhinal neurons is an essential event in the formation of recognition memory (Warburton et al., 2003).

4 THE CHOLINERGIC SYSTEM, NGF/TRKA SIGNALING AND ALZHEIMER'S DISEASE

4.1 Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is clinically characterized by memory and cognitive dysfunction (Mattson, 2004). Although sporadic AD is rare in individuals younger than 60 years of age, the incidence steadily increases with age, affecting up to 40% of those who are more than 85 years old. Brains regions involved in learning and memory processes, including the temporal and frontal lobes, are reduced in size in AD patients as the result of degeneration of synapses and death of neurons. The histopathology of AD brains is characterized mainly by two types of lesions: senile plaques composed of deposits of amyloid-β peptides (Aβ peptides), and neurofibrillary tangles composed of aberrantly phosphorylated tau, an microtubule-associated protein.

Few cases of AD are caused by inherited autosomal dominant mutations. This type of AD is characterized by the early-onset of the disease and is termed familial AD (FAD). The mutations identified so far affect the genes encoding the amyloid precursor protein APP and the proteases presenilin 1 and 2 (Bossy-Wetzel et al., 2004). All these mutations appear to increase the generation of amyloidogenic Aβ peptides.

4.2 Proteolytic APP processing

One central molecular hallmark of AD is the altered proteolytic processing of the amyloid precursor protein (APP). This alteration results in production and aggregation of neurotoxic form of Aβ peptides. APP is a membrane glycoprotein and contains a large extracellular region, a transmembrane helix and a short cytoplasmatic tail (Figure 5a). While the functions of full-length APP are largely unknown, the metabolism of APP is

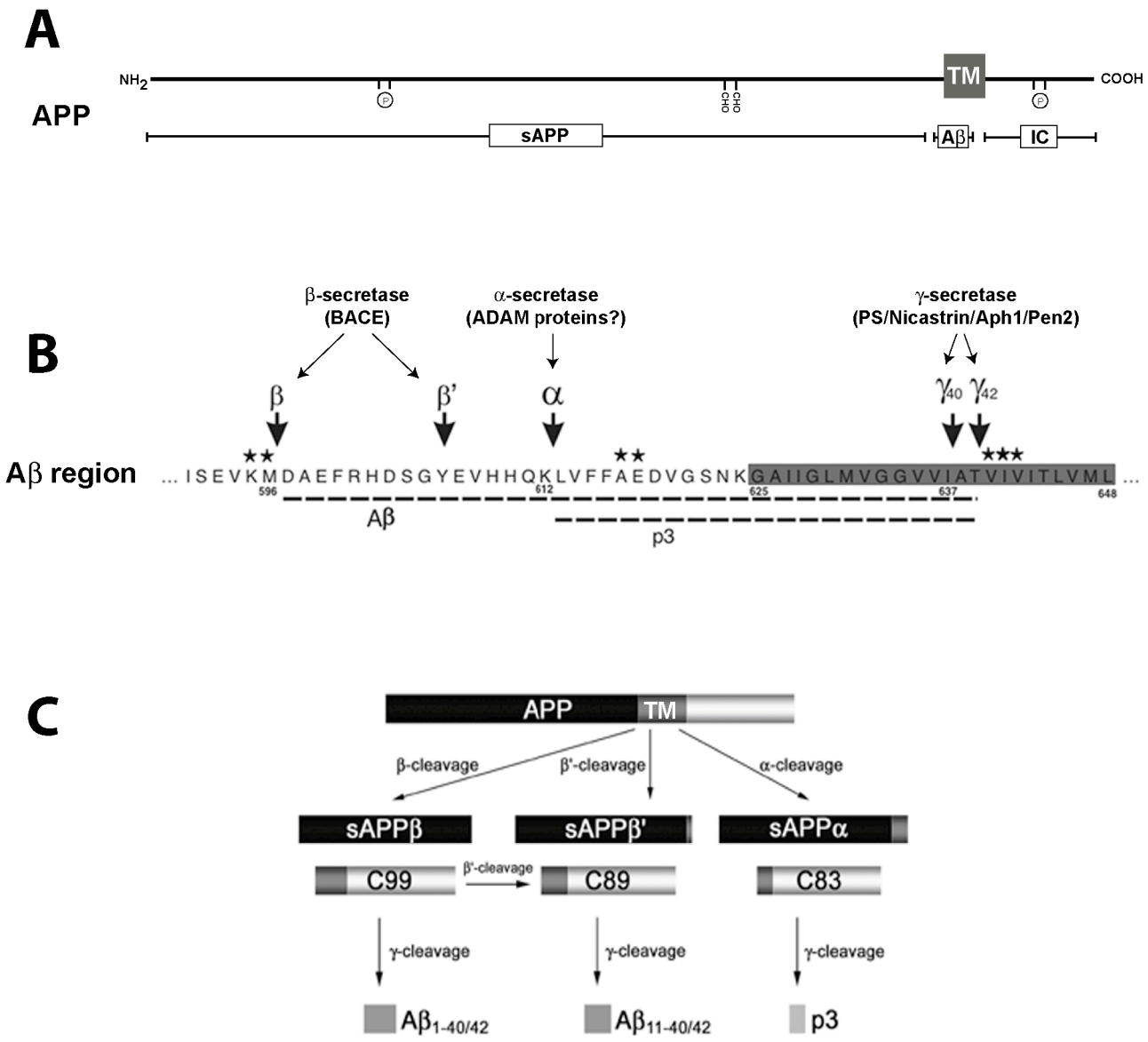

FIGURE 5: Structure and processing of the amyloid precursor protein APP
Panel A shows the overall structure of APP. Phosphorylation sites (P) and glycosylation sites (CHO) are indicated.
In Panel B, the amino acid sequence of the region encompassing the amyloid- peptide sequence is depicted. The major cleavage sites of the different scretases are indicated. The asteriks indicate the locations of mutations causing familial AD.
In Panel C, the cleavage pathways of APP are shown. Cleavage of APP by α-secretase or β-secretase results in a variety of different sAPP and C-terminal APP fragments (C99, C89 and C83). Subsequently, the γ-secretase cleaves the C-terminal fragments and generates different Aβ-peptides of various length and the short P3 peptide.
Figures are modified from De Strooper, B. and Annaert, W. (2000) and from Lee E.B et al (2005)

TM - transmembrane region; sAPP - soluble APP; Aβ - amyloid β peptide; IC - intracellular domain

well characterized (De Strooper and Annaert, 2000). It is mediated by a series of enzymes termed secretases α,β and γ (Esler and Wolfe, 2001)(Figure 5b). Cleavage of APP by α-secretase occurs in the middle of the Aβ domain and precludes the formation of full-length Aβ peptides. α-secretase cleavage releases the extracellular soluble N-terminal APP domain (APPsα), which has neurotrophic and neuroprotective properties, and leaves the intracellular 83-amino-acid carboxy-terminal APP fragment (C83)(Figure 5c). The identity of the α-secretase is still unclear, but various members of the disintegrin-metalloproteases ADAM family are good candidates.

An aspartyl protease, the β-site APP cleaving enzyme BACE, was identified as being the major β-secretase (Vassar et al., 1999). BACE cleaves APP at the N-terminus of the Aβ peptide either at Asp1 or Glu11 (numbering relative to the Aβ peptide) and release soluble APP domain referred as APPsβ and APPsβ', respectively. BACE mediating cleavage at Asp1 leaves a membrane-bound 99-amino-acid carboxy-terminal APP fragment (C99) in the cell. Conversely, BACE cleavage at Glu11 results in the generation of the 89-amino-acid carboxy-terminal APP fragment termed C89.

All three membrane-bound carboxy-terminal APP fragments (C83, C89, C99) are substrates for the γ-secretase complex, which perform an unusual proteolysis in the middle of the transmembrane domain (Iwatsubo, 2004). The precise composition of the γ-secretase complex is still under investigation, but Presenilin 1 (PS1) is very likely to be the active protease into it that is responsible for the generation of Aβ fragments. Other essential members of the γ-secretase complex are Nicastrin, Aph-1 and Pen-2. They seem to be required for substrate recognition, complex assembly and targeting the complex to its site of action. Proteolysis by the γ-secretase complex is heterogenous: Cleavage of the C99 results mainly in a 40-residue peptide (Aβ1-40) and at smaller proportions in a 42-residue peptide (Aβ1-42). Conversely, the γ-cleavage outcome of C89 is mostly represented by the two N-terminally truncated Aβ peptides Aβ11-40 and Aβ11-42. The various Aβ peptides differ in their neurotoxicity and in their amyloidogenic property. Commonly the full-length Aβ1-42 is considered the most amyloidogenic Aβ peptide. On the other hand, cleavage of the C83 by the γ-secretase complex generates a short peptide

named P3. P3 in contrast to the other Aβ peptides is thought to be not amyloidogenic and is not found in amyloid plaques. P3 may have some intracellular signaling properties.
Alteration of the APP cleavage can be caused by a variety of different impairments. For instance, mutations of the APP gene, overexpression or activity alteration of the different secretases, phosphorylation states of the intracellular domain of the APP protein (Perini et al., 2002), alteration of the intracellular APP trafficking (Chyung and Selkoe, 2003; Lee et al., 2005) and disturbances in the metabolism of cholesterol (Wolozin, 2004) have been shown to modify importantly the net outcome of the APP processing.

4.3 Cholinergic neurons and Alzheimer's disease

4.3.1 Cholinergic lesion in Alzheimer's disease

A substantial loss of cholinergic innervation in the cerebral cortex is well accepted as a major aspect of advanced AD. This is most severe in the temporal lobes, including the entorhinal cortex, in which up to 80% of cholinergic axons can be depleted (Geula and Mesulam, 1996). The depletion of cholinergic axons is associated with an equally severe cell loss in the nucleus basalis complex. On the other side, cholinergic innervation of the striatum and of the thalamus remains relatively intact.
The cell and innervation losses are accompanied by defects in the expression of the ACh related enzymes and of the ACh receptors. For instance, decrease of ChAT activity in the cortex was shown to correlate positively with the severity of dementia in AD (Minger et al., 2000; Pappas et al., 2000). In addition, M2 muscarinic receptors are reduced in the brains of individuals with AD (Nordberg et al., 1992). The density of postsynaptic M1 receptors remain unaltered, but there is some evidence for disruption of the coupling between the receptors, their G-proteins and second messengers (Warpman et al., 1993) .
The specificity of the cholinergic lesion in AD remains controversial. AD is associated with substantial variability in the involvement of noncholinergic cortical neurotransmitter like for example serotonin and noradrenaline. In general, however, it seems that cholinergic defects are more consistent than the loss of other transmitters

4.3.2 Links between cholinergic neurotransmission and APP processing

There is good evidence for a link between cholinergic neurotransmission and APP metabolism (Rossner et al., 1998b). For instance, in vitro M1 and M3-mediated

muscarinic stimulation of cortical neurons has been shown to promote the processing of APP by the α-cleavage pathway. Also the use of acetylcholinesterase inhibitors in cell culture assays modifies the APP processing. The effect on the secretion of soluble APP fragments differs thereby between cell types and depends upon which drug was used (Kar et al., 2004). In vivo in rats, it was shown that immunolesions by 192-IgG Saporin result in a significant reduction of secretion of soluble APP fragments without having any significant effect on the mRNA transcription of APP (Rossner et al., 1997).

On the other hand, numerous experiments have also shown that Aβ peptides affect the function of cholinergic neurons, in particular ACh synthesis and the signal transduction events associated with cholinergic neurotransmission (Kar et al., 2004; Zhong et al., 2003). In this regard, it is also of particular interest that Aβ peptides have been shown to bind to the p75 receptor (that is expressed in many cholinergic neurons) inducing apoptotic cell signals (Perini et al., 2002).

It is therefore possible that AD may be associated with a vicious cycle whereby the cholinergic depletion intensifies both the production and neurotoxicity of Aβ peptides which in turn further increases the cholinergic deficits.

4.4 NGF/TrkA expression and function in Alzheimer's disease

There is large number of studies that analyze the expression of neurotrophins and their receptor in post-mortem brains of AD patients. In individuals with AD, there is typically a marked loss of TrkA, which correlates with loss of cholinergic neurons (Chu et al., 2001; Hock et al., 1998; Mufson et al., 1996). Even though there have been some variable results regarding NGF protein levels in different brain regions of AD patients, most recent studies agreed that there are unchanged or increased NGF levels in the hippocampus and cerebral cortex, while the levels in the basal forebrain are decreased compared with age-matched controls (Hellweg et al., 1998; Mufson et al., 1995; Scott et al., 1995). These results suggest that the NGF/TrkA signaling defects in AD may not be due to a problem of NGF synthesis in the target areas of cholinergic innervation; defects in NGF release or an impaired retrograde transport of NGF are more likely to be the cause of the lack of NGF in cholinergic neuronal cell bodies in AD.

A direct link between NGF/TrkA signaling and APP processing was suggested by various in vitro studies that tested the effect of NGF application in cell cultures on the metabolism of APP. For instance, in the pheochromocytoma cell line PC12 it was shown that both APP mRNA expression and secretion of soluble APP was increased by stimulation with NGF (Rossner et al., 1998a). In the same cell line it was shown that APP mRNA expression can be increased also by withdrawal of NGF, an event that drives the cells into apoptotic cell death (Araki and Wurtman, 1998). A similar effect on APP mRNA expression after NGF withdrawal was shown also in primary neuronal cell cultures from the dorsal root ganglia (Nishimura et al., 2003). Interestingly, in these cells it could be also demonstrated, that the increase of APP expression protects cells from death.

A strong in vivo suggestion for the implication of NGF/TrkA signaling in the pathogenesis of AD was provided by the analyses of mice, in which NGF signaling is inhibited by the transgenic expression of an antibody against NGF. Aged anti-NGF mice show increase of APP protein levels and histological signs of β-amyloid plaques in the cortex, hippocampus and thalamus (Capsoni et al., 2002a; Capsoni et al., 2000b). Furthermore, these mice showed hyperphosphorylation of tau proteins, another typical molecular hallmark of AD. Thickness of the cortical layers and of the hippocampus was reduced and many cells showed signs of apoptosis, indicating a loss and atrophy of neurons in these areas. The neuronal impairments in these mice are accompanied by behavioral deficits in spatial learning as analyzed by using the eight-arm radial maze task. Interestingly some of these defects could be rescued by the intranasal application of exogenous NGF or by the injection of the cholinergic agonist galantamine (De Rosa et al., 2005).
The studies of aged anti-NGF mice provided an intriguing insight into a potential direct link between NGF/TrkA signaling and the pathogenesis of AD; they must be, however; interpreted carefully considering the possible side effects of the technical approach used in the anti-NGF mouse model.

5 MAIN AIM OF THIS STUDY

The aim of the here presented study is to analyze the function of NGF/TrkA signaling in the basal forebrain of mice during and after development. Therefore, conditional mutagenesis of the trkA and the ngf gene was performed by using the Cre-loxP system. This approach circumvents the early lethality of the classical knockouts and provides, in contrast to the hitherto used inhibition studies and to the transgenic anti-NGF mouse model, a complete, long-lasting and brain-specific deletion of NGF/TrkA signaling. In addition, the unspecific side effects of the Cre-loxP system are minimal.

The Cre-loxP system is based on the action of the recombinase Cre that is derived from the bacteriophage P1 (Nagy, 2000; Tsien et al., 1996). Cre catalyzes the site-specific recombination between 34 bp long loxP recognition sequences. The loxP sequences can be inserted into the genome of embryonic stem cells by homologous recombination such that they flank one or more exons of a gene of interest (called a "floxed gene"). Mice carrying the floxed gene are crossed to a second mouse that harbors a Cre transgene under the control of a tissue type – or cell type specific transcriptional promoter. In progeny that are homozygous for the floxed gene and that carry the Cre transgene, the floxed gene will be deleted by Cre/loxP recombination but only in those cell types in which the Cre gene-associated promoter is active. Another recombinase used in conditional mutagenesis is the yeast-derived Flp recombinase. Flp works identically as Cre; its consensus recombination site is called Frt sequence.

In this study the generation of the floxed trkA allele will be described. The crossing of the mouse carrying the floxed trkA allele with a transgenic mouse that express the Cre recombinase under the brain-specific Nestin promoter allows the deletion of trkA specifically in the cholinergic neurons of the forebrain. The effects of these deletions on the development, maintenance and function of the cholinergic neurons will be analyzed. In addition, the generation of a floxed ngf allele will be described. This mouse provides a tool for an even more complete analysis of the in vivo function of NGF/TrkA signaling in the cholinergic neurons of the basal forebrain.

IV MATERIAL AND METHODS

1 GENERAL PROTOCOLS AND MATERIALS USED

Standard protocols for molecular biology were taken from Molecular Cloning Laboratory Manual (2nd edition). If not otherwise indicated, all chemicals were purchased from Sigma, Merk and Fluka. All water used to generate solutions and buffers was filtered with the "Milli-Q-Water-System" from Millipore. For DNA preparation: MiniPrep, MaxiPrep, QIAquick PCR purification and Gel extraction kits (Qiagen) were used according to manufacturer instructions.

1.1 Buffers and solutions

10X PBS	1.3M NaCl 70mM Na_2HPO_4 30mM NaH_2PO_4, pH7.2
TE buffer	10mM Tris/HCl 1mM EDTA, pH8
50X TAE	2M Tris-Acetate 50mM EDTA
20X SSC	3M NaCl 0.3M NaCitrate, pH7.0
Tris-buffered saline (TBS)	100 mM Tris-HCl, pH 7.4 150 mM NaCL

TB buffer	50 mM Tris-HCl, pH 7.4
Sodium phosphate buffer (PB), pH 7.4	
	77.4 ml of 1M Na_2HPO_4
	22.6 ml of 1M NaH_2PO_4
Tris-Azide	0.04 M Tris
	0.7% NaCl
	0.01 Na_2HPO_4
	0.01 M NaH_2PO_4
	0.05% NaN3
	Adjust to pH 7.8
LB (Luria-Bertani) medium	10g bacto-trypton
	5g yeast extract
	5g NaCl
	Add H_20 to 1l, pH 7,5
LB plates	LB medium supplemented with 15g/l agar

<u>For Southern blots</u>

Denhardt's reagent	1% Ficoll
	1% polyvinylpirrolidone
	1% BSA
Prehybrization buffer	50% deionized formanide,
	5X SSC,
	5% denhart's solution,
	50 mM Na_3PO_4 pH 7,2,
	1% SDS and 350 µg/ml DNA salmon sperm

Hybridization buffer

50% deionized formanide
5X SSC
5% denhart's solution
50 mM Na_3PO_4 pH 7,2
1% SDS
100 μg/ml DNA salmon sperm,
5% dextran sulfate

For ES cell work

Medium for embryonic stem (ES) cells

Knockout D-MEM with sodium pyrovate (GIBCO BRL) supplied with:
15% Knockout serum replacement (Gibco BRL),
100IU/ml -100μg/ml penicillin-streptomycin (Gibco BRL), 2mM glutamine (Gibco BRL),
1:100 Non-essential Amino Acids (Gibco BRL),
0.1 mM β-mercaptoethanol,
10000 U/ml ESGRO (murine leukaemia inhibitory factor (Gibco BRL).

Freezing medium for ES cells

Knockout DMEM (Gibco BRL) with
20%DMSO
35% fetal calf serum (Gibco BRL)

ES lysis buffer

10 mM Tris, pH 7.5
10 mM EDTA pH 8
10 mM NaCl
0.5% sarcosyl
100 μg/ml proteinase K

For immunoblotting

NP-40 protein lysate buffer	20mM Tris-HCl pH 7.4
	140 mM NaCl
	10% glycerol
	1% NP-40
2xprotein loading buffer	100mM Tris-HCl pH 6.8
	200mM dithiothreitol
	4% SDS
	0.2% bromophenol blue
	20% glycerol

For mice genotyping

Tail lysis buffer	100mM Tris-HCl pH7.5
	1mM EDTA
	250mM NaCl
	0.2% SDS

1.2 Antibodies

Primary antibodies

Anti-ChAT	CHEMICON (Ab143), rabbit polyclonal
	Immunohistochemistry (IHC) 1:500
Anti-ChAT	CHEMICON (Ab144), goat polyclonal
	Immunoblotting (IB) 1:500
Anti-TrkA	Gift from L. Reichardt, rabbit polyclonal
	IHC 1:500; IB 1:1000
Anti-p75	CHEMICON (Ab1554), rabbit polyclonal
	IHC 1:400
Anti-Parvalbumin	SIGMA (Clone Parv-19), mouse monoclonal

	IHC 1:3000
Anti-APP NH-terminal	CHEMICON (Clone 22C11), mouse monoclonal
	IB 1:3000; IHC 1:100
Anti-APP C-terminal	SIGMA, rabbit polyclonal
	IB 1:3000; Immunoprecipitation:1µl per immunoprecipition
Anti-APP Aβ-NT	PRO-SCI Incorporated
	IB 1:500
Anti-ERK1	ZYMED (Clone ERK-6B11), mouse monoclonal
	IB 1:3000
Anti-Tubulin	Sigma, mouse monoclonal
	IB 1:20000

Secondary antibodies

Anti-mouse HRP	JACKSON Immunoresearch
	IB 1:5000
Anti-rabbit HRP	JACKSON Immunoresearch
	IB 1:5000
Anti-goat HRP	JACKSON Immunoresearch
	IB 1:5000
Anti-mouse biotinylated	VECTOR Laboratories
	IHC 1:200
Anti-rabbit biotinylated	VECTOR Laboratories
	IHC 1:100

1.3 Plasmids

The following plasmids were used for subcloning steps in order to generate the targeting constructs:

PLASMIDS	INSERT	PROVIDER
pBluescript II KS	Multicloning sites	STRATAGENE
PTrkA	18 kB genomic locus trkA (Exon 1-17)	L.Tessarollo
PNGF	12 kB genomic locus ngf (Exon 4)	P.Ernfors
PFLRT3	loxP-loxP-FRT-Neo-cassette-FRT	L.Minichiello
pIRES2-EGFP	IRES-EGFP	CLONTECH

2 METHODS

2.1 General mouse work

Mutant and control mice were maintained on a mix genetic background (C57/Black6/129). Young mice were separated from their parents at the age of around 3 weeks; males and females were housed separately. All sacrifices were done by cervical dislocation.

2.1.1 Mice line used

MICE LINE	DESCRIPTION	FIRST REFERENCE
Lox trkA	Conditional targeting of trkA	In this study here
Lox ngf - EGFP	Conditional targeting of ngf	In this study here
Lox trkB	Conditional targeting of trkB	(Minichiello et al., 1999)
Nestin-Cre	Expression of Cre-recombinase under nestin-promoter	(Tronche et al., 1999); (Medina et al., 2004)
Flp-deleter	Ubiquitous expression of Flp recombinase	(Farley et al., 2000)
Cre-deleter	Ubiquitous expression of Flp recombinase	(Schwenk et al., 1995)

2.2 Genotyping of mice

Genetic determination of mice was done by PCR analysis. Tail biopsies were taken from the mice at weaning age of about 3 weeks and mice were ear-tagged using six-digit eartag (Nationalband). The tails were incubated overnight at 56ºC with tail lysis buffer plus 100 μg/ml of Proteinase K (Roche). DNA was then purified using the DNAeasy kit (Quiagen) and diluted in a final volume of 150 μl. PCR reaction was performed using 2 μl of the DNA preparation in a final volume of 50 μl containing 50 pmol of each primer, 200 mM of each dNTPs, 1.5 mM $MgCl_2$, 1X PCR buffer (Applied Biosystem), and 2.5 units of AmpTaq polymerase (Applied Biosystem). 30 μl of the PCR mix after PCR amplification were loaded on an 1% agarose gel.

All primers were ordered from Metabion. The primers used for DNA amplification were the following:

Primer	**Allele**	**Sequence 5' → 3'**
LF	Lox trkA	ACACTGGGTGGCTCAAGGTA
SA	Lox trkA	GTCACTCCCCACATGCCACC
Neo	Lox trkA; lox ngf-egfp	CTTCTATCGCCTTCTTGACG
Ex4	Lox ngf-egfp	GCTTTGATTGCCTCTCTTGA
LA1	Lox ngf-egfp	GGTCCCTGCCTTTCTACTCG
LMAR2DN	Lox trkB	CCAAGGTGATCAACAGCCCAAGTC
LMEX4UP	Lox trkB	TGAAGGACGCCAGCGACAATGCACG
Cre1	Nestin-Cre; Cre-deleter	GCCTGCATTACCGGTCGATGCAACGA
Cre2	Nestin-Cre; Cre-deleter	GTGGCAGATGGCGCGGCAACACCATT
SD 222	Flp-deleter	CCCATTCCATGCGGGGTATCG
SD 223	Flp-deleter	GCATCTGGGAGATCACTGAG

2.3 Generation of transgenic mice

2.3.1 Generation of targeting constructs

All subcloning steps were perfomed by using classical "cutting and ligating" methods. Ligations were performed at room temperature for 2 hrs using T4 DNA ligase from New England Biolabs. All restriction enzymes were purchased from New England Biolabs.
For generating blunt ends in DNA fragment the Klenow fragment of the DNA polymerase I (New England Biolabs) was used. For dephosphorylation of DNA fragments, the Calf intestinal alkaline phosphatase (New England Biolabs) was used. For transfection and amplification of DNA, the E.Coli bacterial strain XL1-blue was used. Ampicillin-resistant bacterial clones were grown in LB medium or on LB plates supplemented with 0.1 mg/ml ampicillin.

2.3.1.1 trkA targeting construct

In the trkA targeting construct the exons 12-14 of the trkA gene are flanked by two loxP sequences (see also Figure 6). The exons 12-14 encode for the major parts of the juxtamembrane domain and of the catalytically active kinase domain of the TrkA receptor. The targeting vector beside the 1.4 kb loxP - flanked segment contains additional 6.5 kb of trkA genomic sequence (4 kb in the long arm on the 5' end of the construct and 2.5 kb in the short arm on the 3'end of the construct). Downstream of the second loxP sequence the targeting vector contains a neo– cassette, in a transcriptional orientation opposite to that of the trkA gene. The expression of the neo-cassette confers resistance to the cytotoxin G418 and is used as selectable marker for successfull integration of the targeting construct. The neo-cassette is flanked by two FRT sequences.

2.3.1.2 ngf – egfp targeting construct

In the targeting construct the complete exon 4 of the ngf gene and fragments of the adjacent introns (in total 2.6 Kb) is flanked by two loxP sequences (see also Figure 18). On the 3' end of the loxP flanked sequence an IRES (internal ribosomal entry site) element and a cDNA cassette encoding for the EGFP fluorescent protein was introduced. EGFP will be expressed under the endogenous ngf promoter only after deletion of the ngf

exon 4. Additionally, a polyA sequence was introduced directly after the 3' end of ngf exon 4. The targeting vector contains furthermore a 3.5 kb 5' homologous arm and a 4.1 kb long 5' homologous arm. Downstream of the second loxP sequence the targeting vector contains a FRT flanked neo-cassette in a transcriptional orientation opposite to that of the ngf genomic sequence.

2.3.2 Targeting of wild-type alleles

Mice E14.1 embryonic stem (ES) cells were plated on confluent mitomycin-C treated mouse fibroblasts in ES media. The ES-cells were expanded for 6-7 days until ready for electroporation.

The targeting vector for the "trkA" transgenic allele was linearized with the SalI restriction endonuclease, while the "ngf-egfp" targeting vector was linearized with the EcoRI restriction enzyme. The DNA was purified by phenol extraction and then precipitated by adding 2 volumes of 96% ethanol and 0.1 volume of 3M Na-acetate pH 4.6. The DNA pellet was washed twice with 70% ethanol, air dried under sterile conditions and resuspended in sterile water.

After linearization, 30 μg of DNA was electroporated into ES cells (10^7 cells) at 240V and 500 μF in a Biorad Gene pulser. The cells were incubated for 20 min on ice and subsequently plated on gelatine coated 10 cm dishes in ES medium. The day after electroporation the media was changed to ES media supplied with 320 μg/ml G418. The media was changed every day until the ES cell colonies were ready to pick (approximately 10 days).

The ES cell clones were picked and placed in a well on a 96-well plate with confluent feeders and ES medium. Media was changed every day and cells were grown until they were confluent (3-4 day).

The ES cells were trypsinised and 2/3 of the cells were frozen in freezing medium, while the rest of the cells were replaced in a gelatinised 96-well plate without feeders and grown until ready for DNA preparation.

2.3.3 Isolation of DNA from ES clones and Southern blot analysis

For screening of the ES cells, the confluent plates were washed with PBS. 50 μl of ES lysis buffer was added to each well and incubated overnight at 55ºC. The DNA was

precipitated using 100 µl EtOH/NaCl, washed three times with 70% ethanol, dried and digested with the appropriate restriction enzyme (as indicated below) overnight at 37ºC. The digested ES cell DNA was run on a 0.7% agarose gel at 30V for 15-18 hrs.

Before blotting, the gels were stained with ethidium bromide, and then depurinated for 15 min in 0.25 M HCl, denaturated with 0.4 M NaOH, 2 times for 30 min, and finally neutralized with SSC 20X for 1 hr.

Gels were blotted overnight onto a GeneScreen Plus membrane (Perkin Elmer) in presence of SSC 20X (capillary blotting). The membrane was baked at 80ºC for 1 hr and then washed for 1 hr in 0.1X SSC and 0.5% SDS at 65ºC to reduce background. Prehybridization was then performed in prehybrization buffer for 2 hrs at 42ºC, followed by hybridization with in hybridization buffer together with labeled probe overnight at 42ºC. The probes were labeled with the radioactive nucleotide α-^{32}P GTP (Amersham) using the Random Primed DNA Labeling Kit (Roche). Following the hybridization, the blots were washed in 2X SSC and 1% SDS, twice at room temperature and once at 50°C, and then washed in 0.1X SSC and 1%SDS, twice for 30 min at 55ºC. Hybridized probes were visualized using the phosphoimager system (Fuji).

The probes used for hybridization were the following (see also Figures 6 and 18):

Probe	Allele	Restriction enzyme	Expected band	
			WT band	Mut. band
TrkA 5’	Lox trkA	BamHI	12 kb	5 kb
TrkA 3’	Lox trkA	BamHI	12 kb	9 kb
Ngf-egfp 5’	Lox ngf-egfp	EcoRV	13 kb	8 kb
Ngf-egfp 3’	Lox ngf-egfp	EcoRI	10 kb	15 kb

All probes used have a length of about 500-600 bp. The probes TrkA 5’, TrkA 3’, Ngf-egfp 3’ were generated by PCR amplification; the probe Ngf-egfp 3’ was generated by digesting the pNGF plasmid with the restriction enzymes EcoRI and SpeI. In addition to the listed probes, an internal probe in the neo-cassette was used. This probe was obtained by digesting the pFLRT3 with the PSTI enzyme.

2.3.4 Injection of ES-cells into C57BL/6 blastocysts

Two clones derived from each construct were injected into C57BL/6 blastocysts following standard protocols. The resulting chimeras were bred for germline transmission. Agouti animals were genotyped, in order to distinguish the heterozygous from wild-type animals, using the southern blot analysis as described for the ES cells screening.

2.4 Analysis of transgenic mice

2.4.1 Preparation of floating sections

Cryostat sections of brains of mice at different ages were used for immunohistochemistry stainings. Therefore the mice were anesthetized with 2% avertin and intracardially perfused first with 10ml cold PBS and then with 10ml cold 4% PFA/0.1M PB. The head of the perfused mice were then left overnight in 4% PFA at C. The next day, the brains were dissected out of the skulls and postfixed for another 12 hrs in 4% PFA. The brains were washed in cold PBS and placed in a 30% sucrose / Tris-Azide solution at 4°C until they sunk to the bottom of the incubation tube. The brains were then shortly dried and placed in embedding molds filled with OCT embedding medium (Sakura Finetek). Subsequently, the embedding molds were placed in isopentane that was in turn submerged in liquid nitrogen for several minutes in order to harden the embedding medium. The frozen brains in OCT were stored at –80°C.

30 μm serial coronal sections of the frozen brains were cut at -20°C on a cryostat. The cryosections were transferred into the wells of a 96-well plate filled with Tris-Azide solution. These floating sections were stored at 4°C.

For the preparation of sections for the staining of DRG neurons, embryos were dissected from the uterus at embryonic day E17.5. The embryos were fixed and embedded in OCT medium as described previously for the brains. 10-16 μm transversal sections of the spinal chord region were cut on a cyrostat at -20°C and transferred directly onto "Superfrost plus" slides (Menzel), and airdried. The slides were conserved at -20°C.

2.4.2 Immunohistochemistry

Immunohistochemistry stainings on floating sections were performed in 24-well plates. All steps were performed on a rocking platform at room temperature, except the incubations with the primary and the secondary antibodies that were performed at 4°C. Sections were washed 3x15 min in PB. To quench endogenous peroxidases, sections were then incubated for 20 min in 2% H_2O_2 and washed 2x15 min in TBS. Unspecific antibody binding was blocked by incubating the sections for 1hr in TBS supplemented with 10% normal goat serum (NGS, Vector laboratories), 3%BSA and 0,4% Triton X-100. Primary antibodies were diluted in TBS supplemented with 1% NGS, 3%BSA and 0,4% Triton X-100 and the sections were incubated with the antibody solution for 40-48 hrs. Subsequently, after 3x15 min washing steps with TBS plus 1% and 0.4% Triton X-100, the sections were incubated for 20-24 hrs with the appropriate biotinylated secondary antibody diluted in TBS supplemented with 1% NGS, 3%BSA and 0,4% Triton X-100. The binding of the biotinylated antibodies was then visualized using the Vectastain ABC kit (Vector Laboratories). Therefore the sections were washed 3x15 min with after 3x15 min washing steps with TBS plus 1% NGS and 0.4% Triton X-100 and then incubated for 1 hr with the Vectastain ABC reagent (containing avidin / biotinylated horseradish peroxidase complexes). After extensive washing first 3x15 min in TBS and then 2x15 min in TB, peroxidase activity was visualized by incubating the sections for 20 min with the substrate diaminobenzidine tetrahydrochloride (in TB at a concentration of 0.5mg/ml) and finally by adding H_2O_2 at a final concentration of 0.3%. When the staining was completed (1-2 min), the color reaction was stopped by washing the sections several times with cold TB. Sections were transferred onto “Superfrost plus” slides (Menzel), airdried, dehydrated in a graded series of alcohols, incubated for 10 min in xylene and coverslipped.

The staining of the spinal chord sections was performed directly on the slides. All staining and washing steps were identical as described for the floating sections. During staining steps, the slides were covered with parafilm in order to avoid drying out of the sections.

2.4.3 Cell counting

The sections were visualized and imaged under a light microscope (Leica DMR) with a digital camera (Leica). Further analysis was done using the Photoshop software (Adobe). The boundaries of the distinct counting areas were defined and cells were counted manually. Only cells with a clearly stained cell body were counted.

The counting criteria and the boundaries of the distinct areas were defined as following. As reference the "Mouse Brain" atlas from Paxinos, G and Franklin, KB (Second edition, Acedemic press) was used (see also Figure 3).

Medial septum: One section every 120 μm of the medial septum throughout its complete rostrocaudally extent was counted. On average a total number of 8-9 sections were counted for each animal. Because only every fourth section was analyzed (every 120 μm), it is unlikely that a neuron would be measured twice. The medial septum was defined as the area triangular with the following anatomical boundaries: *Lateral*: Lines connecting the anterior comissures with the midline of the corpos callosum; *Ventral*: a horizontal line connecting the inferior edges of the anterior commissures. The cells in this area that make part of the nucleus accumbens (which is clearly distinguishable from the cells of the medial septum and is part of the ventral striatum) were not taken into consideration.

Nucleus basalis complex: The nucleus basalis complex was defined as the region containing the cholinergic neurons of the nucleus basalis, the substantia innominata and the globus pallidus. The cells of this region are not clearly distinguishable from the cholinergic neurons of the caudal part of the horizontal limb of the diagonal band. Therefore, in order to avoid counting bias, also the cells of this part of the diagonal band were included in the counting area for the nucleus basalis complex. This definition amounted to counting all cholinergic neurons ventrally from the striatum and the internal capsule. The first section counted was the most rostral section through the decussation of the anterior commissures. Then a section every 120 μm was counted for a total number of 8 sections. On every section the cell number in the nucleus basalis complex on both hemispheres was quantified.

Striatum: The striatal tissue is clearly distinguishable from the surrounding areas. The boundaries are given by the lateral ventricle medially, the external capsule laterally, and

the anterior commissure ventrally. The first section counted was the section where the corpus callosum crossed for the first time completely the midline. Then a section every 240 μm was counted for a total number of 7 sections. On every section the cell number in the striatum on both hemispheres were quantified.

2.4.4 Histochemistry for AChE activity

Stainings for acetylcholinesterase (AChE) were performed using a modified Tago method (Di Patre et al., 1993). The sections were quickly rinsed in 0.05 M Tris-maleate buffer (pH 5.7) and then incubated for 10 min in Tris-maleate buffer containing 6μg/ml promethazine, and washed two additional times in Tris-maleate buffer. Section were incubated with 30 min in a 32.5 mM Tris-maleate buffer containing 5 mM sodium citrate, 3 mM cupric sulfate, 0.5 mM potassium ferrocynide, and 0.52 mg/ml acetylthiocholine iodide, then rinsed five times in 50 mM Tris-HCl (pH 7.6). Sections were incubated for 5 min in 50 mM Tris-HCl containing 0.25 mg/ml diaminobenzidine tetrahydrochloride and 3 mg/ml nickel ammonium sulfate. Hydrogen peroxide (final concentration 0.006%) was added and sections was allowed to incubate for 2-3 more minutes. The reaction was stopped by washing sections 3-4 times with 50 mM Tris-HCl buffer. Sections were transferred onto "Superfrost plus" slides, dehydrated in a graded series of alcohols, incubated for 10 min in xylene and coverslipped.

2.4.5 Preparation of protein lysates

Mice were killed by cervical dislocation and brains were quickly dissected. Total forebrain or specific subareas of the brain were snap-frozen in liquid nitrogen. For preparation of total protein lysates, the tissue was lysed in NP-40 lysate buffer using a dounce tissue homogenizer. The lysate was then cleared from insoluble components by centrifugation at 14000 rpm for 30 min and conserved at –80°C. For detection of soluble APP fragments, the tissue was homogenized with a douncer in 20 mM Tris-HCl pH7.4 (supplemented with 2 mM EGTA and 1 mM EDTA) and ultracentrifuged at 100000 rpm for 1hr. The supernatants were further processed for immunoblotting.

2.4.6 Immunoblotting (Western blotting)

Protein concentrations of the lysates were determined using the Bio-Rad Dc protein assay (Biorad). 50μg of each sample was used for immunoblotting. Proteins were mixed with

equal volume of 2xprotein loading buffer and boiled for 5 min by 95°C. Proteins were usually analyzed on 1 mm thick minigels (Bio-Rad apparatus). The tris-glycine separating gel contained 8% acrylamide (Bio-Rad, stock solution 30% Acrylamide/bis-acrylamide 37.5:1 ratio), 375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% ammonium persulfate and 0.001% N,N,N',N'-tetramethylenediamine (TEMED). The stacking gel contained 4% acrylamide, 125 mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% ammonium persulphate and 0.001% TEMED. The gels were run in Laemmli running buffer (25 mM Tris base, 200 mM glycine, 0.1% SDS) at a current of 20-40 mA per gel. Proteins were transferred onto Protran nitrocellulose (Schleicher & Schuell) using a Hoefer SemiPhor apparatus for 1 hr at a constant mA (1mA/cm^2 gel area) in transfer buffer (20 mM Tris base, 150 mM glycine, 0.1% SDS, 20% methanol). Membranes were stained in 0.2% Ponceau S solution (Serva) and then washed. Unspecific binding was blocked by incubating membranes in 5% non-fat dry milk in PBS containing 0.1% Tween-20 (BioRad) from 1 hr at room temperature.

Primary antibodies were diluted in PBS, 5% milk, 0.1% Tween-20, added to the blots, and incubated overnight at 4°C. Following the antibody incubation the membrane was washed 3-5 times in PBS, 0.1% Tween-20 for a total of 1 hr. Horseradish peroxidase conjugated secondary antibodies were used in a dilution of 1:5000 and incubation of the membranes was performed at room temperature for 1hr. After several additional washing steps for a total time of 1 hr the HRP enzymatic activity of the secondary antibody was revealed by the ECL chemilumescence method (Amersham Pharmacia).

For reprobing blots, membranes were stripped for 20 min at 62ºC in 5 mM PB with 2% SDS and 2mM β-mercaptoethanol.

For quantification, the bands of the developed films were digitalized and quantified by using the NIH Image 1.63 software. This software measures the brightness of each pixel and the total area of the band, and then calculates the mean optical density (O.D.) for each sample. In order to compare the O.D. values of different bands, the values were standardized in regard to protein loading. Therefore on each blot, also expression levels of the ubiquitously and constitutively expressed control proteins as ERK1 or in some cases also tubulin were analyzed and quantified. All the results are indicated as relative

protein levels obtained by calculating the ratio between the O.D. value of the analyzed protein and the O.D. value of ERK1 or tubulin.

2.4.7 Immunoprecipitation

500 μg of protein lysate were immunoprecipitated with 1μg of antibody 22C11 while rocking on a rotating wheel for 12 hrs at 4°C. Immunoprecipitates were collected at 4°C by incubating with protein A-Sepharose CL-4B beads (50 μl of a 1:1 solution in NP-40 protein lysate buffer; Amersham). After 4 washes with NP-40 protein lysate buffer, Sepharose-bound proteins were eluted in 2x protein loading buffer and processed for immunoblotting. For the separation of these immunoprecipitates, pre-cast 4-20% gradient Tris-Glycine gels (Novex gels, Invitrogen) were used. These gels allow a better separation of low molecular weight proteins.

2.4.8 NGF immunoassay (ELISA)

To measure NGF protein, the Chemikine NGF sandwich enzyme immunoassay kit from Chemicon was used. According to the manufacturer's protocol, total forebrains were lysed in 1ml of immunoassay lysate buffer at 4°C. Lysates were centrifuged for 30 min at 14000 rpm and 50 μl of the supernatants were loaded into the microwells of the ELISA plates provided by the kit. Each lysate was assayed in triplicate. The microwells are coated with sheep polyclonal NGF antibodies, which capture NGF from the sample. Captured NGF was detected by mouse monoclonal NGF antibodies and subsequently by peroxidase labeled anti-mouse donkey antibodies. Peroxidase activity was finally visualized by adding substrate solution. The color reaction was stopped after 10 min and the optical density (O.D.) of each well was quantified by a microplate reader using a 450nm wavelength filter. In each assay, also several NGF standards were analyzed and a standard curve was calculated that shows a direct relationship between NGF concentration and the corresponding O.D.s. By using this standard curve, NGF concentration of each forebrain lysate was determined and normalized to the weight of the lysed forebrain.

2.4.9 Statistical analysis

All the experiments were carried out on at least three animals from each genotype (unless otherwise indicated). The immunoblot analyses were repeated at least three times for each sample.

All results are indicated as means ± standard error of the mean. Significance of differences between samples was assessed by using the Student's t-tests. Differences were considered significant when $p<0.05$.

V RESULTS

1 TRKA EXPRESSION IS BRAIN-SPECIFICALLY DISRUPTED IN trkA $^{LOX/LOX}$; NESCRE +/- MICE

We flanked the exons 12-14 of the trkA gene with two loxP sites by homologous recombination in ES cells and derived germline targeted offspring carrying one floxed allele including the neo-cassette (lox neo+ allele) (Figure 6a). This trkA $^{lox\ neo+/+}$ mouse was used to generate mice homozygous for the lox neo+ allele. The homozygous mice trkA $^{lox\ neo+/lox\ neo+}$ were born in expected Mendelian ratio but died in the first three days of life. The phenotype of these mice resembles closely the phenotype observed in complete trkA knockout suggesting that the neo-cassette introduced in the trkA locus interfered severely with the expression of the TrkA protein. In fact, it is known that the intronic insertion of the neo cassette may importantly decrease the level of mRNA produced by the recombinant allele (Meyers et al., 1998; Nagy et al., 1998). The neo sequence could also introduce cryptic splice sites that could lead to truncation of the protein encoded by the gene of interest. The neo cassette that is flanked by two FRT sites was therefore excised in vivo from the recombinant allele by crossing with transgenic mice that express ubiquitously the Flp recombinase (Flp-deleter mice). Successful excision of the neo cassette was verified by Southern and PCR analyses and resulted in the generation of the recombinant neo- allele (Data not shown and Figure 6b). The deletion of the neo-cassette rescued the lethal phenotype of mice homozygous for the recombinant allele indicating that the intronic insertion of the neo cassette in the trkA gene caused fatal decrease of TrkA expression. This effect of the neo-cassette was further verified by immunoblotting of protein lysates of the forebrain of newborn mice with an antibody against TrkA. While no TrkA was detected in the forebrain of trkA$^{lox\ neo+/lox\ neo+}$ mice, TrkA expression was rescued in trkA$^{lox\ neo-/lox\ neo-}$ mice and reached levels undistinguishable from those in wild-type mice (Figure 7a). trkA$^{lox\ neo-/lox\ neo–}$ mice are viable, fertile and display no gross anatomical defects. All subsequent work was done with mice in which the neo-cassette

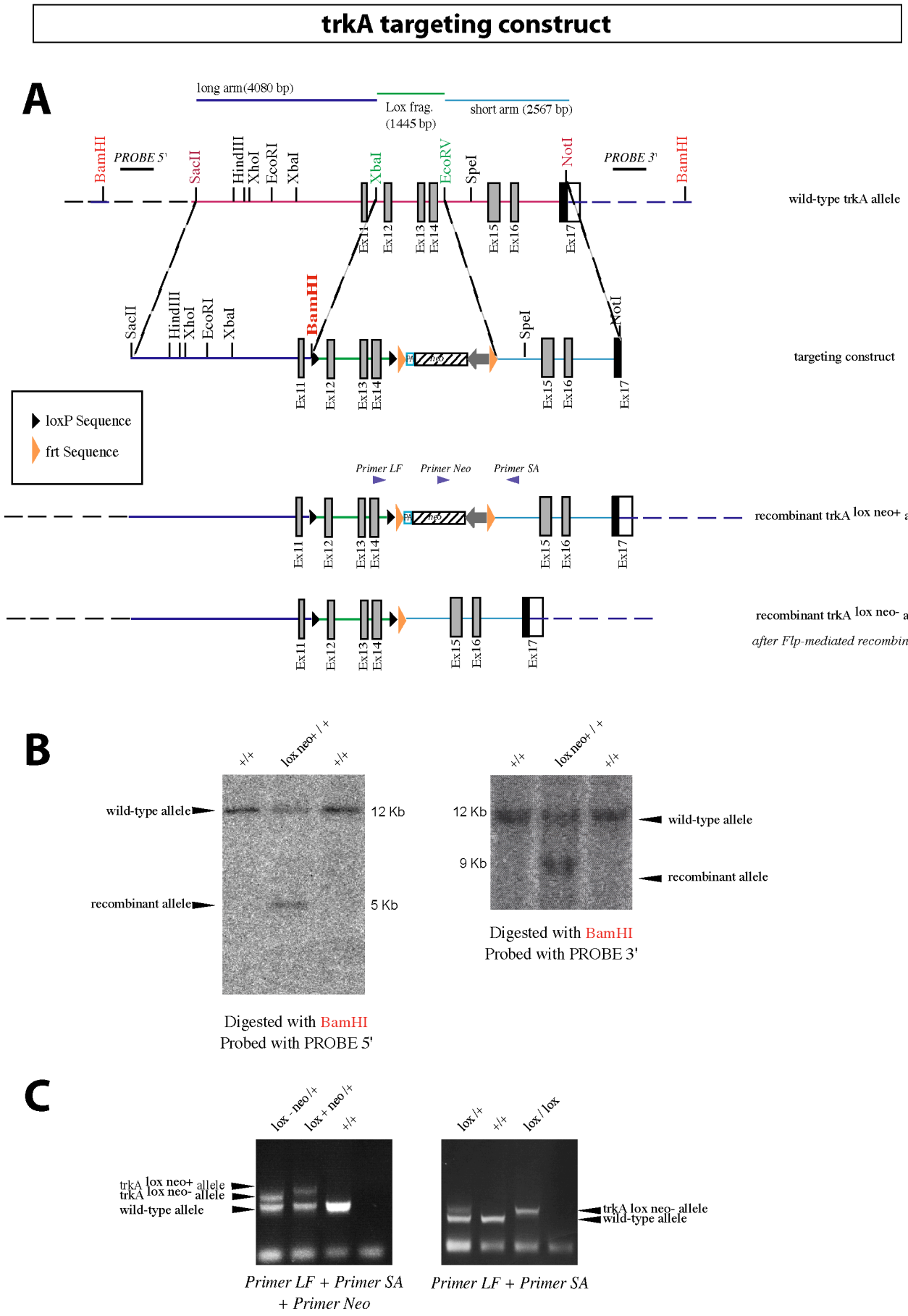

FIGURE 6: Conditional targeting of the trkA exons 12-14
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was deleted and from now on the lox neo- allele is termed simply lox allele. In order to verify that Cre-recombinant mediation delete efficiently TrkA expression in the floxed mice, we crossed the TrkA $^{lox/lox}$ with transgenic mice that express the Cre-recombinase ubiquitously (Cre-deleter mice). Mice homozygous for the floxed allele that express the Cre recombinase die in the first days of life and resemble closely the TrkA knockout mice.

In order to disrupt TrkA expression specifically in the brain, we crossed the mice carrying the floxed TrkA allele with transgenic mice that express the Cre recombinase under the nestin promoter (NesCre+/- mice) in order to generate trkA $^{lox/lox}$; NesCre+/- mice. It was shown previously that Cre activity is detectable in the NesCre+/- mice from early development on throughout all the cells of the neural tube (Medina et al., 2004). This activity results in efficient Cre-mediated loxP recombination in all cells of the CNS. In the PNS, on the other hand, very little Cre activity is detectable in NesCre+/- mice. Only few neurons of the trigmeninal ganglia were found to express Cre, while in others ganglia of the PNS no Cre expression at all was found.

TrkA $^{lox/lox}$; NesCre+/- mice are born in the expected Mendelian ratio and they are viable and fertile. They display no gross anatomical or behavioral abnormalities. Immunoblot analysis demonstrated that TrkA expression is deleted efficiently from the forebrain of trkA $^{lox/lox}$; NesCre+/- mice at the end of the first postnatal week (Figure 7b). We did not

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FIGURE 6: Conditional targeting of the trkA exons 12-14

Panel A shows schematic representation of the exons 11-17 of the mouse trkA locus in the wild-type allele. The targeting construct is depicted below the wild-type allele. It contains the floxed exons 12-14 and the neo-cassette flanked by two frt sequences. Some of the restriction enzyme sites that were used for cloning steps and for further analysis by Southern blotting are indicated. The successful targeting of the wild-type allele with the targeting construct by homologous recombination in ES-cells leaded to the generation of the recombinant trkA $^{lox\ neo+}$ allele. The neo-cassette was excised in vivo with transgenic mice expressing the Flp-recombinase ubiquitously. This resulted in the generation of the recombinant trkA $^{lox\ neo-}$ allele.

Panel B shows Southern blot analysis of successful recombination in the trkA $^{lox\ neo+}$ allele. The position of the two probes at the 5' and 3' site of the targeting construct is indicated in Panel A. Southern blot analysis were done on genomic DNA digested with the restriction enzyme BamHI.

Panel C shows the analysis by PCR of trkA $^{lox\ neo+}$ and trkA $^{lox\ neo-}$ alleles. The position of the primers used for PCR analysis is indicated in Panel A. The PCR reaction shown on the right side with the Primers LF and SA allows to differentiate between wild-type mice, and mice heterozygous or homozygous for the trkA $^{lox\ neo-}$ allele and was used for routine genotyping of the floxed trkA mice.

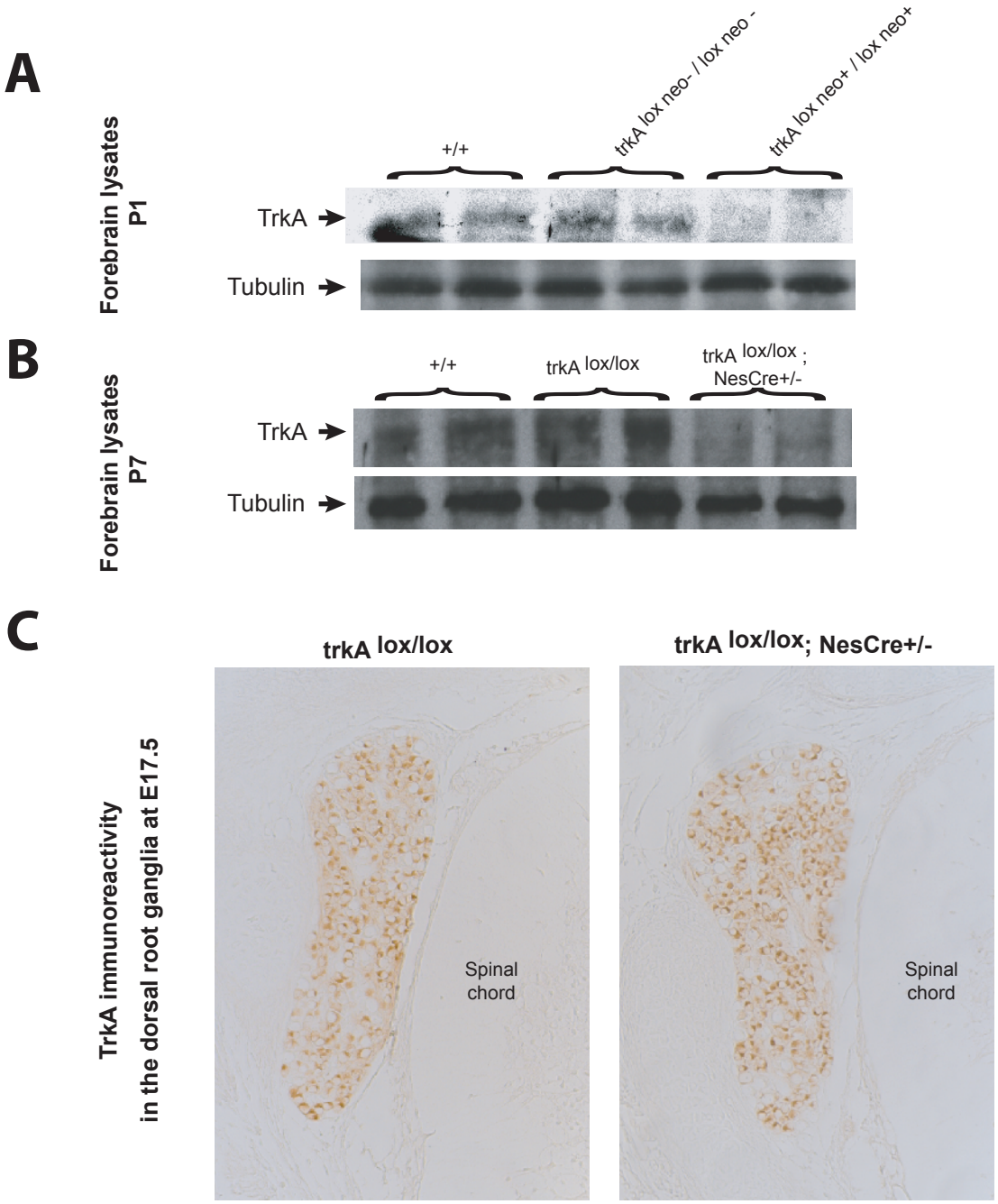

FIGURE 7: Analysis of TrkA deletion in homozygous trkA $^{lox\ neo+}$ and trkA $^{lox\ neo-}$ mice

Panel A: Forebrains of new-born (P1) wild-type, trkA $^{lox\ neo-}$ / lox neo- and trkA $^{lox\ neo+}$ / lox neo+ mice were excised and lysed in protein lysis buffer. 50 μg of each protein lysate were analyzed for TrkA expression by immunoblotting. While in trkA $^{lox\ neo+}$/ lox neo+ mice virtual no TrkA was detectable in the forebrain lysates, TrkA expression levels in trkA $^{lox\ neo}$ / lox neo- mice were undistinguishable from those in wild-type mice. Immunoblotting against tubulin protein demonstrates that in each lane similar amount of total protein lysate was loaded.

Panel B: Forebrains of one week-old wild-type, trkA $^{lox/lox}$ and trkA $^{lox/lox}$; NesCre +/- mice were lysed and analyzed for TrkA expression levels. While expression levels in wild-type and trkA $^{lox/lox}$ mice were identical, no specific TrkA expression was detected in the forebrains of trkA $^{lox/lox}$; NesCre +/- mice.

Panel C: 10-16 mm transversal cryosections of the spinal chord region of E17.5 embryos were transferred directly onto slides and stained with an antibody against TrkA. TrkA staining visualizes clearly the TrkA-positive neurons of the dorsal root ganglia. There were no differences observed between the TrkA expression in the dorsal root ganglia of trkA $^{lox/lox}$ and trkA $^{lox/lox}$; NesCre +/- mice. Thus, NestinCre-mediated deletion of TrkA expression occurs only in the cells of the CNS, but not in PNS neurons.

analyze more in details the time course of TrkA deletion through the nestin-directed Cre transgene, but previous studies performed with the NesCre mouse indicate clearly that Cre is active already from early development on (Medina et al., 2004). Thus, it is very likely that TrkA expression is disrupted in trkA $^{lox/lox}$; NesCre+/- mice already from the stage where in wild-type mice TrkA is first detectable at late embryonic development. On the other hand, as demonstrated by immunohistochemistry analysis, no reduction in TrkA expression was found in PNS neurons of the DRG in trkA $^{lox/lox}$; NesCre+/- mice (Figure 7c). Thus, in trkA $^{lox/lox}$; NesCre+/- mice, TrkA is deleted only in CNS cells where Cre is expressed and not in neurons of the PNS where no Cre activity is detectable.

The TrkA deletion in the basal forebrain of trkA $^{lox/lox}$; NesCre+/- mice was analyzed more in details by immunohistochemistry stainings done on coronal sections of the forebrain (Figure 8). As expected, in wild-type mice expression of TrkA was found in areas of cholinergic cells in the medial septum, in the diagonal band and in the nucleus basalis. TrkA-positive neurons were found also in the striatum. There was no difference between stainings in wild-type mice and in control trkA $^{lox/lox}$ mice (data not shown). On the other hand, expression of TrkA in trkA $^{lox/lox}$; NesCre+/- mice was virtually absent in all the different areas of the basal forebrain. The morphology of the anatomical structures in the basal forebrain in trkA $^{lox/lox}$; NesCre+/- was nevertheless grossly unaffected.

2 TRKA DISRUPTION RESULTS IN A CHOLINERGIC PHENOTYPE IN THE BASAL FOREBRAIN

2.1 TrkA disruption reduces specifically the number of cholinergic neurons in distinct forebrain areas

We next wanted to assess whether the deletion of TrkA results in a modification of the cholinergic phenotype in the basal forebrain. We performed immunohistochemistry stainings for ChAT on coronal sections through the areas of the medial septum and the nucleus basalis complex and through the striatum (Figure 9). In all these areas, groups of strongly stained cell bodies were found. The areas of ChAT immunoreactive cells overlaid the areas where TrkA positive neurons were found. ChAT immunoreactive cells in the striatum were in general slightly smaller than the stained cells of the other forebrain

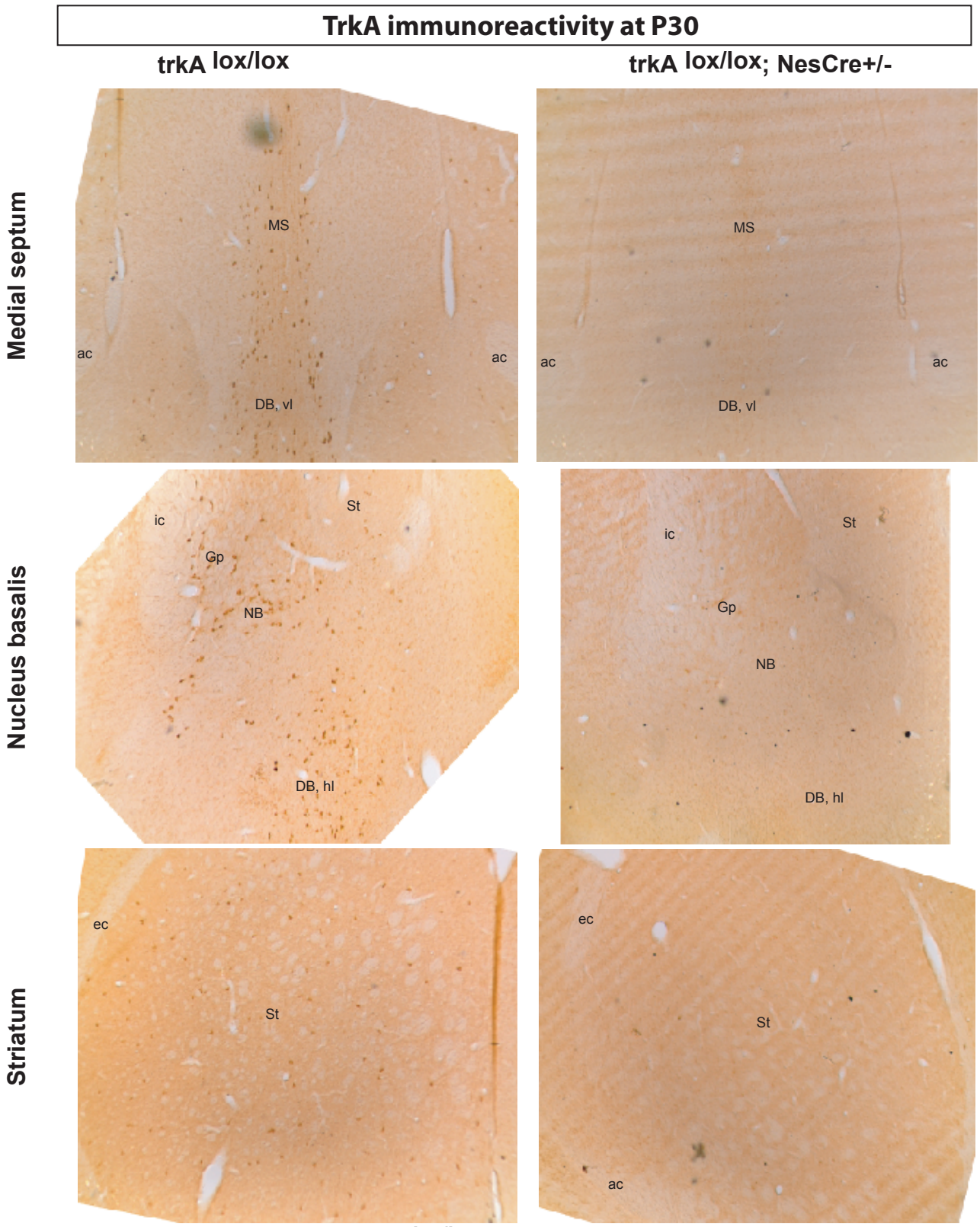

FIGURE 8: TrkA deletion in the forebrain of trkA $^{lox/lox}$; NesCre+/- mice

30μm serial coronal cryosections from the forebrain of trkA $^{lox/lox}$; NesCre+/- mice and trkA $^{lox/lox}$ mice at the age of one month were prepared and kept as floating sections in Tris-Azide buffer. Sections of the areas of the medial septum, nucleus basalis and of the striatum were stained with a polyclonal antibody against TrkA. The sections were transferred on slides and analyzed by microscopy. Representative pictures of the different areas in mutant and control mice are shown. While strongly stained neurons were found in all the indicated areas in trkA $^{lox/lox}$ mice, virtually no stained cells were present in the trkA $^{lox/lox}$; NesCre+/- mice. Some of the most relevant anatomical structures are indicated.

MS - Medial septum; DB,vl - Diagonal band, vertical limb; DB,hl - Diagonal band, horizontal limb; NB - Nucleus basalis; St - Striatum; Gp - Globus pallidum; ac - anterior commissure; ic - internal capsule; ec - external capsule

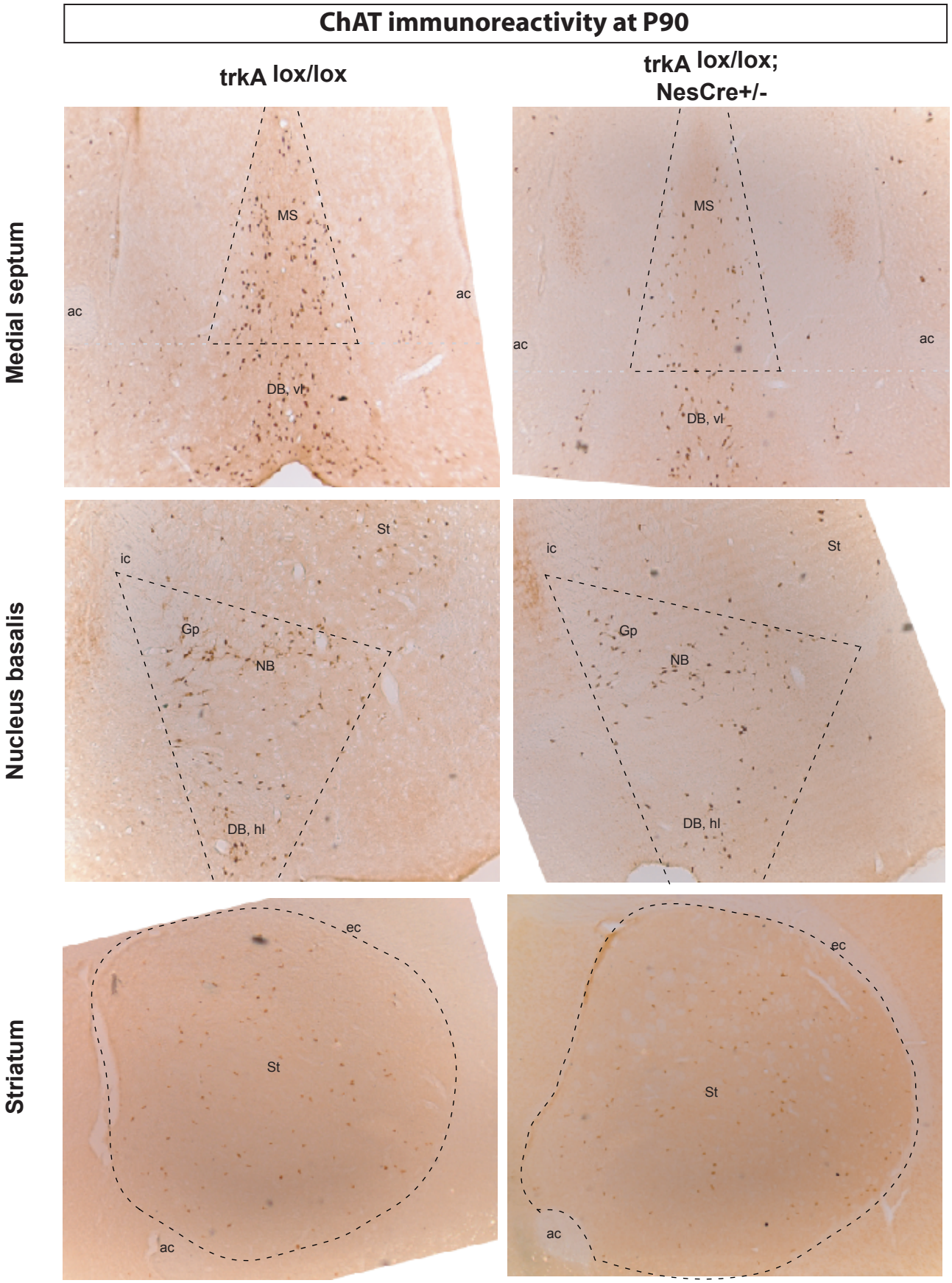

FIGURE 9: TrkA deletion reduces number of ChAT-positive neurons in the forebrain

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MS - Medial septum; DB,vl - Diagonal band, vertical limb; DB,hl - Diagonal band, horizontal limb; NB - Nucleus basalis; St - Striatum; Gp - Globus pallidum; ac - anterior commissure; ic - internal capsule; ec - external capsule

areas. In trkA $^{lox/lox}$; NesCre+/- mice, the number of ChAT-positive cells appears reduced in many sections through the medial septum and the nucleus basalis. ChAT-immunoreactive neurons in the trkA $^{lox/lox}$; NesCre+/- mice seem often hypotrophic and less strongly stained compared to the neurons in control mice.

Next, ChAT-positive cell bodies were quantified as described in the "Material and methods" part. We first analyzed the neurons expressing the cholinergic marker ChAT in the medial septum. At the age of three months (P90), in trkA $^{lox/lox}$; NesCre+/- the number of ChAT-positive cell bodies was significantly reduced by 39.5% ± 4.1% compared to the control trkA $^{lox/lox}$ mice (Figure 10a). However, no significant difference could be found in the number of ChAT-positive cell bodies of the medial septum between wild-type mice, Nestin-Cre transgenic mice and trkA $^{lox/lox}$ mice Thus, neither the expression of the Cre transgene or the lox mutation in the trkA alleles had any effect on ChAT expressing cell numbers. Taken together, these results suggest that deletion of TrkA is reduces specifically the number ChAT-positive neurons in the medial septum.

The counts of the ChAT+ positive neurons indicated strongly that TrkA deletion reduces the number of cholinergic neurons in the medial septum of trkA $^{lox/lox}$; NesCre+/- mice. However, by this method it is not possible to assess whether the reduction of ChAT-positive cells is due to a reduction of cholinergic cells or rather to a downregulation of the expression of the cholinergic marker ChAT. To address this question, we performed immunostainings on the medial septum of P90 animals for another marker of cholinergic neurons, the neurotrophin receptor p75 (Figure 10b). Counts of the p75-positive neurons in the wild-type medial septum at P90 showed that the number of the p75-positive cells is slighter smaller than the number of ChAT-positive neurons. This indicates that only about

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FIGURE 9: TrkA deletion reduces number of ChAT-positive neurons in the forebrain
30µm serial coronal cryosections from the forebrain of trkA $^{lox/lox}$; NesCre+/- mice and trkA $^{lox/lox}$ mice at the age of 3 months were prepared and kept as floating sections in Tris-Azide buffer. Sections of the areas of the medial septum, nucleus basalis and of the striatum were stained with a polyclonal antibody against ChAT. The stained sections were transferred on slides and analyzed by light microscopy. Representative pictures of the different areas in mutant and control mice are shown. The dashed lines indicate the areas that were considered for cell counting of the different structures as explained in "Material and Methods". Some of the most relevant anatomical structures are indicated.

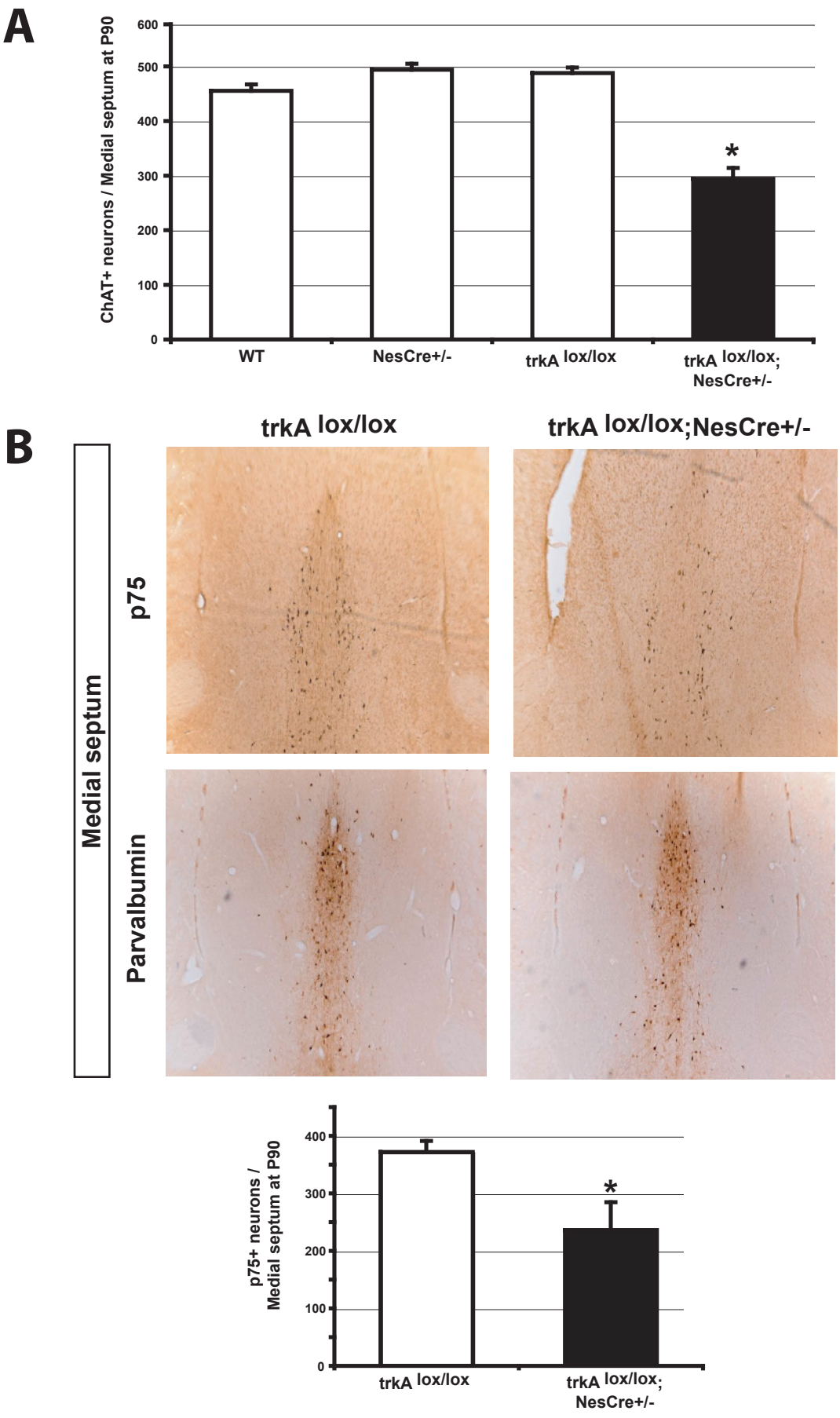

FIGURE 10: TrkA deletion reduces specifically the number of cholinergic neurons

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75% of the ChAT expressing neurons express also p75 and differs from the results of previous studies, which demonstrated that virtually all ChAT positive cells in the medial septum and the nucleus basalis are also p75 positive (Gibbs and Pfaff, 1994; Sobreviela et al., 1994). Alternatively, the difference in the numbers of ChAT- and p75-positive cells in our analysis could be explained also by different sensitivities of the antibodies used for the two proteins. The number of p75-positive was significantly reduced by 33.6% ± 13.1% in trkA $^{lox/lox}$; NesCre+/- compared to control mice. The reduction of p75-positive neurons in the medial septum is very similar to the reduction observed in ChAT-positive neurons. This suggests strongly that TrkA deletion reduces the number of cholinergic cell bodies and not only the expression of cholinergic markers. On the other hand, we performed also immunostainings for parvalbumin, a well-characterized marker of GABAergic neurons (Freund and Antal, 1988) (Figure 10b). GABAergic neurons constitute an important neuronal subpopulation of the medial septum that is distinct from the cholinergic neurons and that do not express TrkA. No difference was found between the expression of parvalbumin in mutant and control mice, indicating that TrkA deletion affects specifically cholinergic cells and not other neuronal subpopulations.

Next, we analyzed whether the effect of TrkA on cholinergic neurons varies during postnatal development. Therefore, we quantified the number of ChAT-positive neurons in the medial septum of control trkA $^{lox/lox}$ and mutant trkA $^{lox/lox}$; NesCre+/- mice at 4 different time points from 2 weeks (P15) until 9 months (P270) after birth (Figure 11a).

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FIGURE 10: TrkA deletion reduces specifically the number of cholinergic neurons

Panel A: Serial cryosections of the forebrain of wild-type, NesCre+/-, trkA $^{lox/lox}$ and trkA $^{lox/lox}$; NesCre +/- mice (at the age of 3 months) were stained with an antibody against ChAT. The ChAT-positive neurons of the medial septum were quantified following the criteria described in "Material and Methods". No significant differences were found between the numbers of medial septum ChAT-positive neurons in all three control mice. The number of ChAT-positive cells in the medial septum of trkA $^{lox/lox}$; NesCre +/- mice were significantly reduced compared to the control mice.

Panel B: Cryosections through the area of the medial septum of trkA $^{lox/lox}$ and trkA $^{lox/lox}$; NesCre +/- mice were stained with polyconal antibodies against the neurotrophin receptor p75 and a marker for GABAergic neurons parvalbumin. Representative pictures of the stainings of the medial septum area are shown. p75-positive cells were clearly reduced in trkA $^{lox/lox}$; NesCre +/- mice compared to trkA $^{lox/lox}$ mice while no differences were found in parvalbumin-positive neurons. The p75-positive neurons in the medial septum were quantified.

"*" Indicates the result of the Student's t-test $p<0.05$

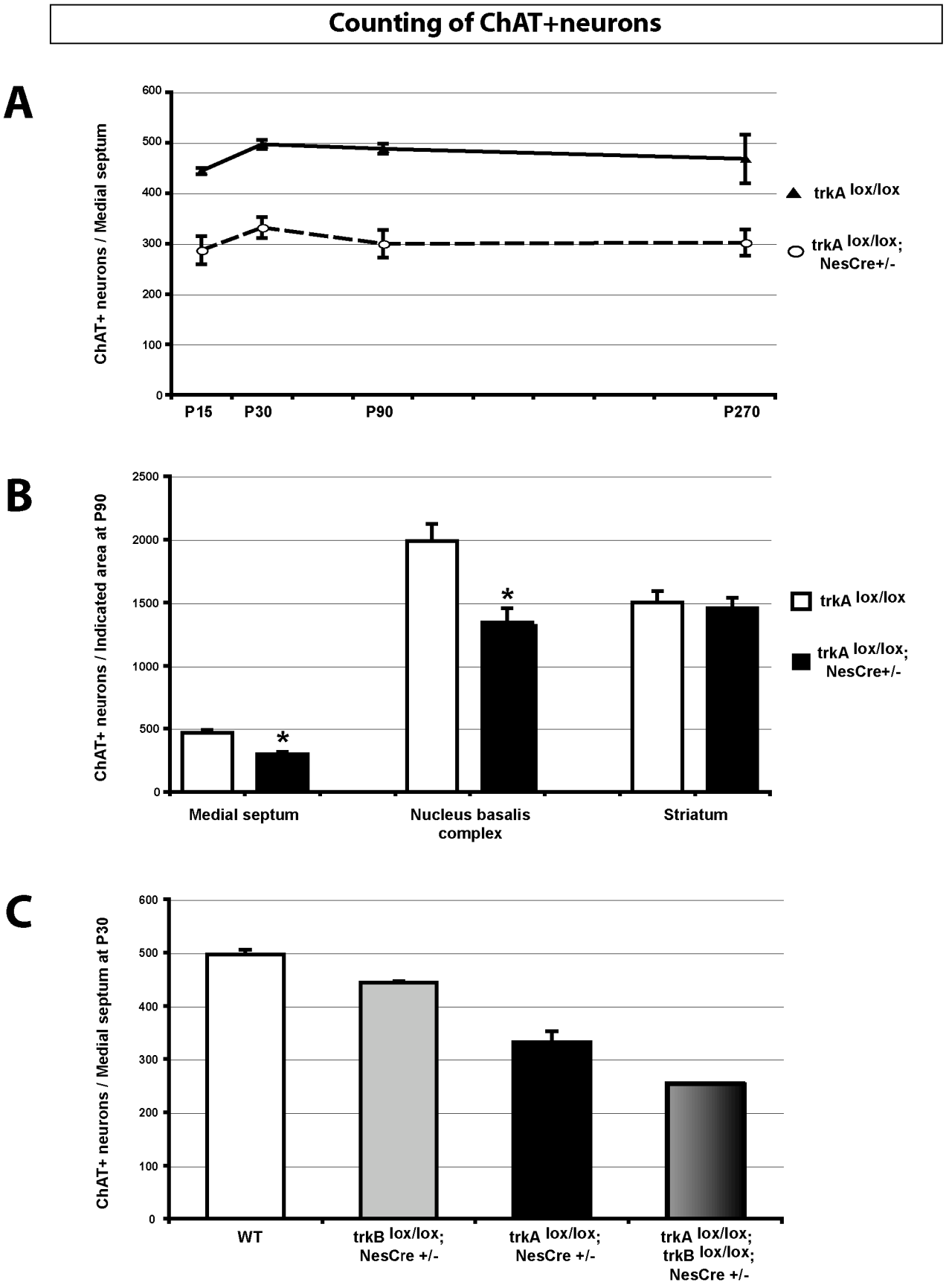

FIGURE 11: Analysis of the cholinergic phenotype in the forebrain of TrkA-deficient mice
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In control mice, the number of cholinergic cell bodies in the medial septum at the earliest time point analyzed (P15) was already very similar to cell numbers in adult mice, indicating that the proliferation of the cholinergic neurons is nearly completed at the end of the second postnatal week. From P15 to P30 there is only a slight increase in the cell number; after this time point it remains relatively stable until P270. At all analyzed time points the differences between control and mutant were highly significant. At P15, the reduction of cholinergic neurons in the medial septum of trkA $^{lox/lox}$; NesCre+/- mice was 35.4% ± 6.3% in relation to control mice. The maximum of reduction (39.5% ± 4.1%) was seen at P90. The reduction of medial septum ChAT-positive neurons in trkA $^{lox/lox}$; NesCre+/- did not increase during aging.

We next assessed whether the reduction of cholinergic neurons in trkA $^{lox/lox}$; NesCre+/- was specific to the medial septum or was common to all cholinergic cell groups in the forebrain. We quantified the ChAT-positive cell bodies in P90 animals in the area of the nucleus basalis and the striatum following the criteria as described in the "Material and Methods" part (Figure 11b). In the nucleus basalis area, ChAT-positive neurons in trkA $^{lox/lox}$; NesCre+/- mice were significantly reduced by 32.4% ± 9.8% compared to control mice to an extent very similar to those observed in the medial septum. On the other hand, no difference between mutant and control mice was observed in the striatum. In this study, the neurons of the diagonal band were not quantified particularly. However,

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FIGURE 11: Analysis of the cholinergic phenotype in the forebrain of TrkA-deficient mice

Panel A: Cryosections of the forebrains of trkA $^{lox/lox}$ and trkA $^{lox/lox}$; NesCre +/- mice of different ages were stained with the antibody against ChAT. ChAT-positive neurons in the area of the medial septum were quantified. The differences between the number of ChAT-positive neurons in the medial septum of trkA $^{lox/lox}$ and trkA $^{lox/lox}$; NesCre +/- mice were significant at all time points analyzed.

Panel B: Forebrain cryosections of trkA $^{lox/lox}$ and trkA $^{lox/lox}$; NesCre +/- mice (at P90) of the areas of the medial septum, nucleus basalis complex and of the striatum were stained with the ChAT-antibody. ChAT-positive neurons in the different areas were quantified following the counting criteria described in "Material and Methods". ChAT-positive neurons were significantly reduced in the medial septum and the nucleus basalis complex in trkA $^{lox/lox}$; NesCre +/- mice compared to trkA $^{lox/lox}$ mice. No differences were found in the striatum.

Panel C: Mice deficient for TrkA were crossed with mice with brain-specific deletion of the neurotrophin receptor TrkB (trkB $^{lox/lox}$; NesCre +/- mice) and brain-specific double-knockout TrkA/TrkB mice (trkA $^{lox/lox}$; trkB $^{lox/lox}$; NesCre +/- mice) were generated. Cryosections of the forebrain of the brain-specific single- and double knockout mice (at the age of one month) were immunostained with ChAT-antibodies. ChAT-positive neurons in the medial septum were quantified.

"*" Indicates the result of the Student's t-test $p<0.05$

the caudal part of the horizontal limb of the diagonal band was included into the counts of the nucleus basalis complex as described in "Material and Methods" and the vertical limb of the diagonal band exhibited by qualitative visual inspection a similar reduction of cholinergic neurons compared to those seen in the medial septum (see also image in Figure 9). Therefore, it is likely that the ChAT-positive neurons in the diagonal band of the mutant mice were reduced to similar extent to the reduction observed in the medial septum and the nucleus basalis. Thus, the effect of the TrkA deletion on the cholinergic neurons in the forebrain appears to be specific to the cholinergic neurons of the groups Ch1-4, while the number of local striatal interneurons remains unvaried (at least at P90).

2.2 TrkB disruption has only a minor effect on cholinergic cell numbers in the medial septum

Cholinergic neurons of the medial septum and of the striatum are known to express the neurotrophin receptor TrkB (Molnar et al., 1998; Richardson et al., 2000; Yan et al., 1997). We wanted to assess whether TrkB signaling is essential for survival of cholinergic neurons in the medial septum and whether this signaling is redundant with the TrkA signaling in the medial septum. We analyzed therefore the cholinergic neurons in the medial septum of animals deficient for TrkB and for both TrkB and TrkA in the CNS. Mice with Cre mediated brain-specific deletion of TrkB (trkB $^{lox/lox}$; NesCre+/- mice) have been shown to exhibit severe defects in cell migration, differentiation, and myelination (Medina et al., 2004). However, until the age of 1 month these mice display no gross anatomical abnormalities. Therefore, we choose to analyze animals at the age of one month. The number of analyzed trkA/trkB deficient mice analyzed up to now is not sufficiently high (for trkB $^{lox/lox}$; NesCre+/- mice n=2 and for trkA $^{lox/lox}$; trkB $^{lox/lox}$; NesCre+/- n=1) for obtaining statistically significant final results. However, the trend of the results allow to get a first insight into the role of TrkB signaling in cholinergic neurons of the forebrain. At P30, mice with brain-specific TrkB deletion show only a slight reduction of about 10.66% ± 0.4% of medial septum ChAT-positive neurons compared to control mice (Figure 11c). We next quantified the cholinergic neurons in the medial septum of mice deficient for TrkB and TrkA in the brain (trkA $^{lox/lox}$; trkB $^{lox/lox}$; NesCre+/- mice). We found an important reduction of

48.7% in mice double deficient for TrkA and TrkB compared to control mice. However, the reduction in trkA $^{lox/lox}$; trkB $^{lox/lox}$; NesCre+/- mice was only about 23.2% compared to trkA $^{lox/lox}$; NesCre+/- mice. Thus, at P30, the TrkB deletion in the cholinergic neurons of the medial septum appears to have only a rather small additive effect compared to the reduction observed after TrkA deletion. In general, the role of TrkB in the cholinergic neurons seems to have minor importance compared to the role of TrkA.

2.3 TrkA deletion decreases the expression of the cholinergic differentiation marker ChAT

Next, we aimed to examine whether TrkA deletion affects also the quantitative expression of the cholinergic marker ChAT. ChAT protein is found not only in the cell bodies of the cholinergic neurons, but also in their axonal and dendritic elongations. Therefore, ChAT protein analysis gives an indication about the state of cholinergic innervation in target areas of cholinergic neurons. First, we determined the ChAT expression levels in the forebrain of wild-type mice during postnatal development. Therefore, total forebrain lysates of wild-type mice at different time points from P1 to P270 were analyzed by immunoblot analysis with a specific antibody against ChAT (Figure 12a). While no ChAT expression was found in newborn mice, ChAT expression reached of about 36.2% ± 2% of its maximum levels at P15 and 78.5% ± 11.2% at P30. ChAT expression was maximal at P90 and remained then relatively stable at P180 and at P270. This time course of ChAT protein expression during postnatal development is similar to those observed previously in other studies (Li et al., 1995). Next, we analyzed the expression of ChAT at 4 different time points in trkA $^{lox/lox}$; NesCre+/- mice compared to wild-type mice (Figure 12b). Already, at P15 ChAT expression trkA $^{lox/lox}$; NesCre+/- was importantly reduced by 29.4% ± 8.3% compared to trkA $^{lox/lox}$ mice. This reduction was further increased during postnatal development and reached its maximum at P180, where ChAT was reduced by 48.3% ± 5.5% in mutant mice compared to wild-type mice. The increase in the reduction between P15 and P180/P270 was significant ($p=0.047$ and $p=0.042$, respectively) indicating that the loss of the cholinergic marker ChAT in TrkA deficient mice is a progressive process during the first months of postnatal development.

ChAT immunoblotting

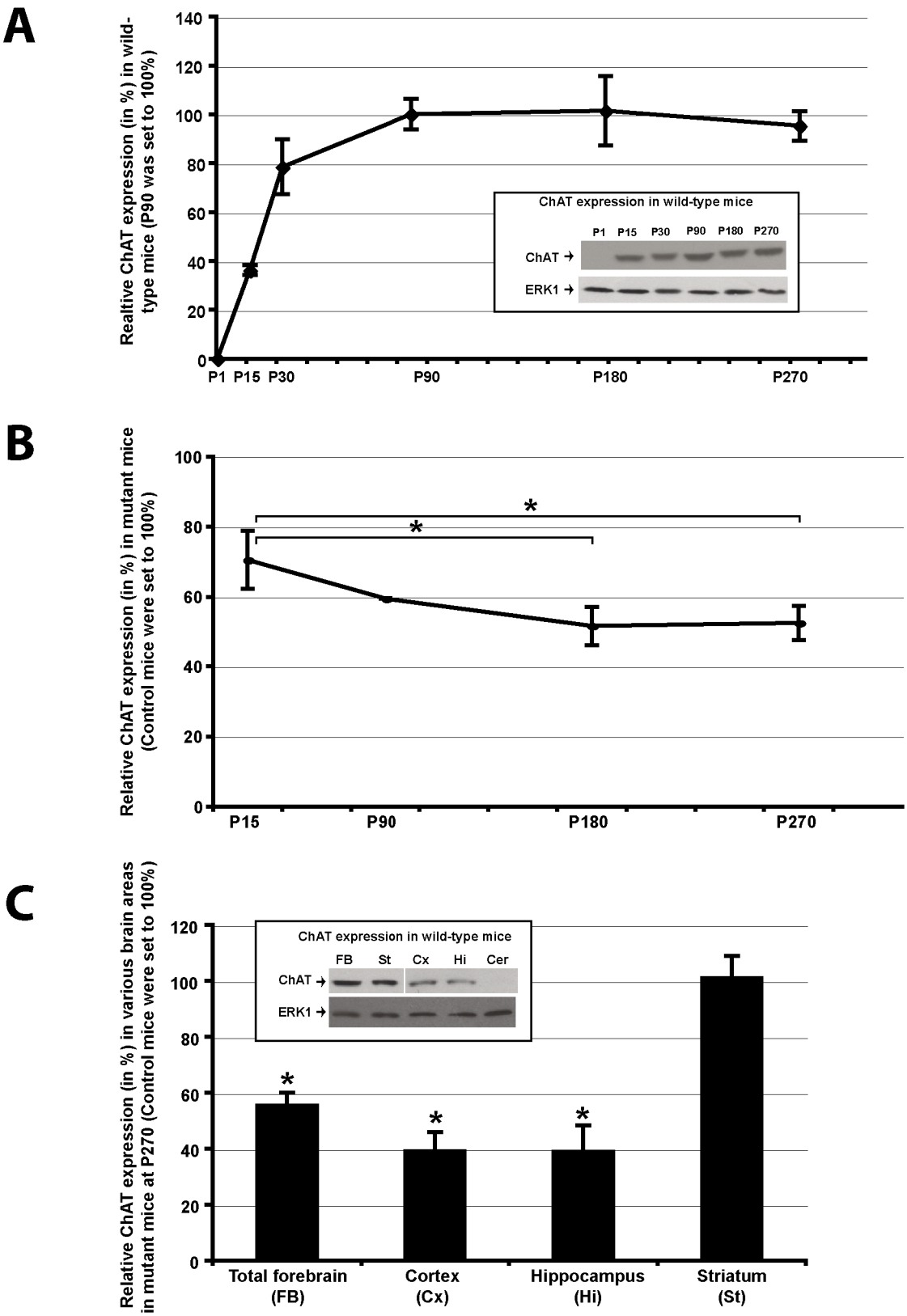

FIGURE 12: TrkA deletion decreases expression of the cholinergic differentiation marker ChAT
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We next analyzed the expression of ChAT protein in different areas of the brain of mice at the age of 9 months (Figure 12c). As expected, ChAT expression in the striatum is relatively high compared to the expression in the hippocampus and in the cortex (see insert in Figure 12c) and virtually no ChAT expression was found in the cerebellum. Next, we analyzed the reduction of ChAT expression in the different brain areas in TrkA-deficient mice versus control mice. We found an important reduction of ChAT expression in the cortex and in the hippocampus. The extent of the reduction was very similar in both areas: 60.9% ± 7.0% in the cortex and 61.3% ± 8.0% in the hippocampus. Remarkably, no difference in ChAT expression was found in the striatum of mutant mice compared to control mice.

Taken together, these data confirmed our results from the cell counting studies, indicating that TrkA deletion importantly affects the cholinergic phenotype of neurons of the cholinergic cell groups Ch1-Ch4, without modulating the ChAT expression of the striatal cholinergic neurons. In addition, they suggested that also the cholinergic target innervation in the hippocampus and in the cortex is strongly reduced in trkA $^{lox/lox}$; NesCre+/- mice.

2.4 Cholinergic innervation of the cortex and the hippocampus is severely reduced in trkA $^{lox/lox}$; NesCre+/- mice

In order to analyze more directly the cholinergic innervation state in trkA $^{lox/lox}$;

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FIGURE 12: TrkA deletion decreases expression of the cholinergic differentiation marker ChAT

Panel A: The forebrains of wild-type mice of different ages were excised and lysed in protein lysis buffer. 50µg of each protein lysate were analyzed by immunoblotting with ChAT-antibodies. The detected bands were quantified by densitometric analysis and normalized to the expression of the ERK1 protein. The ChAT expression at P30 was set to 100%. In the insert, a representative immunoblot is shown.

Panel B: The forebrains of trkA $^{lox/lox}$ and trkA $^{lox/lox}$; NesCre +/- mice of different ages were analysed for ChAT expression by immunoblot analysis. ChAT expression was quantified and normalized to ERK1 expression. At each time point, ChAT expression in control trkA $^{lox/lox}$ mice was set to 100% and relative ChAT expression in trkA $^{lox/lox}$; NesCre +/- mice is indicated. The reductions of ChAT expression observed in trkA $^{lox/lox}$; NesCre +/- mice compared to control mice were significant at all analysed time points. In addition, the reduction of ChAT expression in trkA $^{lox/lox}$; NesCre +/- mice at P180 and at P270 is significantly increased compared to the reduction observed at P15.

Panel C: Distinct forebrain areas were excised in trkA $^{lox/lox}$ and trkA $^{lox/lox}$; NesCre +/- mice at 9 months and analyzed for ChAT expression. ChAT expression was quantified and normalized for ERK1 expression. ChAT expression in the distinct areas in the control trkA $^{lox/lox}$ mice was set to 100% and relative ChAT expression in the trkA $^{lox/lox}$; NesCre +/- mice is indicated. ChAT expression in the cortex and the hippocampus was significantly reduced in trkA $^{lox/lox}$; NesCre +/- mice compared to control mice, while no reduction was observed in the striatum. In the insert, ChAT expression in wild-type mice in total forebrain (FB), striatum (St), cortex (Cx), hippocampus (Hi) and the cerebellum (Cer) is shown.

"*" Indicates the result of the Student's t-test $p<0.05$

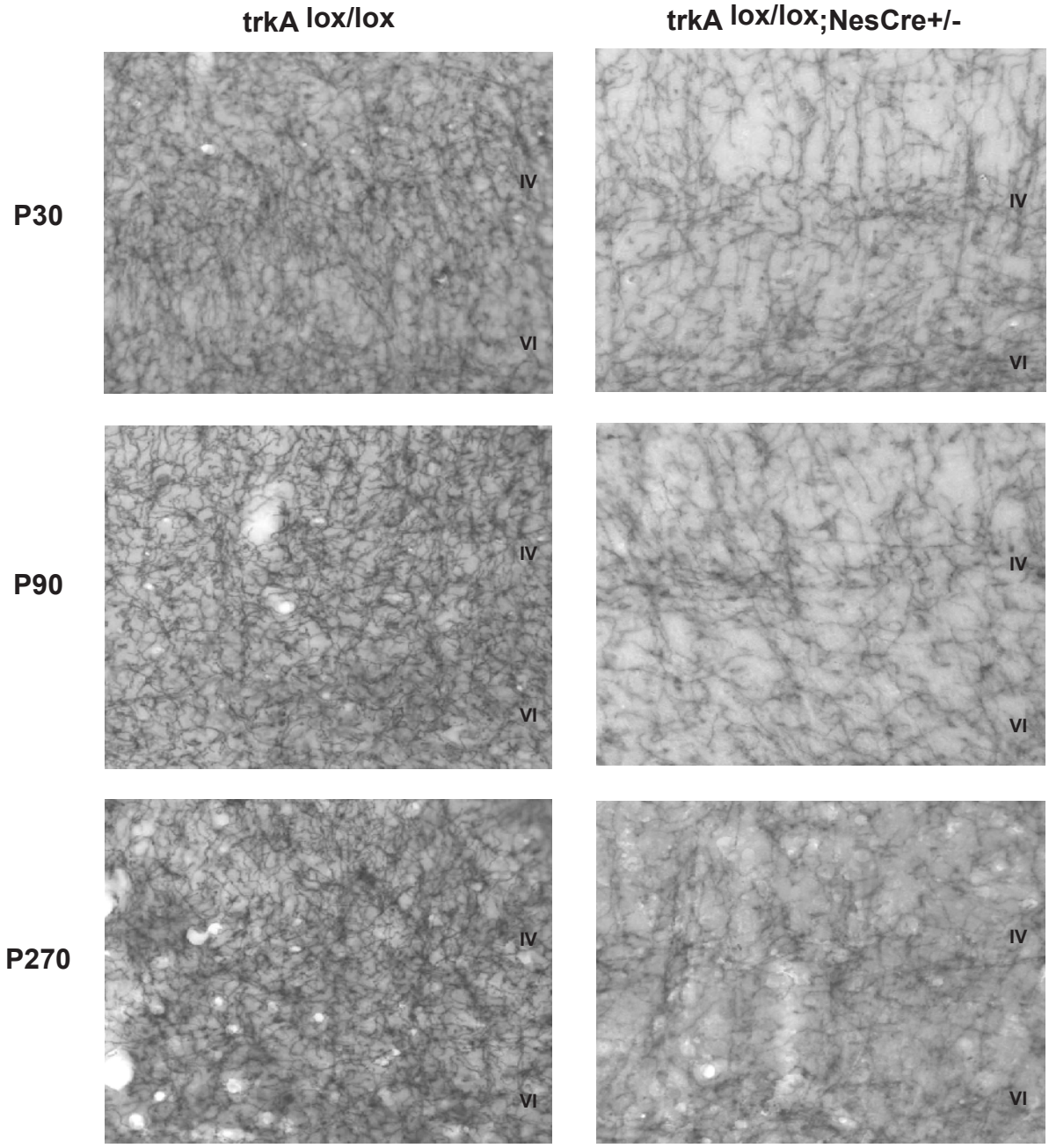

FIGURE 13: Cholinergic innervation of the somatosensory cortex is impaired in trkA $^{lox/lox}$; NesCre+/- mice

30µm coronal cryosections from the forebrain of trkA $^{lox/lox}$; NesCre +/- mice and trkA $^{lox/lox}$ mice of different ages were prepared and kept as floating sections. Sections were stained for the activity of the AChE enzyme as described in “Material and Methods”. AChE staining visualizes elongations of cholinergic neurons. Sections were transferred onto slides and analyzed by light microscopy. Representative images of the somatosensory area of the cortex at different time points are shown.

Approximate localization of the cortical layers IV and VI is indicated

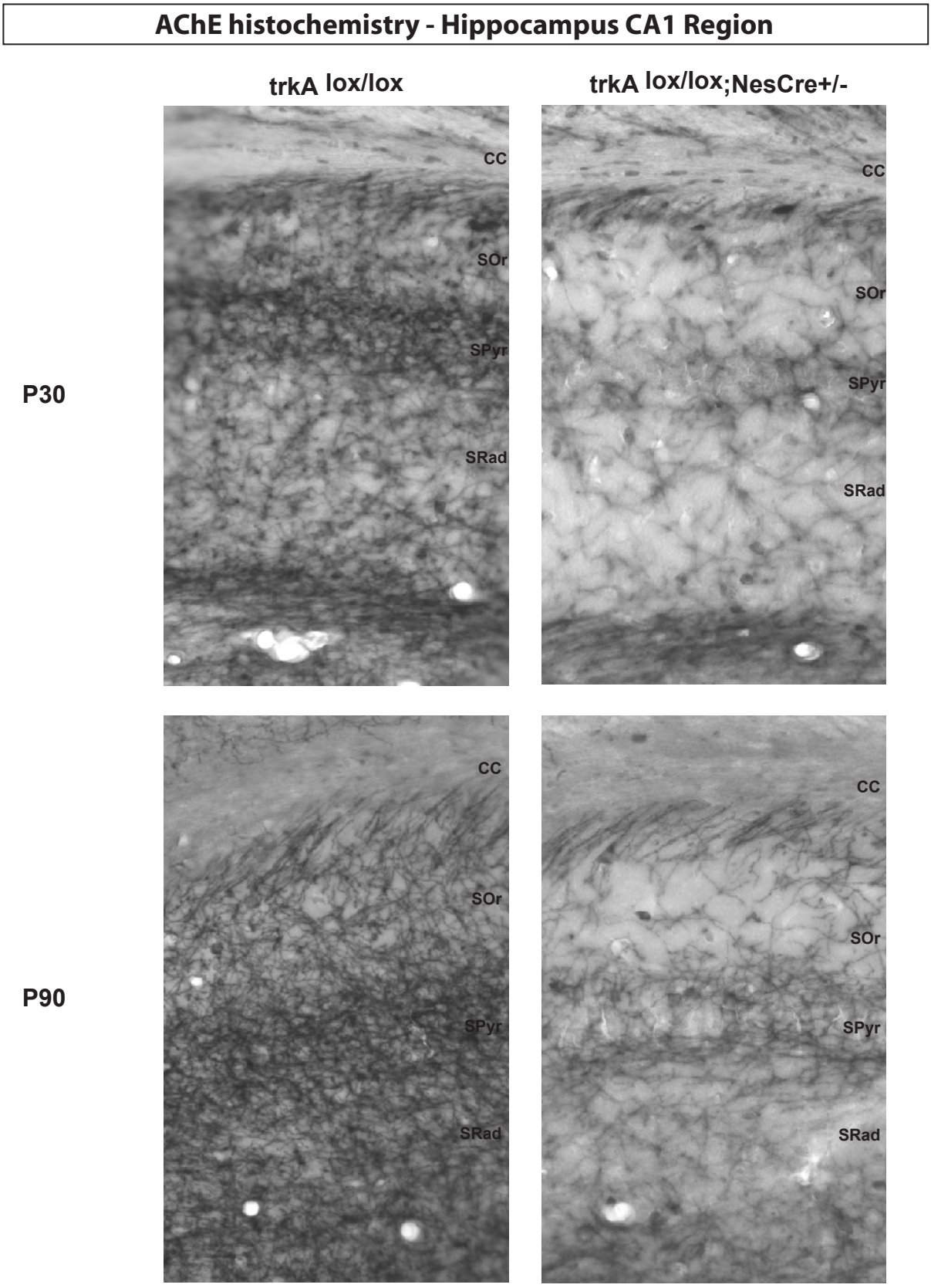

FIGURE 14: Cholinergic innervation of the hippocampus is impaired in trkA $^{lox/lox}$; NesCre+/- mice
Sections were prepared and stained as described in the legend of Figure 13. Representative images of the CA1 region of the hippocampus of mutant trkA $^{lox/lox}$; NesCre +/- mice and control trkA $^{lox/lox}$ mice at P30 and P90 are shown.

cc - corpus callosum; SOr - Stratum oriens; SPyr - Stratum pyramidale; SRad - Stratum radiatum

NesCre+/- mice, acetylcholinesterase (AChE) staining was employed. This staining visualizes the enzymatic activity of AChE. We used coronal sections of mutant and control mice of different ages and stained them for AChE activity. In particular the areas of the somatosensory cortex and the CA1 region of the hippocampus were analyzed. In the wild-type cortex at P30, intense AChE neuronal processes can be observed through all different cortical layers (Figure 13). The major accumulations of cholinergic fibers appear to be localized in the layer IV and VI. A similar expression pattern of AChE in the cortex of wild-type mouse was reported previously (Kitt et al., 1994). The complexity and intensity of AChE fibers further increase during postnatal development. This time course of maturation of the cortical cholinergic innervation correlates with our analysis of ChAT expression levels, where maximum of ChAT expression levels were reached after P30. At P90 and P270, the whole cortex is strongly innervated by AChE positive neuronal processes and laminar distribution is no more recognizable. In trkA $^{lox/lox}$; NesCre+/- cortical cholinergic innervation is markedly reduced already at P30 and this reduction appears to increase further at P90 and at P290. At P290, only a thin band of AChE positive fibers can be observed in the layer IV, while cholinergic innervation in the other cortical layers seem to be nearly absent compared to control mice.

In the CA1 region hippocampus, cholinergic innervation is already clearly detectable in all the different layers in wild-type mice at P30 (Figure 14). Major accumulation of AChE staining can be observed in the stratum pyramidale. Like in the somatosensory cortex, the intensity and complexity of the AChE positive fibers further increase significantly until P90. In trkA $^{lox/lox}$; NesCre+/- mice, cholinergic innervation seem to be delayed and only very few AChE positive neuronal processes can be observed at P30. At P90, cholinergic innervation increases slightly in particular in the region of the stratum pyramidale, but remains severely compromised compared to control mice.

3 TRKA DISRUPTION MODIFIES PROCESSING OF APP

We investigated the effect of TrkA deletion on APP processing in trkA $^{lox/lox}$; NesCre+/- mice at a age of 6 months. First, we analyzed the expression of APP in total forebrain lysates by using the 22C11 antibody Figure 15a). APP expression was significantly

increased by 28.2% ± 4.9% in trkA $^{lox/lox}$; NesCre+/- mice compared to control mice. The antibody 22C11 recognizes full-length membrane-bound APP and the different soluble APP forms. In order to differentiate between these different APP forms we analyzed specifically the expression of the full-length APP by using an APP antibody that recognizes an epitope at the carboxy-terminal end of APP. In the soluble APP fragments, the carboxy-terminal end is cleaved off. We found that the expression of the full-length membrane-bound APP does not differ significantly in TrkA-deficient mice and control mice. Thus, the increase of APP detected by the 22C11 antibody is most likely due to a specific increase of the soluble APP forms. To investigate this hypothesis more directly, we prepared total forebrain lysates that contain only Tris-buffer-soluble proteins. All proteins bound in cellular membranes or enclosed in vesicles were precipitated by ultracentrifugation and only proteins in the supernatants were analyzed (Figure 15b). By using the 22C11 antibody, we detected a 42.6% ± 14.5% increase of soluble APP proteins in mutant mice compared to control mice. As control for proper separation of the soluble proteins from the membrane bound proteins in these protein lysates, we used the APP C-ter antibody. As expected, no bands were detected by this antibody in lysates of soluble proteins at the size of full-length APP (data not shown). Next, we aimed to analyze further the forms of soluble APP fragment that are increased in the TrkA-deficient. We used the antibody Aβ-NT, which recognizes an epitope at the N-terminal end of the Aβ-region. This antibody detects the soluble APP fragments that are cleaved at the α-site and at the β'-site, while it does not detect the soluble APPβ. The expression of these APP forms was slightly increased in trkA $^{lox/lox}$; NesCre+/- mice compared to control mice. At this moment, we are not able to differentiate between the α- and the β'-form of soluble APP, but it appears that the increase of one or of both of these components is partly responsible for the increase of total soluble APP in TrkA-deficient mice.

Next, we wanted to assess whether the increase of soluble APP fragment is accompanied by an altered generation of membrane-bound carboxy-terminal fragments of APP. Therefore, we immunoprecipicated 500μg of total forebrain lysate with the antibody APP C-ter. The immunoprecipitates were immunoblotted with the same APP C-ter antibody (Figure 15c). As a positive control, we used protein lysate from mice that transgenically overexpress human APP (Moechars et al., 1999). We detected three bands in the size of

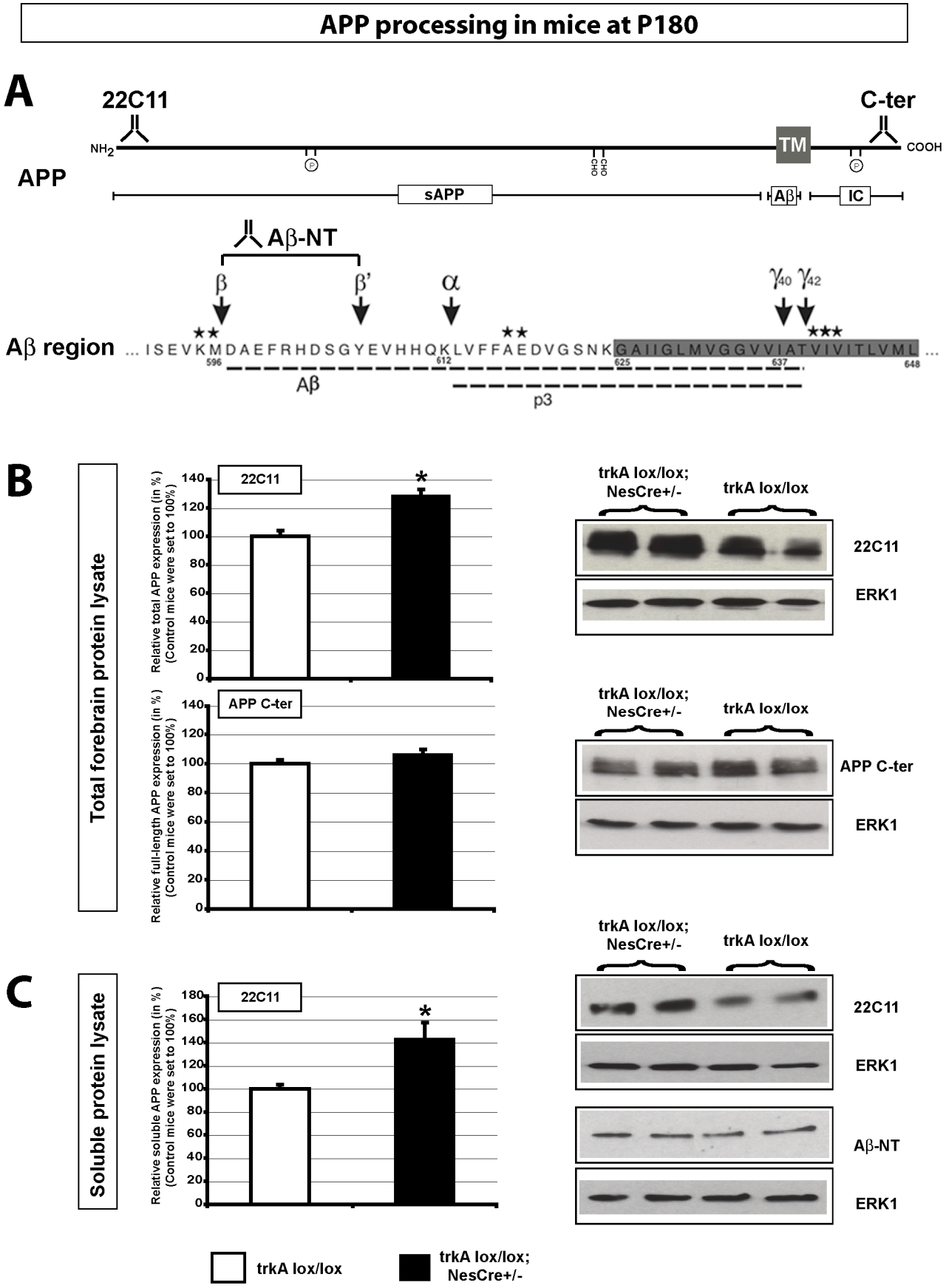

FIGURE 15: TrkA deletion modifies processing of APP
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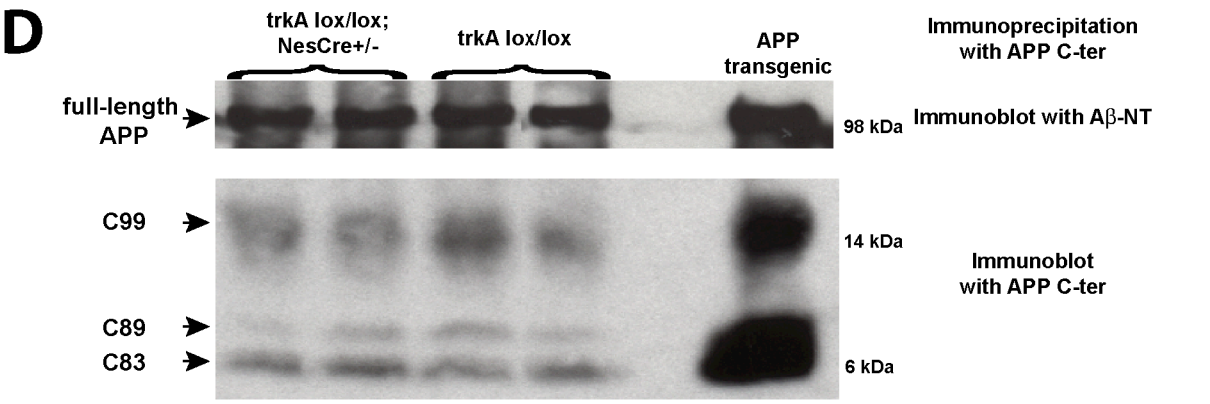

FIGURE 15d: TrkA deletion alters generation of carboxy-terminal APP fragments
The membrane-bound APP carboxy-terminal fragments are generated together with the soluble APP fragments by the α- and the β-secretases. α-cleavage results in the formation of the C83 carboxy-terminal APP fragment, while β-secretase-mediated cleavage at the β- or the β'-site produces respectively C99 and C89 fragments.
Total forebrains were lysed and 500 μg of each protein lysate were immunoprecipitated with the APP C-ter antibody. The immunoprecipitates were separated on a 4-20% gradient acrylamide gel and stained with the APP C-ter antibody. As a positive control, forebrain protein lysates from mice that overexpress human APP were used. In these mice, all APP forms are overexpressed. In trkA $^{lox/lox}$; NesCre +/- mice, expression of the α-cleavage mediated C83 appears to be slightly increased compared to control mice. In contrast, the C99 fragment that is generated by β-cleavage seems less abundant in trkA $^{lox/lox}$; NesCre +/- mice than in control mice. On the same gel, also the full-length APP was visualized by the Aβ-NT antibody. No differences in the expression of full-length APP between control and mutant mice were found.

Legend of the Figure 15a-c (previous page)

FIGURE 15: TrkA deletion modifies processing of APP
Panel A shows schematic representation of the APP protein and of the epitopes recognized by the different APP antibodies used in this study.
Panel B: Total forebrain lysates of 6-months old mice were analyzed by immunoblotting against the APP antibodies 22C11 and C-ter. The 22C11 antibody recognizes both the full-length membrane-bound APP and all the different forms of soluble APP fragments. The C-ter antibody recognizes only the full-length membrane-bound APP. APP expression was quantified and normalized against ERK1 expression. APP expression detected by the different antibodies in control trkA $^{lox/lox}$ mice was set to 100%. While total APP detected by the 22C11 antibody was significantly increased in trkA $^{lox/lox}$;NesCre+/- mice compared to control mice, no significant difference was found in the expression of full-length APP detected by the C-ter antibody. On the right side of the panel, representative immunoblots with the different APP-antibodies are shown.
Panel C: Forebrain were lysed in Tris-buffer and membrane-bound proteins were precipitated by ultracentrifugation. Only soluble proteins in the supernatant were further analyzed by immunoblotting. The 22C11 antibody detects all different forms of soluble APP fragments. The soluble APP expression was quantified and normalized against ERK1 expression. APP expression in control trkA $^{lox/lox}$ mice was set to 100%. The expression of soluble APP shows a significant increase in trkA $^{lox/lox}$;NesCre+/- mice compared to control mice. On the right side of the panel, representative immunoblots with the different APP-antibodies are shown. The Aβ-NT antibody that recognizes the soluble APP forms generated by secretases that cleave at the α- and at the β'-sites detects a slightly increased band in trkA $^{lox/lox}$;NesCre+/- mice compared to control mice.
"*" Indicates the result of the Student's test $p<0.05$

approximately 6-14 kDa that represent the three different forms of carboxy-terminal APP fragments, C99, C89, C83, which are generated by β-, β'-, and α-secretases, respectively. On the same blot, we visualized also expression of full-length APP using the antibody Aβ-NT and not the APP C-ter antibody, which would generate a stronger signal. No differences between mutant and control mice in the expression of full-length APP were observed. This confirmed our previous result and indicated that similar amount of total proteins were used for immunoprecipitation. In addition, the use of the Aβ-NT antibody allowed also to identify precisely the C99 band, because this antibody recognizes C99, but not the other carboxy-terminal fragments C89 and C83 (data not shown). As expected, all APP forms were strongly overexpressed in the transgenic APP mouse. In wild-type mice, the C89 fragment was relatively little expressed compared to the C99 fragment indicating that the β-cleavage site is the preferred cleavage site of the β-secretase in mice. In TrkA-deficient mutant mice, no statistically significant variation in the pattern of carboxy-terminal APP fragments was found. However, there was a certain tendency of increased production of C83 and decreased generation of C99 fragments in mutant mice. Thus, it appears that in TrkA-deficient mice the α-secretase pathway is preferentially activated compared to control mice, while the β-secretase pathway seems to be downregulated. The differences in the generation of carboxy-terminal fragments between control and mutant mice were much less pronounced than the difference observed in the generation of soluble APP fragments. Both kind of fragments are generated by the same proteolytic cleavage pathways, but while carboxy-terminal fragments are further processed by the γ-secretase, soluble APP fragments are more stable and may accumulate in the tissue. For this reason, differences in the generation of soluble APP fragments may be easier to appreciate than altered generation of carboxy-terminal APP fragments. Taken together, our results suggest that the observed increase of soluble APP fragments may be mainly due to increased generation of the soluble APPα form, while the soluble APPβ and β' forms most likely remain unvaried or are even downregulated.

Next, we wanted to analyze, whether the increase of soluble APP is particularly pronounced in specific areas of the forebrain. Therefore, we performed immunostainings of coronal forebrain sections of 9 months old mice with the antibody 22C11. While in

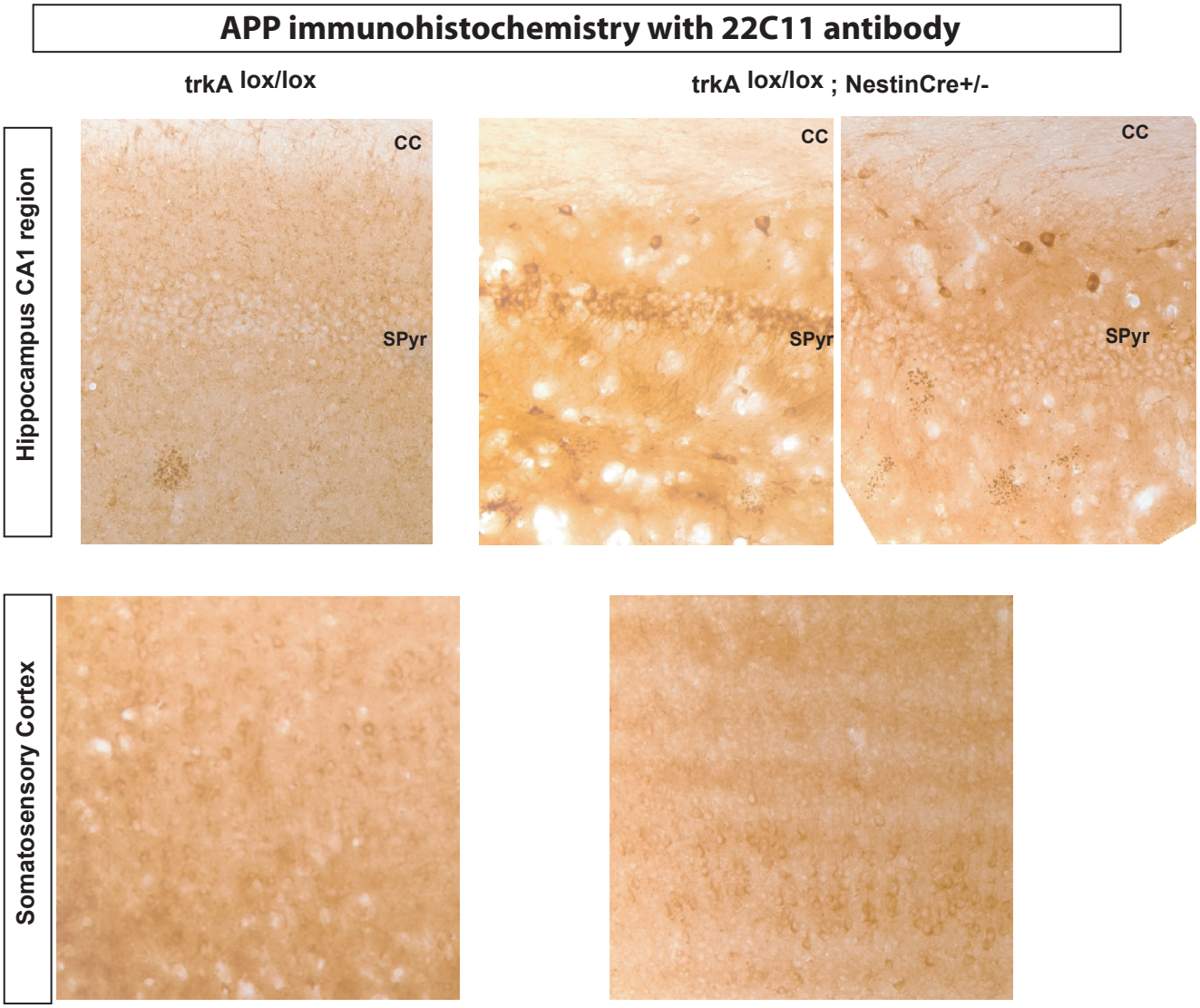

FIGURE 16: APP immunoreactivity is increased in the forebrain of trkA $^{lox/lox}$; NesCre+/- mice
30µm coronal cryosections of the forebrain of trkA $^{lox/lox}$; NesCre +/- mice and trkA $^{lox/lox}$ mice at P270 were stained with the monoclonal antibody against APP 22C11. Many strongly stained cells are found in the hippocampus of trkA $^{lox/lox}$; NesCre +/- mice. Note the stained dots in the hippocampus of mutant mice. These dots can be found also in control mice but at lesser extent than in mutant mice. In some areas of the somatosensory cortex of trkA $^{lox/lox}$; NesCre +/- mice, a slightly stronger APP-immunoreactivity than in control mice can be observed.

cc - corpus callosum; SPyr - Stratum pyramidale

most areas of the forebrain, no obvious staining differences between control and mutant mice were observed, the hippocampi of trkA $^{lox/lox}$; NesCre+/- mice displayed a pronounced increase in immunoreactivity compared to control mice. Many strongly stained cells were found in mutant mice throughout all the areas of the hippocampus. The morphology of these cells suggested that they are most likely neurons. Moreover, in some areas of the hippocampus of the mutant mice, groups of highly stained dots were observed. At the moment, we were not able to characterize more in details these dots, but it appears that they are localized extracellularly. These dots were occasionally observed also in control mice, but in much lesser quantity and staining intensity than in the mutant mice. In addition, also in some areas of the cortex, we found an increase of immunoreactivity in TrkA-deficient mice, although to a lower extent than in the hippocampus.

Next, we aimed to analyze directly by immunoblotting the expression of soluble APP proteins in the hippocampus of TrkA-deficient mice. We prepared lysates of soluble proteins from the hippocampus of mutant and control mice. By using the 22C11 antibody, we confirmed that the deletion of TrkA increases significantly the generation of soluble APP fragments by 80.1% ± 17.7%. Thus, as suggested by the immunohistochemistry analysis, the extent of the increase of soluble APP form was much more pronounced in the hippocampus than in total forebrain lysate (compare Figure 15 to Figure 17). In contrast, cell membrane-associated full-length APP remains nearly unvaried. Remarkably, only a very weak expression of the soluble APPα and β' was found in the hippocampus of control mice as detected by the Aβ-NT antibody. This is in agreement with the result of a previous study that reported only a very low level of soluble APP in wild-type mice as detected by an antibody similar to the Aβ-NT antibody (Stein et al., 2004). However, in the hippocampus of trkA $^{lox/lox}$; NesCre+/- the band detected by the Aβ-NT antibody was strongly increased. As suggested by our previous results, this increase is most likely due to an important upregulation of the soluble APPα - fragment rather than of the APPβ' - fragment.

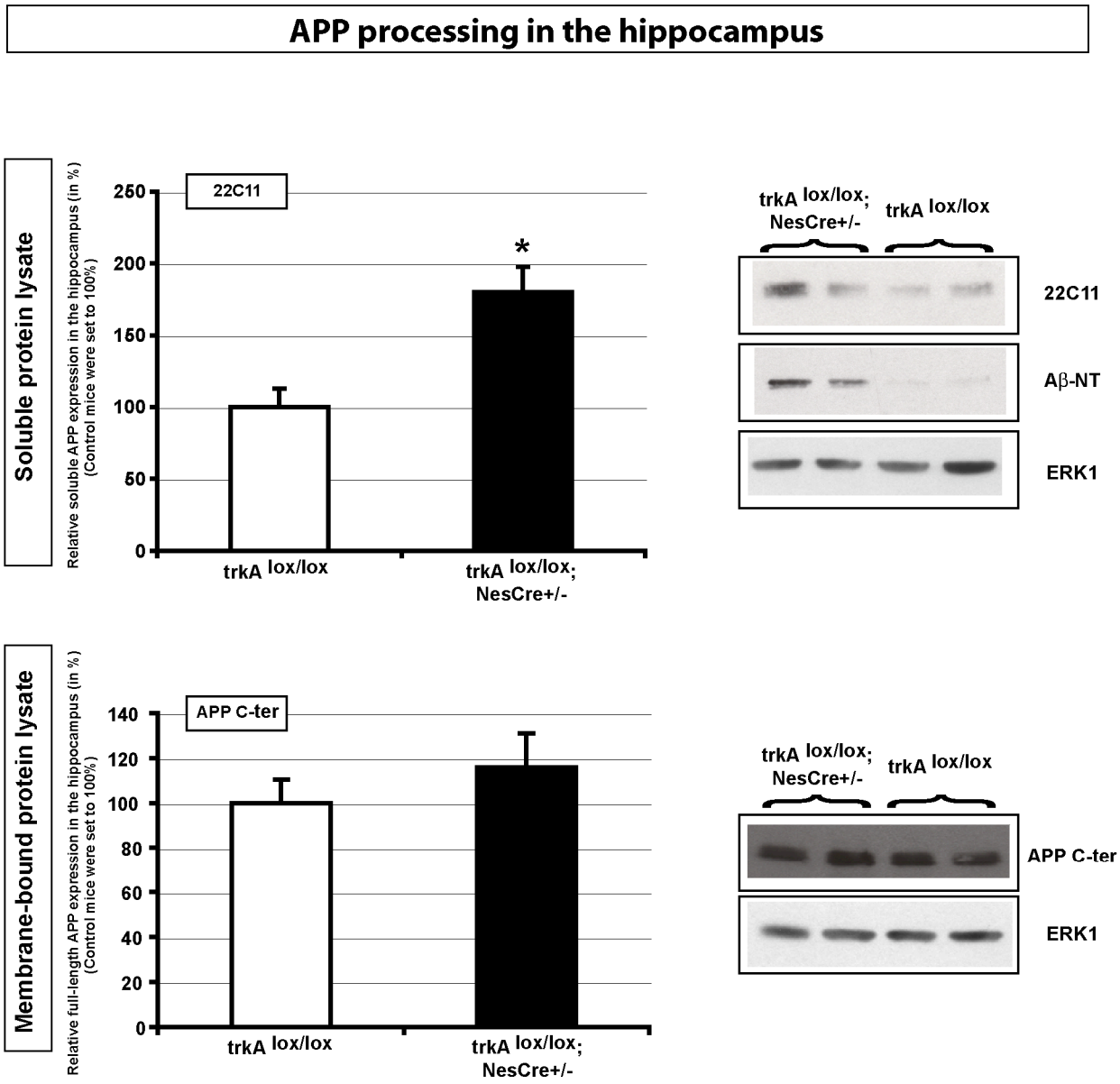

FIGURE 17: TrkA deletion increases generation of soluble APP fragments in the hippocampus
Hippocampus of 6-month old mice were excised and lysed in Tris-Buffer. Membrane-bound proteins were precipitated by ultracentrifugation. The soluble proteins in the supernatants were further analyzed by immunoblotting with the 22C11 antibody and with the Aβ-NT antibody for expression of soluble APP fragments. The precipitated proteins were lysed in detergent-containing protein lysis buffer and analyzed for expression of full-length APP by the APP antibody C-ter. Detected APP protein levels were quantified and normalized against ERK1 expression levels. APP expression in the control trkA $^{lox/lox}$ mice was set to 100%. Expression of soluble APP proteins in the hippocampus was significantly increased in trkA $^{lox/lox}$; NesCre +/- mice compared to control mice, while no differences were found in the expression of full-length APP. On the right side of the panel, representative immunoblots with the different APP-antibodies are shown.
"*" Indicates the result of student t-test $p<0.05$

4 GENERATION OF A FLOXED ngf ALLELE

We have generated a floxed ngf allele by homologous recombination in ES cells. In this floxed allele, the exon 4 of ngf is flanked by two loxP sequences (Figure 18). The ngf exon 4 encodes for the complete NGF protein and deletion of this exon results in the complete disruption of the protein expression (Crowley et al., 1994). Following the second loxP sequence, we introduced a reporter gene, egfp, which is preceded by an IRES sequence that will allow the translation of the egfp transgene. After deletion of the ngf exon 4 by Cre-mediated recombination, the egfp gene will be expressed in cells that previously expressed NGF. We derived germline targeted offspring carrying one floxed ngf allele. These ngf $^{lox\ neo+\ /\ +}$ mice were used to generate mice homozygous for the floxed allele. ngf $^{lox\ neo+\ /\ lox\ neo+}$ mice are viable, fertile and show no gross anatomical defects. In order to delete NGF expression specifically from the brain, we aimed to generate ngf $^{lox\ neo+\ /\ lox\ neo+}$; NesCre+/- mice. Therefore we crossed ngf $^{lox\ neo+/\ +}$ mice with NesCre+/- mice. ngf $^{lox\ neo+\ /\ +}$; NesCre +/- mice from the progeny of this crossing were analyzed for proper recombination of the floxed sequence by Southern blotting (Figure 19b). As expected, Cre-mediated recombination was complete in the forebrain of this mouse, while no recombination was observed in the tail. In parallel, we determined the NGF concentration of the forebrain of a ngf $^{lox\ neo+/+}$; NesCre +/- mouse by a NGF ELISA assay (Figure 19c). In the wild-type forebrain, we found a NGF concentration of 121.4 ± 4.5 pg NGF / g of forebrain. This is in agreement with the result of a previous study that found an approximately three times higher NGF concentration in a wild-type hippocampus (Chen et al., 1997). In deed, it is known that the hippocampus is one of the forebrain areas with the highest NGF concentration (Saporito and Carswell, 1995). In the forebrain of the ngf $^{lox\ neo+\ /\ +}$; NesCre +/- mouse, we found a reduction of the NGF concentration of about 50% compared to the wild-type mouse. This suggest that ngf was successfully deleted from one of two alleles in the ngf $^{lox\ neo+\ /\ +}$; NesCre +/- mouse. In contrast, the forebrain NGF concentration in ngf $^{lox\ neo+\ /\ lox\ neo+}$ mice was unvaried compared to wild-type. Thus, the insertion of the loxP sequences and the neo-cassette into the ngf locus seem not to interfere with the NGF expression.

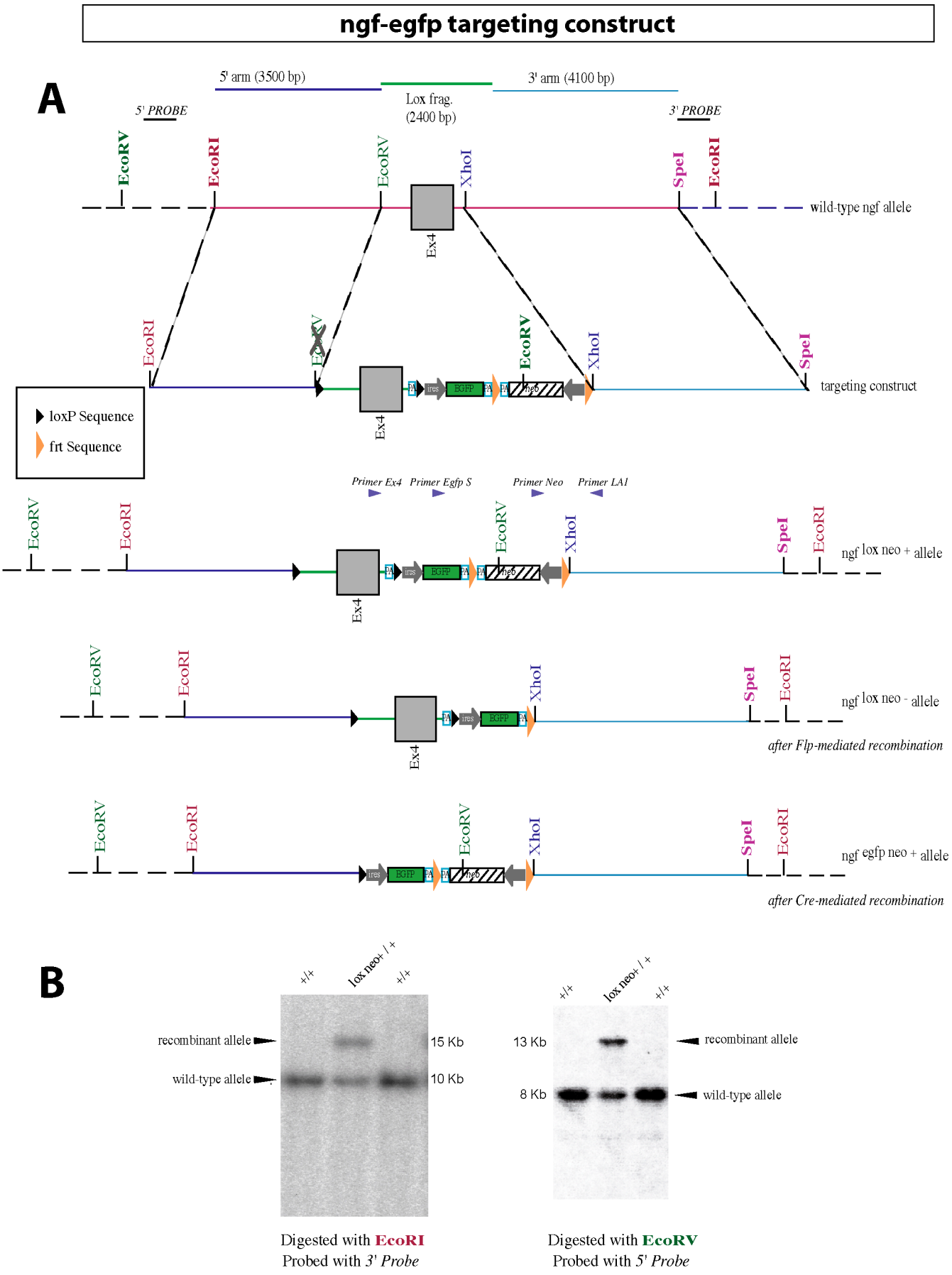

FIGURE 18: Conditional targeting of the ngf exon 4
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Moreover, in the ngf $^{lox\ neo+/+}$; NesCre +/- mouse, we aimed to detect expression of EGFP from the allele in which the ngf exon 4 was deleted. Therefore, we prepared cryosections from the forebrain of the ngf $^{lox\ neo+/+}$; NesCre +/- mouse and analyzed these sections for EGFP expression by using a fluorescent microscope. However, we were not able to detect any EGFP fluorescence in ngf $^{lox\ neo+/+}$; NesCre +/- mice. This may be due to the relatively weak EGFP expression in these mice from only one allele. In addition, the analysis by the fluorescent microscope may be not sensitive enough and other technical approaches may be needed.

Both the normal phenotype and the unaltered NGF expression in the forebrain in ngf $^{lox\ neo+\ /\ lox\ neo+}$ mice strongly suggest that the neo cassette does not reduce expression of NGF in these mice. Nevertheless, we excised the neo-cassette in vivo by crossing ngf $^{lox\ neo+/+}$ mice with Flp-deleter mice. Flp-mediated neo deletion was successful as confirmed by Southern and PCR analyzes (data not shown and Figure 19a).

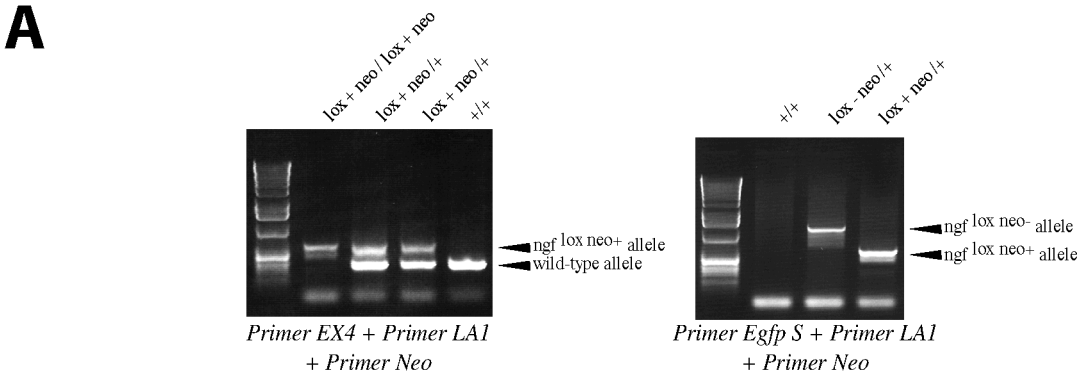
Legend of the FIGURE 18 (previous page):

FIGURE 18: Conditional targeting of the ngf exon 4

Panel A shows schematic representation of the exon 4 of the mouse ngf locus in the wild-type allele. Below the wild-type allele the targeting construct is depicted. The targeting construct contains the floxed exon 4 and the neo-cassette flanked by two frt sites. Some of the restriction enzyme sites that were used for cloning steps and for further analysis by Southern blotting are indicated. Note that the site of the restriction enzyme EcoRV on the 5' side of the exon 4 in the wild-type allele is deleted on the targeting construct. The successful targeting of the wild-type allele with the targeting construct by homologous recombination in ES-cells led to the generation of the recombinant ngf $^{lox\ neo+}$ allele. The neo-cassette was excised in vivo with transgenic mice expressing the Flp-recombinase ubiquitously. This resulted in the generation of the recombinant trkA $^{lox\ neo-}$ allele. Cre-mediated recombination of the ngf lox neo+ allele results in the formation of the ngf egfp neo+ allele. In this allele, the ngf exon 4 is deleted and the green fluorescent protein egfp is expressed under the endogenous ngf promoter.

Panel B shows Southern blot analysis of successful recombination in the ngf $^{lox\ neo+}$ allele. The position of the two probes at the 5' and 3' site of the targeting constructed is indicated in Panel A. The genomic DNA for the Southern blot analysis was digested with the restriction enzyme EcoRI (for the 3'probe) or with EcoRV (for the 5'probe)

PCR analysis of ngf-egfp mice



NestinCre-mediated NGF deletion in ngf-egfp mice

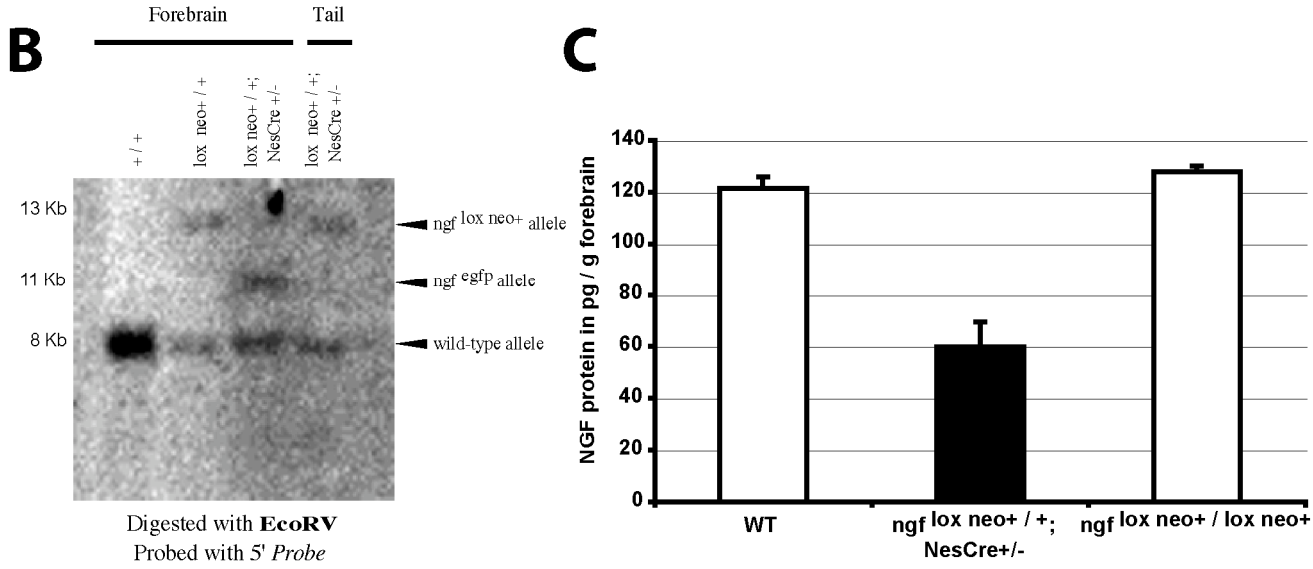


FIGURE 19: Conditional targeting of the ngf exon 4

Panel A shows the analysis by PCR of ngf $^{lox\ neo+}$ and ngf $^{lox\ neo-}$ alleles. Position of the primers used for PCR analysis is indicated in Figure 18a. The PCR reaction on the right side with the Primers EgfpS, LA1 and Neo demonstrates the successful deletion of the neo-cassette in the ngf lox neo- allele.

Panel B: Analysis of the successful NestinCre-mediated deletion of the ngf exon 4 in the forebrain. The Southern analysis with the 5' probe demonstrates that the exon 4 is completely deleted from the genomic DNA of the forebrain of the ngf $^{lox\ neo+/\ +}$; NesCre+/- mouse, while no deletion occurred in the tail DNA of this mouse.

Panel C: ELISA analysis of NGF concentration in the forebrain of wild-type, ngf $^{lox\ neo+/\ +}$; NesCre+/- and ngf $^{lox\ neo+/lox\ neo+}$ mice. Half forebrains were lysed in 1ml lysis buffer and 50 µl of the lysates were analyzed for NGF concentration by using the ELISA microwell plates provided by the Chemikine NGF sandwich enzyme immunoassay kit (Chemicon) The NGF concentration was normalized against the weight of the forebrain. The NGF concentration is reduced by about 50% in the forebrain of the ngf lox $^{neo+/\ +}$; NesCre+/- mouse compared to wild-type mouse, indicating that NGF expression was successfully deleted in one of two alleles in this mouse. In contrast, no NGF reduction was found in the ngf $^{lox\ neo+/lox\ neo+}$ mouse showing that the loxP sites and neo cassette introduced in the recombinant ngf $^{lox\ neo+}$ allele are not interfering with NGF expression.

VI DISCUSSION

1 THE CHOLINERGIC PHENOTYPE IN TRKA DEFICIENT MICE

1.1 Is the cholinergic deficit caused by increased neuronal apoptosis and/or by loss of cholinergic differentiation?

The earliest time point at which we analyzed the loss of cholinergic neurons in the medial septum of TrkA-deficient mice was P15. At this stage, the cell loss in mutant mice compared to control mice has already fully happened and vary only slightly during later development. This indicates that the loss of cholinergic neurons in TrkA deficient occurs mainly during the first postnatal days. We did not investigate whether the cell loss is due to increased apoptotic death in the mutant mice or rather to defects in differentiation or migration. However, there are indications that defects in migration do not contribute to the loss of cholinergic neurons in the forebrain of TrkA-depleted mice. Indeed, at the stage of late development where first TrkA expression in the CNS can be detected, migration and differentiation of cholinergic neurons in the forebrain is already mainly finished (Olsson et al., 1998; Schambra et al., 1989). Therefore, NGF/TrkA signaling is unlikely to be involved in the migration of cholinergic neurons.

However, the question whether the decrease of cholinergic neurons in TrkA-deficient mice results from an increase of apoptotic cell death remains open. Alternatively, the decrease of cholinergic cells could be explained by a severe downregulation of cholinergic markers in these neurons eventually accompanied by cell shrinkage. In the PNS, neurotrophin-dependent neurons die by apoptosis briefly after their formation, when the right neurotrophin support is not provided. Similarly, trkA knockout mice exhibit an important increase of apoptotic cells in the medial septum at P7 compared to control mice (Fagan et al., 1997a). At P13, this increase in apoptosis was no more detectable. This observation would agree with our finding that at P15 the cell loss is already completed in TrkA-deficient mice and does not further increase in later development. However, this kind of increased apoptosis in cholinergic neurons was not

observed in other mouse models where TrkA signaling was depleted (Cattaneo et al., 1999; Ruberti et al., 2000; Smeyne et al., 1994). This may be due to the failure of these studies to detect the right time window where increased apoptosis can be observed. A further argument for the apoptotic cell loss in TrkA-depleted mice is provided by the fact that in this study here and other reports (Ruberti et al., 2000) the decrease of ChAT-positive neurons was accompanied by the decrease of other cholinergic cell marker like p75. However, although being a good indication for the loss of cholinergic neurons, the decrease of p75-positive neurons can not be considered as a definite proof for the death of the cholinergic cells. In fact, it is known that p75 expression can be positively regulated by NGF signaling through TrkA (Verdi and Anderson, 1994).

Finally, many studies demonstrated that cholinergic deficits can be rescued by injection of NGF or are reversible when the inhibition of NGF signaling is eliminated (Bruno et al., 2004; Capsoni et al., 2002b; Cattaneo et al., 1999; Chen et al., 1997; Cooper et al., 2001). The cholinergic deficits in these studies were induced either by genetic or pharmacological manipulation or by physiological aging processes. While in many of these studies, the cholinergic rescue was mainly induced by hypertrophy of the remaining cholinergic neurons and formation of new synapses, in other studies also the total number of cholinergic neurons was rescued by the treatment with NGF (Capsoni et al., 2002b; Cooper et al., 2001). This phenomenon can be explained principally by two different mechanisms: First, NGF may “reactivate” neurons that had downregulated all cholinergic marker due to the absence of appropriate NGF/TrkA signaling or, second, NGF may be capable to induce in vivo de-novo neurogenesis of cholinergic neurons. However, for the latter hypothesis, only first preliminary indications and no clear evidences exist so far (Calza et al., 2003).

At the moment, we have to consider that both cell death and downregulation of cholinergic marker could contribute to the decrease of cholinergic neurons in our mouse model. Further accurate studies will be needed to clarify more precisely the fate of cholinergic neurons in TrkA-deficient mice.

Our studies so far did not allow dissecting definitely whether the decreased cholinergic target innervation in the cortex and the hippocampus of TrkA deficient mice is

exclusively due to the decrease of neurons in the cholinergic cell groups or also to a deficit in axonal outgrowth and expression of cholinergic marker in the cholinergic neurons that survive in the absence of TrkA signaling. However, several results of our study suggest that most likely both kind of defects contribute to the severe impairment in cholinergic target innervation observed in our mouse model. First, the loss of more than 40% of ChAT expression in the total forebrain at P90 is difficult to explain only by the loss of about 40% of the neurons in the cholinergic cell groups Ch1-4, taking in consideration that the total forebrain includes also the large area of the striatum where no neuronal cell loss and ChAT downregulation was detected. Second, the evaluation of the cell numbers in the medial septum of control and mutant mice during different time points of postnatal life indicates that the neuronal cell loss has happened already at P15, while there is no further significant increase of this deficit at least until P270. In contrast, the analysis of ChAT expression demonstrated that the decrease of ChAT expression in the mutant mice is already present at P15, but then is further enhanced during adulthood. Similarly, defects in the cortical and hippocampal innervation by AChE-positive cholinergic fibers are already detectable at P30, but are further increased at P90 and P270. Thus, NGF signaling appears to be essential during the perinatal period to ensure cell survival, while later in life it may play a crucial role in promoting and maintaining proper cholinergic target innervation.

A support for two distinct NGF functions was provided recently by an in vivo study. In this study, the lethal phenotype of the ngf knockout mouse was rescued by the introduction of a ngf transgene that express ngf under the promoter of keratin (Phillips et al., 2004). In these mice, NGF expression in the brain is strongly reduced compared to wild-type mice, but still detectable. The remaining NGF expression is probably due to some aberrant trangene expression and is localized diffusely throughout the CNS without exhibiting the typical endogenous NGF expression pattern. In these mice, no significant cholinergic cell loss was observed in the medial septum, while the cholinergic target innervation in the hippocampus and the cortex was importantly decreased. Thus, the reduced quantity of NGF expression in the CNS of these mice seems sufficient to maintain the survival of the cholinergic neurons of the basal forebrain, while for correct cholinergic target innervation, high NGF expression levels are required. It is possible that

also the precise spatiotemporal regulation of NGF expression is essential for proper target innervation. However, in these mice, it was shown that intraventricular injection of high NGF doses are capable to rescue the impaired cholinergic target innervation. Therefore, the crucial role of NGF in promoting cholinergic target innervation do not depend on the formation of a regional NGF gradient.

1.2 Other neurotrophic factors involved in the development and survival of cholinergic neurons

TrkA deletion decreases the number of the cholinergic neurons in the basal forebrain by up to 40%. However, more than half of these neurons remains intact also in the absence of functional TrkA signaling. This suggest that other neurotrophic factors are also importantly involved in promoting the survival and maintaining the phenotypic differentiation of the cholinergic neurons in the CNS.

One of the apparently most promising candidate molecules for this function appear to be the neurotrophin BDNF and its receptors TrkB. It was shown that TrkB-mediated signaling provides together with the neurotrophin receptor TrkC an essential survival support for distinct non-cholinergic neuron subpopulations in the hippocampus and in cerebrellum. TrkB is expressed after development in most of the cholinergic neurons in the basal forebrain (Molnar et al., 1998; Richardson et al., 2000; Yan et al., 1997). However, the studies that analyzed the survival role of TrkB for cholinergic neurons gave controversial outcomes. While the initial analysis of the trkB and bdnf knockout mice and many subsequent studies did not report any significant loss of cholinergic neurons (Alcantara et al., 1997; Conover et al., 1995; Klein et al., 1993; Minichiello et al., 2002), another study reported a significant loss of about 50% of cholinergic neurons in the medial septum and the striatum of bdnf knockout mice in the first two weeks of life (Ward and Hagg, 2000). The results of the study here presented do not confirm this important role of TrkB in promoting survival of cholinergic neurons in the CNS. We demonstrated that TrkB deletion in the cholinergic neurons results in the loss of only a very small part (of about 10%) of these neurons. The effect of TrkB deletion is only slightly further increase by the deletion of TrkA suggesting that TrkB and TrkA do not cooperate importantly in promoting survival of cholinergic neurons. Our results are in

agreement with the initial observation in TrkB-deficient mice and suggest that the important neuronal loss observed in postnatal bndf knockout mice may be due rather to the overall poor health state and severe weight loss of these mice than to a specific TrkB function in the cholinergic neurons. However, as we have analyzed so far in trkB- and the double trkA/trkB- deficient mice only the neurons of the medial septum at P30, our results do not exclude that TrkB has a more important role in the survival of cholinergic neurons in other cholinergic cell groups like the striatum and / or in more aged mice. Nevertheless, a recent mouse model in which the BDNF production is interrupted in the cortex from early development on suggest strongly that TrkB by itself has no important function in promoting survival of cholinergic striatal neurons also in aged mice (Baquet et al., 2004). In these mice it was demonstrated that cortical BDNF deletion results in aged mice in an important loss of some neuronal subpopulations of the striatum. In contrast, the cholinergic neurons in the striatum in these mice are reduced by only 10%.

Another neurotrophin receptor that may be involved in controlling survival of the cholinergic neurons in the brain is the TrkC receptor. TrkC is expressed in a large amount of the cholinergic neurons of the basal forebrain (Boissiere et al., 1994; Sandell et al., 1998). In vitro experiments show that the main TrkC ligand NT-3 can increase the survival of basal forebrain cholinergic neurons in culture (Ha et al., 1999; Nonner et al., 1996). However in vivo, neither in trkC- nor in NT-3-knockout mice (Ernfors et al., 1994; Klein et al., 1994) any significant impairment of the survival of cholinergic neurons in the perinatal brain was reported. Nevertheless, it is not excluded that NT-3/TrkC signaling has an important role in the cholinergic neurons of the basal forebrain in mature mice. In this regard, it was shown that NT-3 is capable to promote importantly the cholinergic differentiation of sympathetic neurons in the PNS (Brodski et al., 2000). This effect is completely independent of neuronal survival. Thus, it is possible that rather to promote cell survival, TrkC may be involved in inducing and in maintaining the cholinergic differentiation in the cholinergic basal forebrain. Interestingly, mice with brain-specific deletion of NT-3 show no neuronal loss but exhibit an important reduction of neuronal projections from the thalamus to the cortex (Ma et al., 2002). The cholinergic system in the forebrain of these mice was not further analyzed.

Other neurotrophic factors distinct from neurotrophins were also shown to promote the survival of cholinergic neurons in the CNS. For instance, the neuropeptide galanin has been indicated as an essential neurotrophic factor for the basal forebrain cholinergic neurons. Galanin acts through specific galanin receptors and is expressed in many parts of the basal forebrain (Melander et al., 1985; Mufson et al., 2003). In galanin-deficient mice, about 35% of the cholinergic neurons in the medial septum are lost (O'Meara et al., 2000). Interestingly, NGF was shown to induce expression of galanin and therefore some of the neurotrophic function of NGF in the cholinergic basal forebrain neurons may be mediated through galanin (Rokaeus et al., 1998). However, galanin expression is increased also by stimulation with other molecules and at least part of the galanin function in the cholinergic neurons in the CNS may be NGF-unrelated (Ozturk and Tonge, 2001).

Other factors that may be involved in the survival of basal forebrain cholinergic neurons are members of the fibroblast growth factor (FGF) family. FGFs and their receptors have been shown to be expressed in many cholinergic neurons of the forebrain including the interneurons of the striatum (Bizon et al., 1999; Bizon et al., 1996; Yoshida et al., 1994). FGF-2 was shown both to enhance survival of lesioned medial septum neurons and to promote target innervation of cholinergic neurons in the hippocampus (Fagan et al., 1997b; Otto et al., 1989). In addition, FGF-9 was shown to support potently the survival and the upregulation of cholinergic properties in basal forebrain cholinergic neurons in culture (Kanda et al., 2000). In contrast to the NGF/TrkA mediated signaling, FGF seems to exert its effects in cholinergic neurons also in an autocrine manner.

1.3 Why are striatal interneurons resistant to TrkA deletion?

The results of our study suggest strongly that there are functional differences between the TrkA signaling in the basal forebrain cholinergic cell groups Ch1-Ch4 and the cholinergic neurons in the striatum. TrkA deletion reduces the number of the cholinergic neurons Ch1-Ch4, impairs their expression of the cholinergic marker ChAT and affects severely the cholinergic target innervation provided by these neurons; in contrast, the number of cholinergic striatal neurons is not varied in TrkA deficient mice and the expression of ChAT in these neurons remain unaltered. In the original analysis of the

complete trkA knockout mice similar differences were described. In the subsequent analysis of the few TrkA-deficient mice that survive until the end of the third postnatal week a small decrease of cholinergic neurons compared to control mice was observed also in the striatum. This decrease, however, was not statistically significant and was of lower extent (about 20%) than the decrease of cholinergic neurons observed in the medial septum.

What may be the reasons for the different vulnerability of Ch1-Ch4 neurons and of striatal neurons in TrkA-deficient mice?

Striatal cholinergic neurons differ in many points from the other cholinergic neurons of the basal forebrain. One of the most evident molecular difference is the lack of expression of the neurotrophin receptor p75 in the striatum of adult mice, while p75 is expressed in virtually all cholinergic neurons of the Ch1-Ch4 groups during and after development (Gibbs and Pfaff, 1994). p75 function in vivo is linked to neuronal cell death; and it would be conceivable that the lack of the pro-apoptotic p75 signaling in striatal neurons would spare these neurons from cell death also in the case of the deletion of the pro-survival signaling of TrkA. In neurons that express both receptors TrkA and p75, neurotrophins binding to these receptor would activate both pro-apoptotic and pro-survival pathways that would balance each other. In the case however, that one of these signaling pathways is inhibited, the other pathway would take over and the cell would be driven into forced proliferation or cell death. Therefore, deletion of p75 signaling results in an increase of cholinergic neurons (Naumann et al., 2002) while TrkA-deficient mice exhibit less cholinergic neurons (this study here and (Fagan et al., 1997a)). In contrast, striatal cholinergic neurons that do express only TrkA but not p75 would not receive any pro-apoptotic input even in the absence of the anti-apoptic TrkA signaling. One finding that could argue against such a scenario is the observation that some striatal cholinergic express for a very short perinatal period also the receptor p75 (Van Vulpen and Van Der Kooy, 1999). These neurons should be lost in TrkA-deficient mice. However, the expression of p75 in the striatal cholinergic is of very limited quantity and time span and may not be strong enough to drive the cholinergic neurons in the striatum of trkA knockout mice into apoptosis. Another argument against the proposed model is provided by our analysis of the medial septum neurons in TrkA-deficient mice. In fact, we

demonstrated clearly that at least some of the cholinergic neurons that express p75 can survive also in absence of TrkA signaling. Thus, the expression pattern of p75 by itself may not be sufficient to explain the relative resistance of the cholinergic neurons in the striatum against TrkA-deletion.

Interestingly, the cholinergic neurons of the striatum are known to be particular resistant also to other forms of neuronal cell death. For instance, after injection of the neurotoxin quinolinic acid or after induction of cerebral ischemia, cholinergic striatal neurons exhibit less damage than interneurons of the hippocampus or other interneuronal subpopulations of the striatum (Larsson et al., 2001). Similarly, also in the pathological state of Huntington's disease cholinergic interneurons are relatively spared in relation to other neurons of the striatum (Cicchetti et al., 2000). Remarkably, under some circumstances, the resistance of cholinergic interneurons in the striatum accompanied by overexpression of TrkA and p75 in these neurons (Hanbury et al., 2002; Larsson et al., 2001). In this regard, it was shown that the resistance against insults is not mediated or modulated by p75 signaling (Andsberg et al., 2001). However, it is possible that the upregulation of the pro-survival receptor TrkA may confer to the cholinergic interneurons a relative resistance to pathological damages. Therefore, it would be interesting to analyze whether the vulnerability of the cholinergic interneurons in the striatum of TrkA-deficient mice is increased compared to wild-type mice. In general, very little is known about the cellular factors that control the variable vulnerability of the different neuron types in the striatum. For instance, particular expression pattern of specific glutamate receptor subtypes, and intracellular enzymatic activities have been discussed to be responsible for the relative high resistance in cholinergic neurons of the striatum (Calabresi et al., 2000a; Chen et al., 1996; Fass et al., 2000; Medina et al., 1996; Standaert et al., 1996). In addition, distinct regulation mechanisms of the membrane potential may be also involved in the selective vulnerability observed in the striatum (Calabresi et al., 2000b). It is possible that some of these mechanisms are also responsible for the higher resistance of the cholinergic neurons in the striatum in TrkA-deficient mice compared to the other cholinergic neurons in the basal forebrain.

2 THE APP RELATED PHENOTYPE IN TRKA DEFICIENT MICE

2.1 Discrepancies and similarities between the outcome of our work and the results of previous studies

Our analysis of the APP metabolism in TrkA-deficient mice revealed a significant increase of detected total APP in the forebrain and in particular in the hippocampus. This increase is due to an upregulation of the secretion of soluble APP forms. The upregulated soluble APP form could not be definitely identified, but our results suggest strongly that it is the soluble APPα generated by the α-secretase. First, we detected a significant increase of the soluble APP form by using an antibody that recognizes specifically the soluble APP forms α and β', but not the soluble APPβ. Second, by analyzing the intracellular carboxy-terminal APP fragments that are the additional products of the cleavages mediated by α- and β-secretases, we found that in TrkA-deficient mice the β-cleavage pathway remains unaltered or is even downregulated, while the product of the α-cleavage appears to be slightly increased.

The increase of total APP in our mouse model agrees with previous reports that analyzed APP expression in cells or animals with reduced NGF/TrkA signaling or cholinergic activity. Most interestingly, the transgenic anti-NGF mouse where NGF signaling is inhibited by expression of a neutralizing NGF-antibody exhibits an important increase of total APP expression (Capsoni et al., 2000b). The nature of the increased APP was not further characterized. Similarly, in vitro it was shown that the withdrawal of NGF from NGF-dependent cells increases importantly the expression of total APP protein (Araki and Wurtman, 1998; Nishimura et al., 2003). This increase was accompanied by an upregulation of APP mRNA transcription and has a potent anti-apoptotic effect. In addition, also the inhibition of cholinergic activity in mice or rats by pharmacological modulation or by immunolesions resulted in an increase of APP protein expression (Rossner et al., 1997; Seo et al., 2002). Conversely, administration of muscarinic agonists decreases APP protein levels.

On the other side, our finding that the generation of the soluble APPα is increased in TrkA-deficient mice is rather surprising. Generally, the issue of the effect of NGF/TrkA signaling and cholinergic activity on the secretion of soluble APP is discussed in a very

controversial manner in the literature. For instance, NGF signaling was shown in cell culture studies to increase the secretion of soluble APP fragments (Rossner et al., 1998a). This effect is most likely mediated through both the TrkA and the p75 receptors. Similarly, activation of cholinergic pathways is to induce in vitro potently the secretion of soluble APP forms. This effect is transmitted mainly through muscarinic receptors and involves the activation of the protein kinase C (PKC), which is known to be an important inducer of soluble APP secretion (Nitsch et al., 1992; Pakaski et al., 2000). In vivo, overactivation of the PKC enzyme increases secretion of soluble APP fragments (Caputi et al., 1997). In addition, an animal model with impaired cholinergic activity demonstrated that decreased cholinergic activity correlates positively with decrease of secretion of soluble APP fragments (Rossner et al., 1997). However, the in vivo outcome of the inhibition of the cholinergic system on secretion of soluble APP is controversial. Under some circumstances, the administration of procholinergic agents results in the decrease of generation of soluble APP fragments (Haroutunian et al., 1997; Lahiri et al., 1998; Shaw et al., 2001). Similarly, rats that had received lesions of the nucleus basalis showed an important increase of soluble APP secretion in the cortex (Wallace et al., 1995). Remarkably, the effect on APP secretion in these rats was accompanied by slight increase of the carboxy-terminal APP fragment that are generated by α-secretase. This confirms the results presented in our study here. Another intriguing outcome from the latter study with immunolesioned rats was the observation that the effect of cholinergic depletion on the secretion of soluble APP is time-dependent. While in a short period directly after lesioning, the secretion of APP is first inhibited, at later time points (from 1 week after lesioning on) the secretion is then importantly enhanced. The secretion is further increased during aging. In this regard, in vitro it was also shown that the increase of APP secretion observed after pharmacological activation of cholinergic pathways is only transitory (Racchi et al., 2001). Thus, the discrepancies between the different outcomes of the inhibition of the cholinergic system on secretion of soluble APP protein may be at least partly explained by the different time points at which the animals were analyzed.

2.2 What may be the mechanisms involved in the modification of APP processing after TrkA deletion?

From the analysis we have done up to now, we can only speculate about the mechanisms that lead to the alteration of APP processing after TrkA deletion. First of all, we have no clear evidence whether the APP processing is modified directly by the lack of functional NGF/TrkA signaling or rather by a secondary effect due to the cholinergic deficit. The finding that we detected cells with increased APP immunoreactivity mainly in the hippocampus and in the cortex and not in the areas of the cholinergic cell groups indicates that the increase of APP is preferentially observed in areas with impaired cholinergic innervation. However, it is not to exclude that also the lack of NGF signaling in the axons of the cholinergic neurons in the hippocampus and the cortex contribute directly to the APP-related phenotype.

One of the easiest explanations of the observed increase in total APP protein levels in the TrkA-deficient would be the contemporary increase also of transcription of the APP gene in these mice. For instance, in cell culture experiments, the withdrawal of NGF resulted in the upregulation of APP mRNA (Araki and Wurtman, 1998). However, our finding that the full-length membrane-bound APP remains unvaried in TrkA-deficient mice makes it difficult to explain the APP protein increase only by upregulated APP gene transcription. In this case, the upregulation of the APP transcription should be accompanied by an important alteration of the APP cleavage activities in order to give a final outcome of unvaried full-length APP and increased soluble APP. Finally, the hypothesis of an upregulated APP gene transcription in TrkA-deficient mice is not supported by the result of a previous study that showed that cholinergic depletion in rats has no significant effect on APP mRNA transcription levels (Apelt et al., 1997).

One more likely reason for the observed APP processing alteration in the TrkA-deficient mice would be the upregulation of the secretases responsible for the α-cleavage and/or the downregulation of the β-secretase BACE. At the moment however, none of these kind of links between NGF/TrkA signaling, cholinergic pathways and expression of α- and β-secretases have been so far reported in the literature. In rats with immunlesioned cholinergic system, no significant changes in the expression of the BACE-secretase have been observed (Lushchekina et al., 2002).

Alternatively, rather than by changes in the expression of the secretases, the APP phenotype in our mouse model could be induced also by alteration of the activity of the different secretases. In cell culture studies it was shown that the pharmacological activation of the cholinergic pathway leads to an increase of the activity of the α-secretase candidate ADAM10 (Zimmermann et al., 2004). However, this finding that would be in disagreement with our model was observed only in short-term experiments and was not verified in in vivo models. More interestingly, it was shown in vivo that the activity of the BACE secretase can be importantly modified by phosphorylation of the intracellular part of the APP protein (Lee et al., 2003). Phosphorylation of the T668 site in APP was shown to facilitate the BACE cleavage and to increase the generation of Aβ-peptides. Inhibition of this phosphorylation increases cleavage by α-secretase. In this regard, it is particularly interesting that TrkA signaling was shown to be capable to phosphorylate the intracellular part of the APP protein (Tarr et al., 2002a; Tarr et al., 2002b). The preferential phosphorylation site so far identified for TrkA signaling in APP (T682) is distinct from T668, but is also importantly involved in the regulation of APP processing. Indeed, mutation of the T682 site in vitro results in the decrease of endocytosis of full-length APP (Perez et al., 1999). In turn, this leads to the increase of soluble APP secreation and the decrease of formation of full-length Aβ-peptides. Thus, it is particular intriguing to speculate that the lack of NGF/TrkA signaling in TrkA-deficient mice results in a changing of the APP phosphorylation state and in turn in the preferential cleavage of APP by α-secretase.

Finally, it is noteworthy that α- and β- secretases have been shown to compete in the same cellular compartments for intracellular APP cleavage (Skovronsky et al., 2000). Thus, any kind of inhibition of β-secretase in the TrkA-deficient mice would also result in the concomitant increase of α-cleavage and viceversa.

2.3 What could be the consequences of the alteration of APP processing in TrkA-deficient mice?

The preferential cleavage of APP by α-cleavage instead by β-cleavage in TrkA-deficent has two main consequences:

First, the soluble APPα is importantly increased in TrkA-deficient mice. The α-form of soluble APP is known to have neurogenic, neurotrophic and neuroprotective properties (Caille et al., 2004; Mattson, 1997). It was shown to protect neurons from death by glutamate toxicity and by neurotoxic Aβ peptides. Moreover, it may provide a protective support against cell death by neurotrophin withdrawal (Nishimura et al., 2003). In this regard, it is possible that the increase of soluble APPα in TrkA-deficient mice may be part of an anti-apoptotic response from cholinergic neurons that suffer from the lack of pro-survival TrkA-signaling. Alternatively, the increase of soluble APPα may be also generated by non-cholinergic cells in order to provide a neurotrophic support to the cholinergic neurons. It is tempting to speculate that the increase of soluble APPα may contribute to the neuroprotective effect that allow a large part of the cholinergic neurons to remain intact even in the absence of TrkA signaling.

Second, the preferential α-cleavage of APP in TrkA-deficient mice may result also in the reduction of neurotoxic Aβ peptides. We did not analyze directly the generation of Aβ peptides in our mouse model. However, the slight decrease of carboxy-terminal APP fragment generated by β-secretases in TrkA-deficient mice may suggest that the β-cleavage pathways are downregulated in these mice. Such a reduction of β-cleavage is often associated with a decrease of generation of the neurotoxic and amyloidogenic Aβ-peptides. A support for such an anti-amyloidogenic effect of TrkA deletion was provided by a recent study that investigated the effect of cholinergic depletion in transgenic mice overexpressing APP protein (Boncristiano et al., 2002). The very high levels of APP in these mice result in a strong upregulation of Aβ peptides and frequent formation of amyloid plaques. Interestingly, the inhibition of cholinergic innervation of the cortex by immunolesions results in these mice in the decrease of amyloid plaques and downregulation of Aβ-peptides. Thus, the cholinergic deficit in TrkA-depleted mice may provide protection against the formation of amyloidogenic Aβ-peptides. An additional indication for an anti-amyloidogenic effect of TrkA-deletion has been provided by the finding that the acetylcholinesterase enzyme can form complexes with Aβ-peptides (Alvarez et al., 1998; Inestrosa et al., 1996). These complexes display higher amyloidogenic and neurotoxic properties than Aβ-peptides complexes without

acetylcholinesterase. Thus, the strong reduction of acetylcholinesterase in the cortex and the hippocampus of TrkA-deficient mice may protect these mice additionally from the aggregation of Aβ-peptides in amyloid plaques.

On the other hand, the transgenic anti-NGF mice have been shown to display an important amount of pathological signs that resemble closely those observed in Alzheimer's disease. These include formation of amyloid plaques and neuronal cell loss in the brain. How is this phenotype in mice with inhibited NGF signaling reconcilable with the APP-related changes observed in the TrkA-deficient mice?

First of all, the animals we analyzed in this study were importantly younger than the anti-NGF mice that show the Alzheimer's disease-like phenotype. While the anti-NGF transgenic mice that displayed pathological signs of Alzheimer's disease had an age of 15-17 months, the animals we analyzed for the APP-related phenotype were 6-9 months old. It is possible that further aging of these mice will lead to important changes of the so far observed phenotype. Such changes could be due to distinct phenomenons.
First, it is possible that the primary neuroprotective effect of the increased APP cleavage by α-secretases may be transformed in aged mice to a rather neurotoxic effect. In this regard, it was demonstrated that the neuroprotective function of soluble APPα is importantly mediated through the upregulation of neuroprotective genes like insulin-like growth factor 2 and transthyretin (Stein et al., 2004). Inhibition of the transthyretin expression eliminates the neuroprotective effect of soluble APPα and leads to neurodegeneration in transgenic APP mice, even if the level of soluble APPα is high. Thus, it is possible that in aged TrkA-deficient mice, the neuroprotective effect of soluble APPα may be neutralized by the concomitant downregulation of genes that mediate the neurotrophic function of soluble APPα. In addition, it was shown that full-length Aβ-peptides are not the only products of APP processing that exhibit neurotoxic properties. Indeed, also small 31-amino acid fragments of the carboxy-terminal end of APP (termed C31) are potent inducers of apoptosis (Lu et al., 2000). These fragments are generated by the proteolytic activity of different caspases. Interestingly, all carboxy-terminal APP fragments (including these fragments produced by α-cleavage) can serve as substrates for

the generation of C31-fragments. Thus, the upregulation of α-cleavage in TrkA-deficient mice may result in the increase of neurotoxic C31-fragments that could potentially induce increased cell death (as observed in the transgenic NGF-mice).
Aditionally, it is possible that the preferred cleavage of APP by α-cleavage in 6-months old TrkA-deficent mice could switch in older mice for so far unknown reasons to preferred β-cleavage of APP. This may result in aged mice in the upregulation of generation of neurotoxic and amyloidogenic full-length Aβ peptides similar to the situation observed in aged transgenic anti-NGF mice.

Finally, it is important to point out that although our TrkA-deficient mouse and the transgenic anti-NGF mouse both display inhibited NGF/TrkA signaling, these mice are not identical. First, in TrkA-deficient mice, the inhibition of NGF/TrkA signaling results from the depletion of the TrkA receptor. This means that all signaling of TrkA is completely abolished. On the other side, NGF in these mice is still present and can still bind to the p75 receptor. In contrast, the modulation of NGF/TrkA signaling in transgenic anti-NGF mice is produced by the blocking of NGF actions. Thereby, NGF-mediated activation of both the TrkA and the p75 receptors is eliminated in these mice. However, TrkA is still expressed and can be eventually activated by other ligands like the NT-3 neurotrophin. In addition, the deletion of TrkA in our mouse is complete and occurs from early development on, while blocking of NGF in the anti-NGF mice is only partial and starts at a not precisely defined time point after development. Finally, the forced expression of NGF-antibodies in transgenic anti-NGF mice could be accompanied by unspecific toxic effects that may alterate the APP-related phenotype in these mice. All these differences could importantly contribute to differences in the APP-related phenotypes in TrkA-deficient mice and in anti-NGF transgenic mice

VII OUTLOOK

The further analysis of the cholinergic phenotype of TrkA-deficient mice will be mainly focused on the functional consequences of the deletion of cholinergic target innervation in these mice. In this regard, we will investigate whether the behavioral phenotype of the TrkA-deficient mice is altered due to the deficits of cholinergic innervation in the hippocampus and the cortex. Mainly behavioral phenotypes related to spatial learning and attention functions will be investigated. Furthermore, the behavioral analysis will be combined with the measurement of electrophysiological parameters in specific areas of the brain. Together these two experiments will help understanding impairments in synaptic activity in trkA mutant mice.

The APP-related phenotype in TrkA-deficient mice will be characterized in more details. In this regard, we will focus our study on understanding what could be the mechanisms that lead to the increased α-secretase-mediated APP cleavage in TrkA-deficient mice. This will include also the expression analysis of genes involved in the APP processing. In addition, we will analyze more directly the generation of amyloidogenic Aβ-peptides in our mouse model. Finally, we will study whether the APP processing is further modified in more aged TrkA-deficient mice.

In addition, mice carrying the floxed ngf gene will be crossed first of all with two different transgenic Cre- expressing mice. The use of the transgenic mouse that express Cre under the nestin-promoter will permit to delete NGF expression from neuronal precursors in the CNS. The analysis of this mouse will allow to confirm the phenotypes observed in the Nestin-Cre mediated TrkA-deleted mouse. Eventual differences between the NGF- and the TrkA-deficient mouse may give insights into the potential role of p75 and / or NT-3 signaling in controlling the cholinergic and the APP-related phenotype. Finally, we will cross the floxed ngf mouse also with a transgenic mouse that express the Cre recombinase under the promoter of the calcium-calmodulin-dependent protein kinase II (CaMKII) gene (Minichiello et al., 1999). In this mouse, Cre is expressed in principal

CNS neurons subpopulations but only from the third postnatal week on. The use of the CaMKII-Cre transgene will allow investigating precisely whether NGF/TrkA signaling has any specific role in the forebrain also after development.

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MARKUS MÜLLER

Tabellarischer Lebenslauf

30.10.1971	geboren in Berlin-Steglitz
1990	Erlangung der deutschen Hochschulreife am Französischen Gymnasium, Berlin
1991 – 1998	Studium der Humanmedizin an der Freien Universität, Berlin und an der Humboldt-Universität, Berlin
1998	Erfolgreicher Studiumabschluß (Staatsexamen) mit der Gesamtnote “gut”
1998 – 2001	Wissenschaftliche Tätigung an der Universität Turin (Italien) unter der Leitung von Frau Dr. C. Ponzetto
Mai 2001-heute	Doktorarbeit am EMBL-Monterotondo (Italien) unter der Leitung von Frau Dr. L. Minichiello Seit Wintersemester 2003 immatrikuliert für die Promotion an der LMU München, Fakultät Biologie

Wissenschaftliche Publikationen

Müller, M., Dietel, M., Turzynski, A., and Wiechen, K. (1998). Antisense phosphorothioate oligodeoxynucleotide down-regulation of the insulin-like growth factor I receptor in ovarian cancer cells. Int J Cancer *77*, 567-571.

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